Genetic Improvement of Underutilised Legumes

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ABSTRACT

In today's world of rapidly changing climates, ever-increasing human population growth and increased competition for space, the production of energy and high-quality food to meet increasing demands presents an enormous challenge to the related industries. It has been predicted that global food production alone, must increase by 70% by 2050 to prevent worldwide starvation. The application of plant biotechnology offers a promising alternative over classical crop improvement techniques in the development of breeding programmes that could respond rapidly to future demands for high-yielding and nutritionally-enhanced crops.

Adopting such strategies to underutilised legume crops represents a viable avenue for crop improvement, especially in developing countries with a high incidence of poverty and malnutrition, where local environmental conditions frequently prove too challenging for effective cultivation of commercial cultivars. The main objective of this thesis was to generate fundamental data regarding the adoption of tissue culture-based transformation strategies for the genetic improvement of two such crops, jicama [*Pachyrhizus erosus* (L.) Urban] and Bambara groundnut [*Vigna subterranea* (L.) Verdc.].

The work carried out on jicama investigated the regeneration potential of this plant *in vitro* as a basis for future biotechnological techniques. Preliminary tissue culture experiments identified explant types and plant growth regulator (PGR) matrices and concentrations that produced optimal regenerative responses *in vitro*. Histological analysis of regenerated tissue revealed that shoot formation occurred *via* somatic embryogenesis. Possible avenues for further optimisation of the explant-to-plant regeneration protocol, for downstream molecular genetic applications, were explored and discussed.

For Bambara groundnut, this study initially focused on developing a novel explant-to-plant regeneration system, previously not reported for landraces DipC, Uniswa-Red and S19-3, through the adoption and modification of techniques

specific for distinct Bambara landraces. This micropropagation protocol employed whole zygotic embryos as starting explants, which formed the basis for subsequent transformation studies. Genetic transformation was attempted *via* biolistic- and *Agrobacterium*-mediated approaches, using transformation vectors pVDH65, pBI121, harbouring the *npt*II selectable marker and *uidA* (*gus*) reporter genes, while pBI121-LeB4-Ber e1 additionally carried the methionine-rich *ber e1* gene. T-DNA transfer in transformed explants was confirmed *via* histochemical GUS analyses, while PCR assays identified putatively transformed regenerated shoots. Explant viability was significantly compromised following bombardment or inoculation, resulting in reduced shoot regeneration, and thus limited the production of transgenic plants. Improvements in transformation efficiency and regeneration frequency were made following protocol optimisation, and the potential for further improvements to efficiently produce well-developed, healthy transgenic shoots were explored and discussed.

The results presented in this thesis describe the optimisation of innovative tissue culture and regeneration protocols, which form a fundamental prerequisite to future transformation-based experiments for jicama and Bambara groundnut. Additionally, previously unpublished data generated from transformation studies in Bambara groundnut has revealed its potential for genetic manipulation. These results have established the foundation of novel micropropagation and transformation systems with the potential to be further optimised to genetically enhance and exploit the enormous agronomic potential of these underutilised legume crops.

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LIST OF ABBREVIATIONS

ABA	Abscissic acid
AFLP	amplified fragment length polymorphism
Asp	Aspartate
BAP	N^6-benzylaminopurine
BM	basal medium
BN2S	Brazil nut 2S albumin (protein)
BSR	Bambara shoot regeneration
bp	base pair(s)
CA	California
CaMV	Cauliflower Mosaic Virus
CIP	Calf intestinal phosphatase
cm	centimeter(s)
Co.	Company
Corp.	Corporation
Cys	Cysteine
d	day(s)
DipC	Diphiri cream
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetra-acetic acid
e.g.	*exampli gratia* (Latin; for example)
et al.	*et alia* (Latin; and others)
FAA	Formaldehyde-acetic acid
FAO	Food and Agriculture Organization
Fig.	figure
FSA	food security assessment
Gly	Glycine
GM	genetically modified
GmBH	Gesellschaft mit Beschränkter Haftung (limited liability company)
gus	beta-glucuronidase gene

GUS	beta-glucuronidase protein
h	hour(s)
HT	herbiside tolerant
kb	kilobase(s)
L.	Linnaeus
LB	Luria broth
LB.	left border
Leu	Leucine
Ltd.	Limited
MCS	multiple cloning site
ME	Maine
Met	Methionine
mA	milliampere(s)
mg	milligram(s)
mgL^{-1}	milligram(s) per litre
min	minute(s)
ml	millilitre(s)
mm	millimeter(s)
mM	millimolar
MM	Materials and Methods
MMT	million metric tonnes
MS	Murashige and Skoog medium (Murashige and Skoog, 1962)
MS0	Murashige and Skoog medium with 0.8% (w/v) agar
NAA	α-Naphthaleneacetic acid
nm	nanometer(s)
NN	Nitsch and Nitsch
nos	nopaline synthase
No.	number
***npt*II**	neomycin phosphotransferase II gene
OD	optical density
PCR	polymerase chain reaction
PGR	plant growth regulator
RAPD	random amplified polymorphic DNA

RB	right border
rDNA	recombinant DNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
sec	second(s)
SSA	Sub-Saharan Africa
TAE	Tris acetate EDTA
T-DNA	Transferred DNA
Ti	tumour inducing
T_m	melting point
***uid*A**	beta-glucuronidase gene
UK	United Kingdom
USA	United States of America
UV	ultra violet
V	volt(s)
var.	variety
Verdc.	Verdcourt
vir	virulent gene(s)
v/v	volume to volume ratio
WI	Wisconsin
wk	week(s)
w/v	weight to volume ratio
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
®	Registered
™	Trade mark
°C	degree(s) Celsius
%	percent/percentage
x	time(s) (magnification factor)
X	time(s) (multiplication factor)
µg	microgram(s)
µl	microlitre(s)
µmol $m^{-2}s^{-1}$	micromole per metre squared per second

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CHAPTER 1. GENERAL INTRODUCTION

1.1. PLANT BIOTECHNOLOGY: AN OVERVIEW

Traditional crop improvement approaches, such as selective breeding and hybridisation through the controlled pollination of plants, have been used for centuries by scientists and farmers to produce plants with desirable traits (Brown and Thorpe, 1995) since the manipulation of genetic variability is fundamental to any successful plant breeding programme. However, in the face of unprecedented global human population growth, unparalleled demands on energy, increasing competition for space and rapidly changing world climates, it has become evident that such classical plant breeding approaches are inadequate and insufficiently rapid to adapt to such requirements (Graham *et al.*, 2007; Rao, 2008; Stewart and Ow, 2008; White and Broadley, 2009; Beddington, 2010; Tester and Langbridge, 2010).

Plant biotechnology is an extension of such traditional breeding strategies and offers a promising alternative to these classical, sometimes inefficient crop improvement methods. It encompasses techniques which permit the manipulation of genetic information in a more precise and controlled manner. Such novel approaches include those for the study of genetic variability and diversity within and among different germplasm collections, tissue culture coupled with recombinant DNA and genetic transformation technologies, as well as techniques employed in the molecular characterisation of putative transgenic plants (Stewart and Ow, 2008).

Faced with such immediate and global challenges, the successful application of these technologies to crop improvement is paramount to ensure its eventual acceptance and integration. Developments and improvements in molecular techniques and in particular, sequencing technologies, have helped merge plant biotechnology approaches with classical crop improvement techniques. For instance, recent advancements in Next Generation sequencing approaches such as FLX 454 (Roche), Solexa (Illumina) and SOLiD (Applied Biosystems) can

produce >1 Gbp of *de novo* sequences per day and have therefore surpassed traditional Sanger sequencing technologies (producing 1Mbp per day) (Sanger *et al.*, 1977) in terms of efficiency and throughput (Nyrén, 2007; Meyer *et al.*, 2008; Pettersson *et al.*, 2009). Such developments have facilitated high-throughput applications including serial analyses of gene expression and whole genome sequencing, while similarly initiating the development of new techniques, such as ultra-deep amplicon sequencing (Meyer *et al.*, 2008). Moreover, such molecular advancements have been shown to produce important and tangible benefits to crop improvement, including developments in crop resistance to herbicides and pests, an expansion into biofuel production, progression of plant metabolite production and perhaps most importantly, increases in crop productivity and nutritional content (Rao, 2008).

1.1.1. Applications of plant biotechnology

Throughout the initial period of developing plant biotechnology, the majority of research focused on adding traits to improve the production or culture of crops, referred to as 'input' traits. More recently, however, research has developed further to encompass the development of plant-based products which are beneficial to customers, referred to as 'output' traits (Korth, 2008).

1.1.1.1. Crop development for biofuel production

The efficient production and development of bio-ethanol and bio-diesel has become vital in the face of rising fuel costs and increasing awareness of our dependence on non-renewable fossil fuels (Korth, 2008; Rao, 2008). Ethanol is biodegradable, renewable and emits less pollution during combustion than the majority of fossil fuels. Current strategies involve the transformation of complex carbohydrates from corn or sugar cane to simple sugars before yeast driven fermentation produces ethanol. Major concerns with this approach centre on the high levels of nitrogen fertiliser, fuel and pesticides required to produce corn and to a lesser extent sugar cane crops (Korth, 2008). Therefore, investigations into the conversion of high-cellulose materials, such as wood chips and perennial grasses into ethanol are gathering momentum. Although currently an inefficient

process, conversion of large biomass crops such as switchgrass and fast-growing trees to ethanol has the advantage of requiring comparatively little agronomic input and thus represents a more sustainable approach to biofuel production (Korth, 2008). Genetic engineering of plants containing altered cell wall architectures leading to transgenic crops which are more amenable to efficient ethanol production through cellulose enzymatic digestion is thus being developed (Rao, 2008). This sector is currently experiencing rapid development and progress in isolating individual cellulases with improved thermal stability and modified enzymatic activities in bioreactors, which show potential as one possible sustainable alternative to fossil fuel consumption (Rao, 2008).

1.1.1.2. Plant engineering for metabolite production

Plants have been acknowledged as one of the most suitable media to manipulate and produce novel metabolites for the benefit of sectors including health and nutrition (Rao, 2008). For instance, the structure-based engineering of strictosidine synthase, which catalyses the condensation of tryptamine and secologanin and leads to the synthesis of numerous monoterpenoid indole alkaloids in higher plants, has been shown to generate novel alkaloid libraries with potential applications in pharmacology (Loris *et al.*, 2007). Also, the natural diversity of compounds such as plant cytochromes P450 provide an enormous potential resource to exploit and engineer beneficial pharmaceuticals, develop crop protection compounds, and enhance phytoremediation (Morant *et al.*, 2003; Jackson *et al.*, 2007). Further to this, the production of plant-manufactured pharmaceuticals (PMPs) such as human and animal oral vaccines and antibodies in plants has been proposed as a sustainable and cost-effective alternative to the administration of traditional medicines (Korth, 2008). Vaccination could be effected by consumption of food sources containing an immunogenic protein which is easily stored and grown *e.g.* clinical trials have shown an immune response in people consuming transgenic potatoes containing the surface antigen of the hepatitis B virus (Korth, 2008). Producing such pharmaceutical proteins in plants present advantages over conventional *Escherichia coli*, yeast and mammalian cell cultures since large quantities of biomass expressing the product of interest can be generated on minimal input. Additionally, risks associated with

the transfer of infectious diseases to humans are minimised in plants, unlike mammalian cultures, since plants typically contain no animal pathogens (Korth, 2008). However, issues relating to public acceptance, human allergic reactions due to plant glycosylation and altered immunogenicity due to plant sugar moieties added to proteins have been encountered during PMP manufacture (Korth, 2008).

1.1.1.3. Improving crop insecticidal properties

Modern crops are increasingly under attack from a growing range of pests which has subsequently resulted in the need to develop innovative crop protection strategies (Beddington, 2010). This problem has been worsened by increased mobility of people and agricultural material which has thus introduced new and highly destructive pests and diseases, as well as permitting a more rapid mixing and evolution of virulent diseases including the catastrophic "Ug99" strain of stem rust in wheat (Levine and D'Antonio, 2003). This disease currently threatens 20% of global wheat in Central and North Africa, the Middle East and Asia (Beddington, 2010). Pesticide applications have helped to significantly increase crop production since the 1950s. However crop losses due to pest and disease remain relatively high, ranging from 26 – 29% for soybean and wheat, and 30 – 40% for maize, rice and potatoes (Oerke, 2006). Although usage of pesticides may continue, innovative strategies such as integrated pest management techniques, natural plant defence stimulation and the application of 'semiochemicals', such as insect pheromones to dissuade pest attack could also be employed concurrently (Cook *et al.*, 2007; Pickett *et al.*, 2007; Khan *et al.*, 2008). Another approach to pest control involves the generation of genetically manipulated (GM) crops expressing the Cry family of insecticidal proteins originating from the bacteria *Bacillus thuringiensis* (Bt crops) (Brookes, 2008; Rao, 2008). Although successfully employed in major global crops such as maize, cotton and wheat, laboratory trials have observed insect resistance to this entomocidal toxin by evolving a physiological response to bypass toxin activity (Rao, 2008). Modern strategies to prevent the establishment of such resistance involve the use of directed evolution techniques. This process is essentially composed of two steps, whereby a library is created of individuals producing

mutant proteins, and a selection process to identify protein variants which offer the most suitable pesticidal properties (Arnold and Georgiou, 2003a, 2003b). These variants are created by protein fragmentation and reassembly from a particular gene sequence, or through the introduction of random base pair changes into the gene sequence by employing error-prone polymerase chain reaction (PCR) (Rao, 2008). These methods enable producers to reduce the risk of significant pest damage while also saving time and energy on performing field surveys and applying insecticides (Brookes, 2008). Additionally, it has been demonstrated that Bt maize suffers 5 – 10 fold less fungal damage from *Fusarium* infection and contains lower mycotoxin levels than non-Bt maize. These lower mycotoxin levels result in more acceptable and safer human food or animal feed products and so are considered superior to non-Bt maize and are thus more marketable (Brookes, 2008). Coupled with this, the health and safety for farmers is improved since they are exposed to fewer pesticides, which has also enhanced the populations of beneficial insect populations, while crop growing seasons have become shorter resulting in the production of two crops in one season (Brookes, 2008).

1.1.1.4. Inducing crop herbicide resistance

As with Bt crops, the increase in productivity of many crops can be attributed to the integration of heterologous proteins conferring resistance to specific herbicides including glyphosate, sulfonylurea, and imidazolinones (Rao, 2008). The ESP synthase gene originating from the glyphosate insensitive bacterium, *Agrobacterium tumefaciens*, has been employed to create glyphosate resistant crops. As with the development of pest and disease resistance, herbicide resistance has been previously demonstrated under laboratory conditions and therefore protein engineering, through directed evolution, has been employed to produce variants with superior properties to the parent ESP synthase enzymes (Rao, 2008). In rice (*Oryza sativa*), Zhou *et al.* (2006) isolated a mutant ESP synthase gene by error-prone PCR mutagenesis which conferred glyphosate tolerance in tobacco (*Nicotiana tabacum*). Eleven rounds of directed evolution were similarly employed on three glyphosate N-acetyltransferase (GAT) genes from *Bacillus licheniformis* and resulted in an enzyme which detoxified

glyphosate 10,000-fold more efficiently than the parental enzymes, but maintained 77% identity with the original amino acid sequence (Castle *et al.*, 2004). The cultural benefits of such crops primarily centre on an increased flexibility in herbicide applications. For conventional crops, control of post emergent weeds regularly involves herbicide applications to poorly established plants which often results in delayed crop maturity (Brookes, 2008). These issues are avoided with GM herbicide tolerant (HT) crops, which further facilitates the employment of no-tillage approaches which represent significant time and financial efficiencies with a reduction in labour, fuel and ploughing costs (Brookes, 2008). Due to their HT, risks associated with residual herbicides in soil for proceeding crops have been eradicated, while HT crops with fewer competing weeds have demonstrated a significant reduction in time to harvest, an improvement in harvest quality and an associated increase in financial reward (Brookes, 2008).

1.1.1.5. Reducing global malnutrition through crop improvement

1.1.1.5.1 Current status of global human nutrition

In order to remain healthy, humans require at least 22 mineral elements and 22 amino acids, of which 8 are essential and must be supplied by diet (Graham *et al.*, 2007; FAO, 2010; Bouis and Welch, 2010). Currently, however, 1 billion people suffer from caloric and protein malnutrition (FAO, 2010; Henley *et al.*, 2010). Supply of essential amino acids such as methionine, cysteine, histidine is particularly low in some staple crops which can result in reduced bioavailability and deficiencies of crucial nutrients such as Zn and Fe in the human diet (Graham *et al.*, 2007; Bouis and Welch, 2010). Additionally, amino acid deficiencies lead to reduced protein levels and emaciation in humans (Bouis and Welch, 2010).

A recent declaration by the World Summit on Food Security concluded that global food production must increase by 70% by 2050 in order to prevent global starvation (FAO, 2009). This represents a mean annual increase of 44 million metric tonnes (MMT) per annum and a 38% increase over production from conventional breeding strategies (Fig. 1.1.).

Such an intense scale of increased global food production is unparalleled, and will require revolutionary changes to current breeding strategies, agronomic processes and crop improvement techniques (White and Broadley, 2005, 2009; Beddington, 2010; Tester and Langridge, 2010).

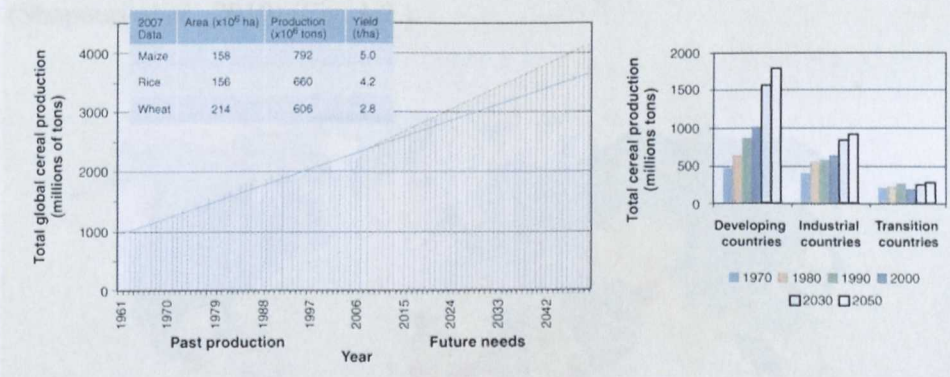


Fig. 1.1. Global cereal production (Left) has experienced an increase in production from 877 million metric tons in 1961 to 2351 million metric tons in 2007 (blue). This increase must continue to 4000 million metric tons (MMT) by 2050 (red) to achieve predicted global requirements. Thus the rate of yield increase must grow by 38% *i.e.* from 38 MMT (blue line) to 44 MMT (red dotted line) per annum to achieve this goal. The inset table shows 2007 FAO data for the three major cereals (Tester and Langridge, 2010). The appended graph (Right) illustrates that developing countries will have the greatest demand for future yield increases. Figure adapted from Tester and Langridge (2010).

The need for improved crop production had been made clearly apparent as recently as 2007 and 2008, where wheat and maize commodity prices rose three-fold above their 2005 levels, and those of rice rose further still (IMF, 2008). Moreover consumers were rapidly subjected to these increases, sparking civil unrest in developing and developed countries, including strikes in Italy and riots in Haiti (Beddington, 2010). Furthermore, growth in food prices increased global malnutrition by 75 million people in 2007, representing a regression to levels of the previous decade (Beddington, 2010). However, this situation was shown to improve slightly between 2009 and 2010, due to an economic recovery for 70 developing countries examined by the food security assessment (FSA), resulting in a decrease in the number of food-insecure people from 953 to 882 million (Shapouri *et al.*, 2010). Nevertheless, the report highlighted that food security remained precarious, and that just a brief economic slowdown or food production

shock could result in mass malnutrition and food insecurity. The report demonstrated that the population in Sub-Saharan Africa (SSA) represented <25% of all 70 developing countries assessed, however the region accounted for 44% of the total food-insecure people. Furthermore, evidence exists of hazardous dietary deficiencies in micronutrients (White and Broadley, 2009) and protein intake (Henley *et al.*, 2010) among populations in SSA with future predictions indicating that food insecurity in this region would further deteriorate after 2010 (Shapouri *et al.*, 2010) (Fig. 1.2.).

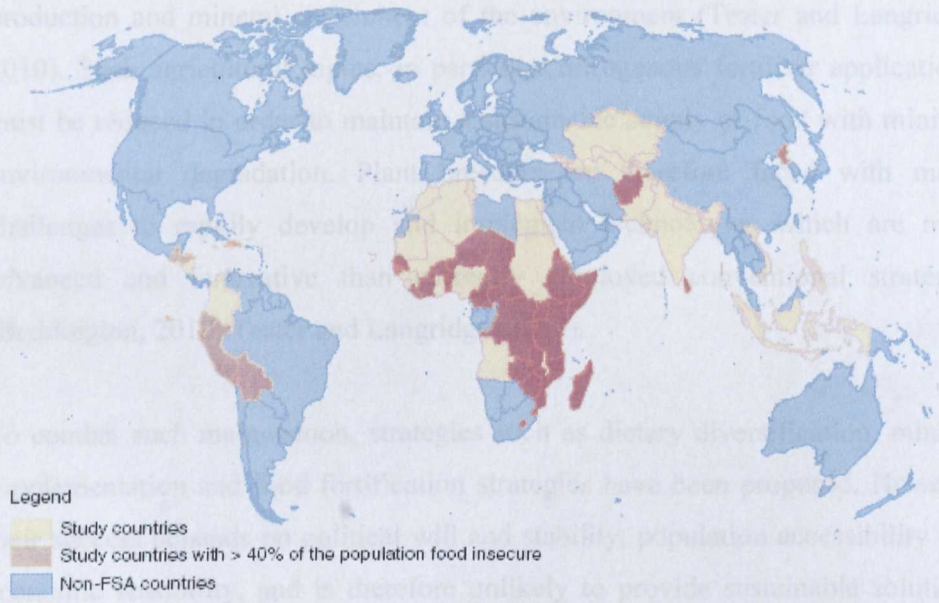


Fig. 1.2. Global map illustrating 70 developing countries which were assessed by the food security assessment (FSA). In 37 (out of 70) developing countries, over 40% of the population were estimated to be food insecure. Source: Shapouri *et al.*, 2010.

1.1.1.5.2 Sustainable approaches to alleviate malnutrition

In order to provide an adequate amount of high quality food to the rapidly expanding global human population, crops need to produce higher yields with increased nutrition content (White and Broadley, 2005, 2009; Beddington, 2010; Bouis and Welch, 2010; Tester and Langridge, 2010). It has already been observed that the global human population has exceeded the carrying capacity of traditional and low input agriculture (Graham *et al.*, 2007). It has therefore been necessary to apply expensive modern inorganic fertilisers to obtain current crop

yields to prevent global starvation (Graham *et al.*, 2007; White and Broadley, 2009). However, supplies of the majority of these essential mineral elements will become limiting in the future *e.g.* zinc (Zn) and copper (Cu) reserves are estimated at 480 and 940 million tonnes respectively (US Geological Survey, 2007) and their supply will become exhausted within 60 years at current rates of consumption (Cohen, 2007; Kesler, 2007). Coupled with this, mineral fertilisers must be applied regularly, and can be costly to manufacture, distribute and supply. Moreover, the manufacture and use of certain inorganic fertilisers results in significant environmental damage, including increases in greenhouse gas production and mineral enrichment of the environment (Tester and Langridge, 2010). Such agricultural inputs, in particular nitrogenous fertiliser applications, must be reduced in order to maintain a sustainable supply of food with minimal environmental degradation. Plant breeders are therefore faced with major challenges to rapidly develop and implement technologies which are more advanced and innovative than currently employed conventional strategies (Beddington, 2010; Tester and Langridge, 2010).

To combat such malnutrition, strategies such as dietary diversification, mineral supplementation and food fortification strategies have been proposed. However their success depends on political will and stability, population accessibility and economic feasibility, and is therefore unlikely to provide sustainable solutions for many impoverished developing countries (Bouis *et al.*, 2003; Mayer *et al.*, 2008; Bouis and Welch, 2010). Crop genetic biofortification through increasing the nutrient quality of staple crops, has been regarded by many as a more sustainable direct solution to human dietary malnutrition (Bouis *et al.*, 2003; Mayer *et al.*, 2008; Bouis and Welch, 2010).

White and Broadley (2005) defined biofortification as the process of increasing "the bioavailable concentrations of an element in edible portions of crop plants through agronomic intervention or genetic selection". Genetic selection can occur either through plant breeding methods, which involves selection following natural genetic variation within a species, or *via* genetic engineering approaches whereby a desired trait is introduced into the crop. Staple foods, which are heavily consumed by poorer populations, make ideal targets for biofortification

using genetic engineering methods. This approach has been successful in the past, with the production of ferritin-Fe enriched rice grain (Goto *et al.*, 1999) and β-carotene enriched 'golden rice' (Ye *et al.*, 2000). This system offers economic advantages, since the main expense lies towards the establishment of nutritionally enhanced lines which subsequently can be introduced into current agronomical systems with relatively little extra cost (Welch and Graham, 2002; Bouis *et al.*, 2003; Timmer, 2003; Stein *et al.*, 2007). The process of genetic selection using genetic modification strategies makes use of a wide range of laboratory-based tools and techniques, as detailed in Section 1.1.2.

1.1.2. Common techniques in plant biotechnology

1.1.2.1. Plant regeneration *via* tissue culture

Plant tissue culture encompasses techniques designed to maintain and grow explants such as cells, tissues, organs or even whole plants *in vitro* and under aseptic conditions. Tissue culture relies on the fact that plant cells are totipotent, *i.e.* each living cell has the ability to regenerate a whole plant (Sharma *et al.*, 2005). Whole plant regeneration, which is a prerequisite for most *in vitro* applications of biotechnology, can occur *via* two main morphogenic pathways, namely organogenesis and somatic embryogenesis (Jiménez, 2001; Phillips, 2004).

Both organogenesis and somatic embryogenesis can occur either directly or indirectly. The direct pathway is termed adventitious regeneration, and occurs without a transitional callus stage, while the indirect pathway, also known as the *de novo* pathway, takes place following an intermediate callus stage (Phillips, 2004).

1.1.2.1.1. Components of the tissue culture medium

The growth and development of explants *in vitro* are highly influenced by the composition of the culture medium. Many tissue culture media have been developed over the years to suit different types of explants of different species. A typical culture medium is composed of a carbon source, trace elements, inorganic

salts and organic compounds, as well as plant growth regulators such as auxins and cytokinins, at appropriate concentrations. One of the most commonly used basic tissue culture media is that prepared to the formulation of Murashige and Skoog (1962; MS), a 'high salt' medium because of its relatively high potassium and nitrogen content (Beyl, 1999).

The addition of plant growth regulators (PGRs) to culture media stimulate cell division and expansion and regulate shoot and root regeneration and development from cultured explants (Beyl, 1999). Auxins and cytokinins are two main classes of PGRs. Auxins are involved in developmental processes, such as apical dominance, cell elongation and root formation, while cytokinins play a role in cell division and shoot initiation and elongation *in vitro*. One of the most commonly used auxins in tissue culture experiments is the synthetic compound α-naphthaleneacetic acid (NAA), while a common synthetic cytokinin is N^6-benzylaminopurine (BAP).

1.1.2.1.2. Callus formation, organogenesis and somatic embryogenesis

Callus tissue is made up of a mass of unorganised and dedifferentiated parenchymatous cells which are actively dividing. Explant sources for the induction of calli include cotyledons, stems, leaves and immature embryos. During callus formation, the cells dedifferentiate both in morphology and in metabolism, and as such, lose their ability to photosynthesise. Extensive studies on several plants, ranging from food crops to ornamentals, have shown that an optimised culture medium which contains a carbon source, growth regulators, vitamins and minerals is necessary to maintain the healthy proliferation of callus cells (Haliloglu, 2002; Roy and Bannerjee, 2003). Callus lines are an important source of material in plant biotechnology, as the callus cells can be cultured on medium with growth regulators to redifferentiate and regenerate whole plants (Chang and Chang, 2000).

In vitro organogenesis is the formation of organs such as shoots, roots or flowers, from a cultured explant *e.g.* a leaf segment or a zygotic embryo. An important feature of this developmental process is the presence of vascular tissue which

connects the regenerating organ to the explant tissue (Jiménez, 2001). Culture conditions, particularly the type and concentration of growth regulators present in the medium, affect the type of organ formed (Jiménez, 2001). In direct organogenesis, where organs form without the presence of intervening callus, the cells that first form in response to hormones in the medium are relatively few in number. While they may be dedifferentiated at first, they quickly become meristematic and differentiate into the initials of new organs. During indirect organogenesis, the formation of callus first occurs, followed by the initiation of organ formation within the callus tissue.

Somatic embryogenesis is an asexual form of plant propagation that mimics many of the events occurring during the development of zygotic embryos (Jiménez, 2001; Vicient and Martínez, 1998). It refers to the initiation of embryos from previously differentiated somatic cells. Under defined conditions, almost all plant cells have the capacity to become embryogenic, a characteristic which relies on reprogramming of gene expression and triggers many structural changes similar to those found in normal, zygotic embryos (Jiménez, 2001). Since the discovery of somatic embryos in carrot (*Daucus carota*) cell cultures by Steward and co-workers (1958), the developmental process of somatic embryogenesis has been documented extensively (Kitamiya *et al.*, 2000).

Somatic embryos are bipolar structures with both root and shoot apices (Phillips, 2004) and develop without any vascular connection to the parent tissue (Jiménez, 2001). Stages in their development include the globular, heart and torpedo stages, similar to zygotic embryo development, and their *in vitro* formation can be induced from cultured explants by manipulating the culture conditions and medium compostion (Zimmerman, 1993).

1.1.2.2. Plant histology

Plant histology can be broadly defined as the study of the morphology of cells and their assembly into tissues and organs. Histological techniques are important in the study of the culture and regeneration of plants *in vitro* as they provide essential information about the developmental processes occurring in the parent

explants which lead to the regeneration of specific structures such as somatic embryos or adventitious shoots (Trigiano *et al.*, 1999).

A typical protocol for processing plant tissue sections for histological studies includes incubating the tissue in a fixative solution that will kill the cells but preserve the structure of the tissue, followed by dehydration of the tissue using a graded solvent series (*e.g.* ethanol). The next step is to replace the anhydrous solvent with a support medium such as molten paraplast or paraffin. This infiltration process is slow and can take several days. Once infiltration is complete, the support medium is solidified to encase the tissue section into a block, a process which is known as embedding (Ruzin, 1999).

The embedded tissue is then sectioned thinly using a microtome, and mounted on glass microscope slides which are preferably pre-coated with an adhesive such as polylysine to help the sectioned tissue adhere more firmly to the slide (Ruzin, 1999). The mounted section is then cleared with xylene or Histo-Clear (Fisher Scientific, Loughborough, UK) solution, to remove the embedding matrix, and the tissue is stained using chemical dyes such as methylene blue.

Once stained, the tissue section is protected from the external environment by mounting beneath a coverslip onto it. Coverslips can be permanently mounted onto slides using mounting cements such as Canada balsam or Histomount. The slide is now ready to be observed under the microscope, and can be stored for several months at room temperature without any damage to the structure of the tissue.

1.1.2.3. Marker-based systems for analysis of genetic variance

In any breeding programme, knowledge of genetic diversity is critical since any information about genetic variation in the germplasm could help breeders in sampling available collections and choosing better parents for the production of crops with desirable agronomic traits (Azam-Ali *et al.*, 2001). A traditional method of assessing genetic variation is *via* the use of morphological and physiological markers. More recently, molecular techniques have been developed

to study the correlation between genotype and phenotype. These techniques include the use of genetic markers to investigate the molecular variations within and among plant species and cultivars.

Genetic markers are DNA sequences which can be readily detected and whose genetic inheritance can be monitored (Newbury and Ford-Lloyd, 1999). Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers have been used successfully to construct molecular genetic maps for numerous plant species.

The development of the PCR-based marker system known as random amplified polymorphic DNA (RAPD) analysis offered scientists a cost- and time-effective approach to evaluate genetic variation in germplasm collections (Penner *et al.*, 1993). Single 10-mer oligonucleotide primers of arbitrary sequences are used in this system to amplify an informative number of DNA regions across the genomic DNA of the test parents (Williams *et al.*, 1990). The amplified PCR products obtained from each test sample are separated by electrophoresis on an ethidium bromide-stained agarose gel and compared. Any visible differences in the banding patterns are interpreted as polymorphisms in the genotype. These polymorphisms function as genetic markers. The incidence of polymorphisms can be due to several factors. Nucleotide insertions or deletions within the genome can modify the size of the amplified fragment, while some insertions can make the priming sites too far apart to support any possible amplification (Janick, 1992). Also, single base substitutions or deletions within the priming sites will not result in amplification, and hence no visible band. Sets of RAPD primers are commercially available at a relatively low cost as they are inexpensive to synthesise.

In contrast to RFLP markers, analysis of RAPD markers does not require DNA cloning, Southern blotting, or DNA hybridizations with labelled DNA probes; markers are simply scored from an ethidium bromide stained agarose gel following electrophoresis of the PCR amplification products. While RFLPs are frequently expressed as codominant genetic traits, allowing the identification of individuals heterozygous for the polymorphism, RAPDs are most often

expressed as dominant traits, and therefore cannot be used for the identification of heterozygotes.

1.1.2.4. Recombinant DNA technology and molecular cloning

One of the most important advances in molecular biology was the development of recombinant DNA (rDNA) technology, which greatly facilitated the study of genes. The technology involves the isolation of a gene of interest, and its insertion into a foreign DNA molecule which acts as a vehicle or vector that can be replicated in living cells. The result of the combination of these two DNA molecules of different origins is known as recombinant DNA. The recombinant DNA molecule is then placed into a host cell of either prokaryotic or eukaryotic origin, which upon replication, produces a clone carrying the recombinant DNA. Cloning is necessary to produce large amounts of the rDNA, which can then be purified for further study.

1.1.2.4.1. Restriction endonucleases and DNA ligase

Two major categories of enzymes are important in the isolation of genes of interest and the preparation of recombinant DNA. They are the restriction endonucleases and the DNA ligases. Restriction endonucleases recognise and cleave short nucleotide sequences known as restriction sites on a double-stranded DNA molecule. DNA ligase binds together pieces of DNA by forming phosphodiester bonds.

Restriction endonucleases, also known as restriction enzymes, are categorised into three major classes, namely Type I, Type II, and Type III, based on the three following characteristics, namely, the types of sequences that they recognise, the nature of the cut made in the DNA, and the enzyme structure (Pingoud, 2004). All three types of restriction enzymes can be further categorised into subtypes. Type I and Type III restriction enzymes cleave DNA at non-specific locations away from the recognition sites, thus producing random cleavage patterns. They are not useful in gene cloning experiments. The majority of Type II restriction enzymes are typically composed of two identical polypeptide subunits that join

together to form a homodimer. They recognise short and specific, usually palindromic DNA sequences of between 4 – 8 base pairs (bp) in length, and cleave both DNA strands at these specific sites only. Type II restriction enzymes are thus an important tool in molecular biology research. Examples of such Type II restriction enzymes are *Bam*HI, discovered in *Bacillus amyloliquefaciens* (strain H), and *Hin*dIII from *Haemophilus influenza* (strain d).

During restriction digestion, the restriction enzyme cuts double-stranded DNA by breaking the covalent phosphodiester bond between the phosphate group of one nucleotide and the sugar of the adjacent nucleotide, thus giving rise to free 5′-phosphate (PO_4) and 3′-hydroxyl (OH) ends (Pingoud, 2004). When cleaving DNA, Type II restriction enzymes can generate either one of two types of cuts, namely 'staggered' or 'blunt' cuts. A staggered cut occurs through a catalytic reaction whereby phosphodiester bonds in the palindromic DNA sequences are hydrolysed to produce single-stranded complementary nucleotide tails called 'sticky' or 'cohesive' ends. A blunt cut occurs when, through a similar catalytic reaction, the restriction enzyme cleaves both strands of the DNA at the same position to generate ends with no unpaired nucleotide.

DNA ligase is an enzyme which can essentially join together two pieces of double-stranded DNA *via* the catalytic formation of a covalent phosphodiester bond between the 5′-PO_4 terminus of one DNA fragment and the 3′-OH terminus of the other (Rossi *et al.*, 1997), to generate a circular DNA molecule. This process is known as ligation and takes place in the presence of ATP as a co-factor. One of the most widely used ligases is T4 DNA ligase, derived from bacteriophage T4. Its activity is ATP-dependent (Rossi *et al.*, 1997). T4 DNA ligase is an essential component of recombinant DNA technology, as it is extensively used to ligate restriction-digested DNA fragments carrying genes of interest into cloning vectors.

1.1.2.4.2. Cloning vectors

Cloning vectors are circular double-stranded DNA molecules designed as carriers for recombinant DNA molecules containing genes of interest. They contain several unique restriction sites that occur only once in the vector, and can independently replicate themselves, as well as the foreign DNA fragments that they carry (Brown, 2006). Cloning vectors also carry a selectable marker gene, usually in the form of an antibiotic resistance gene, to allow for the easy selection of host cells that have successfully been transformed with the vector. A large number of cloning vectors have been successfully created for use in the bacterial host *E. coli*.

The different types of cloning vectors currently available to the molecular scientist include plasmids, phages and cosmids. These vectors differ in the size of insert that they can accommodate, with plasmids being able to take up a DNA fragment of up to 10 kb in length. Phages (*e.g.* bacteriophage λ) and cosmids (a plasmid carrying a phage λ *cos* site) can take up a maximum of 20 kb and 45 kb of foreign DNA respectively.

Plasmids are extrachromosomal double-stranded circular DNA molecules which carry an origin of replication. They are naturally-occurring and replicate independently within bacterial cells. Plasmids are named with a system of uppercase letters and numbers, where the lowercase 'p' stands for 'plasmid'. For example, in the case of pBR322, the letters 'BR' identify the original creators of the vector, Bolivar and Rodriguez, and '322' represents the identification number of the specific plasmid (Brown, 2006). Plasmid vectors are generally modified to carry a selective antibiotic resistance gene, as well as a multiple cloning site (MCS) which contains a number of unique restriction sites. The MCS is usually targeted for the insertion of foreign DNA to produce a recombinant plasmid DNA molecule.

1.1.2.4.3. Reporter genes

Reporter genes are nucleic acid sequences which encode for easily assayed protein activity (Drake, 1996). Often fused with other marker genes and introduced into plasmid vectors, they are used in plant transformation systems to facilitate the selection of transformed tissue.

An extensively used reporter gene system is the β-glucuronidase (*uidA*) gene reporter system, developed by Jefferson *et al.* (1987). The *uidA* gene, isolated from the bacterium *E. coli* (Jefferson *et al.*, 1987; Papadopoulou *et al.* 2005), encodes the β-glucuronidase (GUS) enzyme. Putatively transformed plant tissues which have incorporated the *uidA* gene can thus be assayed for GUS enzyme activity to confirm the presence of the transgene in the cells.

1.1.2.4.4. Selectable marker genes

Since the transformation efficiency for many crop plants is usually relatively low, the use of selectable marker genes is necessary to screen for and select transformed plants (Puchta, 2003). Selectable marker genes are nucleic acid sequences which are introduced into plasmid vectors *via* recombinant-DNA technology. Following plant transformation, their expression in putatively transformed tissues provides an effective screening and selection system.

The neomycin phosphotransferase II (*npt*II) gene is one such selectable marker gene, originally isolated from *E. coli* as a component of transposon Tn5 (Beck *et al.*, 1982; Jelenić, 2003). It confers resistance to the aminoglycoside antibiotics neomycin and kanamycin (Miki and McHugh, 2004). During plant transformation, cells which have incorporated the *npt*II gene become resistant to these antibiotics. They will survive on media supplemented with either kanamycin or neomycin and can therefore be selected as transgenics.

1.1.2.4.5. Construction of recombinant plasmid DNA

The construction of a recombinant plasmid DNA molecule carrying a gene of interest begins with the cleavage of the circular plasmid vector with a restriction enzyme. This results in a single cut, creating a linearised plamid. A foreign DNA molecule carrying the gene of interest is also cut with the same enzyme. This DNA molecule, known as the insert, can then be joined to the linearised vector in a ligation reaction, to produce a circular recombinant plasmid DNA. In reactions where only one restriction enzyme is used, ligation reactions are not 100% effective, because the two ends of the plasmid vector can be easily ligated back in a process known as self-ligation. Treating the linearised vector with the enzyme phosphatase before the ligation step decreases the occurrence of self-ligation, as the enzyme removes the terminal 5′-phosphate from the vector, making it impossible for the vector to form a phosphodiester bond and recircularise in the presence of ligase. The vector can thus ligate only to the 5′-phosphate group of the insert to become circular again.

Another strategy involves the use of two restriction enzymes to cleave both the vector and the insert DNA. This generates non-complementary sticky ends on both the vector and the insert, thus inhibiting self-ligation and promoting the ligation of the insert to the vector in the correct orientation, to produce the recombinant plasmid.

1.1.2.4.6. Bacterial host transformation and recombinant selection

The next step in a gene cloning experiment is to introduce the recombinant plasmid into a living host, usually bacteria, which rapidly grow and replicate to produce clones. This allows for a large number of the recombinant DNA molecules to be produced from a limited amount of starting material (Brown, 2006). One of the most common bacteria strains used for transformation is *E. coli* DH5α. This mutant strain has no restriction-modification system, and therefore cannot degrade foreign DNA following transformation. The traditional transformation method involves incubating the bacterial cells in a concentrated calcium chloride solution to increase the permeability of the cell membrane,

making the cells 'competent' (Hanahan *et al.*, 1995). To this day, it is not completely understood how this $CaCl_2$ treatment works (Brown, 2006).

Following this treatment, the recombinant plasmid is added to the competent cells, and the mixture is kept on ice. It is believed that the $CaCl_2$ in the solution allows the bacterial membrane to become more receptive to the recombinant plasmid DNA (Brown, 2006). At this stage, the DNA becomes loosely associated to the bacterial membrane, but is not being transported into the cell. The actual movement of DNA into the competent cells is facilitated by subjecting the mix to a heat-shock at the higher temperature of 42°C for 2 min.

The competent cells are then plated onto semi-solid medium containing a selection agent, to isolate cells that have successfully incorporated the recombinant plasmid. The recombinant plasmid would have been designed to carry a selectable marker gene which codes for an antibiotic such as ampicillin or kanamycin. Each bacterial cell which has been successfully transformed will grow on the selective medium to produce a single colony. Individual colonies can be tested for successful plasmid integration through PCR analysis *via* a process called colony PCR. Colonies which test positive are then grown in large volumes of liquid medium to recover the cloned DNA through purification methods. Glycerol stocks can also be produced for long term storage of the transformed bacteria at this stage, and the stocks stored at -80°C.

The purified recombinant plasmid DNA is now available in sufficient amounts to be used in plant transformation experiments.

1.1.2.5. Plant genetic transformation

A common technique in molecular biology, transformation is the genetic modification of a cell, which results from the introduction, uptake and expression of foreign genetic material. Plant transformation is an important research tool in biology, as well as a practical tool in modern agriculture. The successful production of transgenic plants is dependent on the smooth integration of

techniques such as gene transfer, transgene expression strategies, selection of transgenics and tissue culture systems (Birch, 1997).

The methods of gene delivery which have been developed for plant transformation and which are used routinely today are numerous and can be biological or non-biological (Darbani *et al.*, 2008). Biological systems are based on naturally-occurring infectious agents such as bacteria and viruses, while non-biological systems use chemical or mechanical methods to promote DNA uptake into plant cells (Darbani *et al.*, 2008).

1.1.2.5.1. Biolistic particle delivery

Biolistic particle delivery, also known as particle bombardment, is a mechanical procedure whereby microparticles coated with DNA are accelerated to a high velocity to penetrate different plant and animal cell types (Rao *et al.*, 2009). The DNA elutes off the particles that lodge inside the cells, and there exists a possibility that it then gets stably incorporated in the host chromosomes, thus producing transformed cells (Kikkert *et al.*, 2004). It has been reported that transgene expression only occurred in bombarded cells where the microparticles were delivered directly inside the nucleus of the targeted cells (Yamashita *et al.*, 1991; Hunold *et al.*, 1994). The efficiency of biolistic-mediated transformation is therefore heavily dependent on the number of microparticles which get lodged in the cell nucleus following bombardment. Hunold *et al.* (1994) reported that stable transgene integration was efficient in tobacco cells which had received microparticles directly in their nucleus and which survived the following 48 h.

The most widely used device for plant transformation is the Biolistic® PDS-1000/He Particle Delivery System marketed by Bio-Rad Laboratories. This system employs high-pressure helium released by a rupture disc to propel a macrocarrier sheet loaded with DNA-coated metal particles (microcarriers) towards target cells. A stopping screen halts the macrocarrier, and the microcarriers continue towards the target and penetrate the cells.

Because of its physical nature and simple methodology, the biolistic process can be used to deliver substances into a wide range of intact cells and tissues from a diversity of organisms. In plant research, the major applications have been transient gene expression studies, production of genetically transformed plants, and inoculation of plants with viral pathogens.

1.1.2.5.2. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation is one of the most extensively employed methods in plant genetic modification systems (Rakoczy-Trojanowska, 2002). In nature, *Agrobacterium tumefaciens*, a common soil bacterium, causes crown gall disease by transferring some of the DNA from its tumour inducing (Ti) plasmid into the plant host.

Agrobacterium-mediated transformation is an indirect transformation method which involves introducing genes of interest into the bacterial Ti plasmid, and using the bacterium to harmlessly insert the desired gene, *via* the plasmid vector, into the plant genome. It is usually carried out by inoculating aseptically cultured plant material with *Agrobacterium*, co-culturing the inoculated explant on regeneration medium for 2 – 3 days, and subsequently subculturing the regenerated callus and/or shoots onto medium containing antibiotics, to select for putative transgenics (Drake, 1996).

Studies on the mechanism of T-DNA transfer have shown that several genetic elements are essential to make up the *Agrobacterium* transfer machinery (Veluthambi *et al.*, 2003). They are the *Agrobacterium* chromosomal virulence (*chv*) genes, the T-DNA itself, which has its integration site flanked by 25bp imperfect repeats (known as the right and left borders), and the plasmid virulence (*vir*) genes, found on a *vir* region (Hellens *et al.*, 2000, Veluthambi *et al.*, 2003). The removal of the oncogenic region within the T-DNA was found to disarm the bacteria, without disturbing its mechanism of transfer. This, together with the discovery that the physical separation of the T-DNA and virulence regions onto different plasmids does not affect T-DNA transfer provided both plasmids are

present in the same *Agrobacterium* cell, formed the basis for the development of binary Ti plasmid vectors (Hellens *et al.*, 2000).

Agrobacterium-mediated transformation has several advantages. It is widely available and hence cost effective. It also allows for a precise and linked transfer of genes into the plant genome, a stable integration of relatively long (>150kb) stretches of T-DNA, a higher frequency of stable transformation with single copy insertions, as well as a relatively low incidence of transgene silencing (Veluthambi *et al.*, 2003).

1.1.2.6. Molecular characterisation tools

Following explant transformation, the regenerated callus and/or shoots which survive on selective medium are designated as putative transgenics. Several molecular techniques are available to confirm that those tissues have, in fact, been successfully transformed.

1.1.2.6.1. Histochemical GUS assay

In plant transformation systems which use *uidA* as a reporter gene (Section 1.1.2.2.), the β-glucuronidase (GUS) enzyme activity in putatively transformed tissue can be assayed histochemically and visualized using the cyclohexylammonium X-Gluc (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid) salt as substrate, which yields an insoluble blue precipitate in the presence of β-glucuronidase (Leydecker *et al.*, 2000).

The histochemical GUS assay is a popular molecular tool in the analysis of plant gene expression because it is a simple, rapid and sensitive assay (Kirby and Kavanagh, 2002).

1.1.2.6.2. Polymerase chain reaction analysis

Polymerase chain reaction (PCR) is a molecular technique designed to exponentially amplify specific DNA segments *via* enzymatic action. It is an

important tool in plant biotechnology, as it is used to detect and confirm the presence of transgenes in putatively transformed tissues.

A typical reaction tube will contain extracted genomic DNA (from plant tissue) containing the segment to be amplified (usually a reporter or selectable marker gene segment), DNA polymerase, oligonucleotide primers, a mixture of deoxynucleotide triphosphates (dNTPs), and a buffer solution containing magnesium. The amplification reaction is carried out in a thermocycler, where the reaction mixture is subjected to a number of replication cycles. Each cycle consists of a denaturation phase, during which the DNA becomes single-stranded; an annealing phase, during which the primers bind to complementary sequences on either side of the target sequence, and an extension phase, when the polymerase uses the original DNA strands as templates to synthesise two new strands of DNA. The number of the target DNA sequence doubles following each amplification cycle.

PCR products are usually electrophoresed on agarose gels containing ethidium bromide to separate the amplified DNA strands from the rest of the mixture on the basis of size and electric charge. Ethidium bromide binds to DNA and fluoresces under UV light, thus making it possible to determine the size of the amplified DNA band against a standard DNA ladder of known sizes which is usually run in a well flanking those containing the PCR products.

Conventional PCR methods, however, can only explain the presence of transgenes in putatively transformed plants, but not gene integration. Inverse PCR (iPCR) is a technique that can be used to confirm T-DNA integration in the host cell genome *via* the amplification of junction fragments (*i.e.* the plant genomic DNA sequences which flank the known T-DNA sequences) (Does *et al.*, 1991). This technique involves the restriction digestion of target DNA, followed by circularisation of the restriction fragments and finally amplification using primers that direct DNA synthesis away from the core region of the known T-DNA sequence (*i.e.* in the opposite direction of primers used in a conventional PCR reaction) (Triglia *et al.*, 1988; Jong *et al.*, 2002). Following gel

electrophoresis, the presence of amplified bands corresponding to the expected DNA fragment size confirms the integration of T-DNA within the host genome.

1.1.2.6.3. Reverse-transcription PCR

A technique known as reverse-transcription PCR (RT-PCR) is often employed to investigate genomic DNA transcription into messenger RNA (mRNA), thus confirming gene expression. In this method, a reverse transcriptase enzyme converts mRNA into complementary DNA (cDNA). The cDNA is then amplified by PCR and the products analysed by gel electrophoresis. The presence of bands corresponding to the correct size confirms that the gene of interest has been integrated into the plant genome and is being expressed at the transcription level.

1.1.2.6.4. SDS-PAGE and western blotting

To confirm transgene expression at the translational level, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting are popular detection methods. Total protein is typically collected from tissue samples in the form of a lysate and subjected to SDS-PAGE in order to separate the proteins by their molecular weight. SDS-PAGE is a powerful technique for resolving protein mixtures. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures the secondary and non–disulphide–linked tertiary structures of proteins, while also applying a negative charge to each protein in proportion to its mass. By unfolding the protein and giving a near uniform negative charge along the length of the polypeptide, SDS treatment therefore eliminates the effects of differences in shape so that chain length, which reflects mass, is the sole determinant of the migration rate of proteins in SDS-polyacrylamide electrophoresis.

Western blotting involves the detection of a target protein by using monoclonal or polyclonal antibodies which specifically bind to the target protein. Following electrophoresis, the separated protein bands are transferred from the gel onto a solid supporting membrane [*e.g.* nitrocellulose or polyvinylidene difluoride

(PVDF) membrane] in order to make the proteins more accessible to antibody detection.

After an initial blocking step with non-fat dry milk to reduce non-specific binding of antibodies, the membrane is washed and incubated in a buffer solution containing the primary antibody. The primary antibody is specific for the protein of interest, and when used at the correct concentrations, should not bind to other proteins on the membrane. Following this incubation, the membrane is washed to remove any unbound primary antibody, and incubated in a buffer solution containing a secondary antibody. The secondary antibody binds to the primary antibody and creates a protein-antibody-antibody sandwich. The secondary antibody also typically carries a horse radish peroxidase enzyme which converts a luminol substrate to a light releasing substance. After this incubation, the membrane is washed once more, and developed in a buffer solution containing the luminol substrate. Bands corresponding to the detected protein of interest will appear as dark regions on the developed film. Band densities in different lanes can also be compared to extract information on the relative abundance of the protein of interest.

1.2. THE LEGUMINOSAE

Members of the family Leguminosae (Fabaceae), commonly known as legumes, make up the third largest family of flowering plants, with an estimated 20,000 species classified into 727 genera (Cronk *et al.*, 2006). The taxonomy of legumes is primarily based on morphological features and consists of 3 major subfamilies, namely the Papilionoideae, the Cesalpinioideae and the Mimosoideae. The Papilionoideae is the largest of the subfamilies, with 476 genera and approximately 14000 species, most of which are herbaceous (Singh *et al.*, 2007).

Broadly characterised by their unusual flower structure, podded fruit, and ability to nodulate in the presence rhizobia, legumes include important grain, and forage crops, as well as ornamental, medicinal and agroforestry species (Singh *et al.*, 2007). Legumes are economically important for several reasons. Cultivated grain and forage legumes occupy between 12% – 15% of the earth's arable land

(Graham and Vance, 2003). Grain legumes are second only to cereals in food crop production (Cronk, 2006). They are a rich source of dietary proteins and account for more than 35% of the world's processed vegetable oil (Graham and Vance, 2003). Major cultivated grain legumes include cowpea (*Vigna unguiculata* (L.) Walp), chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.), common bean (*Phaseolus vulgaris* L.), groundnut (*Arachis hypogea* L.) and soybean (*Glycine max* (L.) Merr). Forage legumes are an important source of animal fodder since they provide a cheap and readily available supply of high quality protein necessary for the healthy development of farmed monogastric and ruminant animals. Examples of important forage legumes include alfalfa (*Medicago sativa* L.), bird's foot trefoil (*Lotus corniculatus* L.) and clover (*Trifolium pratense* L.). Lesser known, but still economically important legume crops include fenugreek (*Trigonella foenum-graecum*), and grasspea (*Lathyrus sativus* L.; Singh *et al.*, 2007; Lambein *et al.*, 2008). Recent investigations have highlighted the possible medicinal properties of fenugreek. Amin and co-workers (2005) reported that fenugreek extracts may possess chemopreventive properties against breast cancer. Fenugreek also contains the alkaloid trigonelline, which has been suggested to support neuronal network regeneration in human cells (Tohda *et al*, 2005). Grasspea is a grain legume whose agronomic properties make it the most affordable pulse in Bangladesh, as it is relatively more tolerant towards prolonged drought conditions, poor soils and other biotic and abiotic stresses than other legumes (Lambein *et al.*, 2008).

Nitrogen is a macronutrient essential to all plants for their growth and development, and is used in the form of nitrogen-containing compounds mainly for the synthesis of amino acids, proteins and other bio-organic compounds. Plants normally use nitrogen in its oxidised form as nitrate (NO_3^-) ions, or in its reduced form as ammonium (NH_4^+) ions. Most plants are not adapted to produce nitrate and ammonium ions by themselves and, therefore, these ions are supplied to them in the form of fertilisers. Legumes, however, can circumvent this need for the nitrogen-containing fertilisers since they are adapted to form a symbiotic relationship with nitrogen-fixing bacteria such as Rhizobia, a type of gram-negative soil bacteria (Gonzales *et al.*, 2005; España *et al.*, 2006), to directly reduce atmospheric nitrogen into ammonia (Oldroyd and Downie, 2004).

The establishment of symbiosis is highly host-specific. Briefly, plants secrete compounds known as flavonoids or isoflavonoids, which are then detected by compatible bacterial strains. Bacterial NodD proteins become activated, and result in the activation of nodulation (*nod*) genes. *nod* genes encode proteins which synthesise lipochito-oligosaccharides called Nod factors (NFs), which are responsible for many of the early events in the root infection process (Chang *et al.*, 2009). Host plant recognition of NFs causes the plant root hair to curl and trap the rhizobial bacteria. The plant cell wall degrades and the cell membrane invaginates to initiate an intracellular tubular structure called the infection thread. The bacteria then induce root cortical cell division *via* cell reprogramming which results in the establishment of a nodule primordium. The infection thread grows towards the root cortex, and once it reaches the cells of the nodule primordium, the bacteria are released *via* endocytosis. Inside the cell, the bacteria differentiate into bacteroids and are enclosed within vacuole-like structures known as symbiosomes, where they convert N_2 to NH_4^+, the conjugate acid of ammonia (NH_3). Ammonium ions provide the plants with the form of nitrogen that they require to synthesise important compounds, such as amino acids and nucleic acids. This symbiotic relationship greatly reduces the need for the application of nitrogen-based fertilisers to agriculturally important legume crops, and also allows the crops to thrive on poor soils (Stacey *et al.*, 2006; Chang *et al.*, 2009).

Legumes are cultivated extensively in areas with semi-arid and humid climates, where extreme conditions such as inconsistent rainfall and long dry seasons prevail. Besides making up the bulk of the staple foods that feed tens of millions of people daily and being a nutritious source of forage for livestock, they also protect sloping lands against erosion due to their deep rooting system. However, legume productivity and adaptability can be hampered by extreme abiotic stresses such as drought and waterlogging (Dita *et al.*, 2006). This can make the plants vulnerable towards pests and diseases, and can cause significant losses in yield (Dita *et al.*, 2006). Also, despite the fact that legumes are a rich source of dietary protein, they are generally deficient in their content of sulphur-containing amino acids cysteine and methionine (Lambein *et al.*, 2008). These shortcomings

highlight the constant need for the development of research programmes with a focus on the genetic improvement of legumes.

1.2.1. Model legume species

Certain developmental processes in legumes (*e.g.* nodulation) cannot be studied and understood using *Arabidopsis thaliana* as a model plant. Therefore, over the past two decades, scientists have begun to search for a legume species that could replace *A. thaliana* and become the model plant for the genetic study of the Leguminosae. Some of the best characterised legumes are soybean (*Glycine max*), pea (*Pisum sativum*) and alfalfa (*Medicago sativa*), but these could not be chosen, as they either have genomes which are too large, exhibit complex ploidy, or are difficult to genetically transform and regenerate *in vitro* (Cook *et al.*,1997; Cook, 1999). Most of the major economically important crop legumes, including the ones mentioned above, belong to the subfamily Papilionoideae, and fall into two clades, namely Galegoid and Phaseoloid (Zhu *et al.*, 2005). Studies based on genome comparative mapping have also revealed that members of the Papilionoideae subfamily showed extensive genome conservation (Zhu *et al.*, 2005)

Following the logic behind the selection of *A. thaliana* as the model plant, the two legume species selected as model plants to study legume biology and genomics were *Medicago truncatula* (n=8, ~ 500Mb) and *Lotus japonicus* (n=6, 472.1Mb), both belonging to the Galegoid clade. They are diploid species with short life-cycles (2-3 months), are amenable to forward and reverse genetics and have genomes that are only three to four times bigger than the *Arabidopsis* genome (Cook *et al.*, 1997; Zhu *et al.* 2005; Sato *et al.*, 2010). Investigations in the study of *M. truncatula* and *L. japonicus* were directed at different facets of legume biology. Initial studies focused on the identification of genes involved in the nodulation process (Sagan *et al.*, 1995; Kapranov *et al.*, 1997). Genetic transformation of *M. truncatula* was achieved *via Agrobacterium*-mediated and vacuum infiltration methods (Cook, 1999). In the late 1990s, the generation of genomic data began, and tools for map-based cloning were developed, including the construction of bacterial artificial chromosomes (BACs) and expressed

sequence tag (EST) libraries, and the production of detailed genetic maps (Cook,, 1999; Young *et al.*, 2005; Sato *et al.*, 2007). By 2005, genome sequencing projects for both model plants were progressing satisfactorily, with 122Mb of completed sequence available between the two sequences (Young *et al.*, 2005). In 2006, following the development of new technologies, a project to sequence the entire soybean genome (approximately 1.1Gb) using a method known as shotgun sequencing was undertaken by the Department of Energy (DOE) Joint Genome Institute in the USA (Schmutz *et al.*, 2010). In February 2011, Sanchez and co-workers reported that the soybean, *M. truncatula* and *L. japonicus* genomes have been sequenced.

An important role of model plants is to serve as a source of accumulated knowledge to be transferred and applied to other plant species. Publicly available genomic data is a key resource in comparative studies investigating genome-scale synteny among plant species. Synteny refers to the conservation of co-localised genes on choromosomes of different species. Macrosynteny is the conservation of common DNA markers or large homologous sequences between species, while microsynteny is the conservation of smaller gene content and order over a short and physically defined DNA contig (Zhu *et al.*, 2005).

Legume macrosynteny was investigated in *M. truncatula*, alfalfa, pea, mungbean and chickpea, using comparative genomic approaches which revealed that synteny is correlated to the phylogenetic distance between the species (Zhu *et al.*, 2005). There was an almost perfect synteny between the genomes of *M. truncatula* and alfalfa, with highly conserved nucleotide sequences between the two species. On the other hand, approximately 76% of the markers mapped between *M. truncatula* and mungbean confirmed the presence of conserved gene order, suggesting that synteny was limited to small genetic intervals between more distantly related legumes (Zhu *et al.*, 2005).

The level of microsynteny between *M. truncatula* and soybean was investigated, as reported by Young and co-workers (2005), by comparing two genomic sequences (approx. 1Mb) from soybean with the *M. truncatula* genome. The analysis revealed syntetic regions which carried approximately 500 predicted

genes, and that 75% of the soybean genes were collinear with their *M. truncatula* homologs (Young *et al.*, 2005).

Model legume crops are of great importance in translational genomic studies, where information gathered from available genomic databases is applied towards crop improvement using genome-based technologies. The tetraploid and out-crossing forage crop alfalfa is susceptible to a disease known as anthracnose, caused by the fungal pathogen *Colletotrichum trifolii*. Alfalfa is generally recalcitrant to genetic manipulation, making research towards producing anthracnose-resistant alfalfa a tedious and time-consuming task. Since alfalfa and *M. truncatula* share a high level of macrosynteny, the genomic data available from *M. truncatula* can be exploited towards alfalfa crop improvement. Yang and co-workers (2008) reported that map-based cloning was successfully employed to clone *RCT1*, an anthracnose-resistant gene from *M. truncatula* and that the transfer of this gene to disease-susceptible alfalfa plants conferred broad-range resistance to anthracnose. Knowledge accumulated from model plants can thus be applied towards crop improvement for the study and development of agronomically important traits.

1.2.2. Legume seed crops

Legume seed crops, also known as grain legumes, are an important component of food systems throughout the world. Grain legumes contain reserves of proteins, lipids (in the form of triacylglycerols) and carbohydrates (in the form of starch) (Boulter, 1982; Gallardo *et al.*, 2008). Agronomically, grain legumes are considered important rotational crops because they have the ability to fix nitrogen, reduce soil pathogens and produce nutritionally valuable seed for human and livestock consumption.

During their development and maturation, legume seeds accumulate reserves of proteins, oils and carbohydrates for use in the initial stages of seedling germination and establishment (Gallardo *et al.*, 2008). The largest component of those reserves is made up of storage proteins, which accumulate in the cotyledonary parenchyma cells in membrane-bound organelles known as storage

vacuoles or protein bodies (Duranti, 2006). The dry weight protein content of legume seeds can range from 20% in peas and beans to as much as 40% in soybean and lupin, making legume seed crops one of the richest sources of food protein in the world (Duranti, 2006; Gallardo *et al.*, 2008). Based upon the extraction and solubility of the proteins in water or other solvents, the majority of seed storage proteins can be grouped into three classes, namely the albumins (soluble in water), globulins (soluble in salt/water solutions) and prolamins (soluble in ethanol/water solutions) (Shewry *et al.*, 1995; Duranti, 2006). The globulins are the most abundant fraction in legume seeds, while the prolamins are most abundant in cereals. Globulins are further classified as 7S (vicilin-like) and 11S (legumin-like) globulins, based on their sedimentation coefficient (S) (Duranti, 2006). Another important, but less abundant, class of proteins in grain legumes are the 2S albumins, defined by their low molecular weight, a sedimentation coefficient of approximately 2 and their compact globular structures with conserved cysteine residues (Shewry *et al.*, 1995; Moreno and Clemente, 2008). Investigations into the amino acid composition of 2S albumins revealed their relatively high content of the sulphur-containing amino acids cysteine and methionine (Youle and Huang, 1981; Moreno and Clemente, 2008). While grain legumes are generally deficient in their cysteine, methionine and tryptophan amino acid content (the latter two being essential amino acids), they contain relatively high levels of lysine and threonine, essential amino acids which are normally lacking in cereals. On a nutritional level, grain legumes and cereal crops complement each other in terms of dietary requirements. When consumed together as part of a balanced diet, they provide a good source of essential amino acids and minerals (Duranti, 2006; Lambein *et al.*, 2008). However, this type of balanced diet is not readily available to all parts of the world, and efforts are currently under way to investigate strategies to improve the nutritional qualities of legume and cereal crops individually, so that each crop type is eventually self-sufficient in its essential amino acid content.

Recent scientific studies have aimed at improving legume seed crops in terms of their nutritional value, and resistance to disease and environmental stresses. There has been a focus on the application of plant tissue culture and transformation technologies to improve important agronomic characteristics of

several legume species of economic importance including soybean (Dang and Wei, 2007) and chickpea (Chakraborti *et al.* 2009). Modern technologies such mutagenesis has been employed to generate nematode-resistant soybean cultivars in Japan (Singh and Hymowitz, 1999). Transcriptional profiling has been carried out in several chickpea genotypes to identify genes which are responsible for cold tolerance and which could potentially be used as candidate genes in future genetic transformation studies (Mantri *et al.*, 2007). This will benefit chickpea productivity in Australia, currently the largest exporter of this pulse (Mantri *et al.*, 2007), since the plant is not well adapted as a winter annual and cannot set pods under cool conditions. Most of these efforts, however, have been directed at legume crops cultivated on a large scale, with an already well-established breeding history. There is an untapped resource of legume crops with comparable agronomical and nutritional qualities (particularly in their areas of cultivation) which do not receive the same level of attention from the scientific community. They are known as underutilised legumes, and are further discussed in Section 1.2.3.

1.2.3. Underutilised legume crops

Although nutritionally important legume crops such as soybean, cowpea and chickpea are cultivated on a large scale worldwide, the poorer parts of the developing world are still struggling to source and supply nutritious and wholesome food to expanding populations. Protein-energy malnutrition is a major problem currently affecting populations with a starch-based diet (Sridhar and Bhat, 2007; Bhat and Karim, 2009). Underutilised or neglected crops are crops which have attracted relatively little attention from scientific communities and funding organisations across the world, and as a consequence, the potential for these crops to become part of sustainable agricultural systems has not been fully exploited. Many of these neglected species have adapted to grow in stressed environmental conditions such as salt-affected or arid areas. The nutritional qualities of underutilised legumes are comparable to those of major cultivated crops, and they contain adequate levels of proteins, essential amino acids, polyunsaturated fatty acids, dietary fibre as well as vitamins and minerals (Bhat and Karim, 2009). Underutilised crops are cost-effective alternatives to

importing nutritionally comparable food cultivated and produced abroad, and have the potential to become an important component of a nutritionally well-balanced diet, especially in their areas of cultivation.

Recent studies investigating several underutilised crops from India, Africa and China have unearthed valuable information about the nutritional properties and functional aspects of the crops. Examples of such crops include African yam bean (*Sphenostylis stenocarpa* Hochst.), and velvet bean (*Mucuna* sp.). African yam bean is a legume which has adapted to grow in acidic and leached sandy soil, and is cultivated in parts of central eastern and western Africa for its seeds and tubers (Aletor and Aladetimi, 1989; Bhat and Karim, 2009). The nutritional qualities of the seeds were found to be comparable to soybean, with lysine and methionine levels of 9.28g/ 16g N and 1.16g /16g N respectively (Bhat and Karim, 2009). The seeds also contain high levels of crude protein (21% - 29%) and carbohydrates (50%) (Eromosele *et al.*, 2008). A study by Machuka and Okeola (2000) into the analysis of seed protein extracts of *Mucuna* revealed that, following one- and two-dimensional polyacrylamide gel electrophoresis, N-terminal sequencing of resolved polypeptide bands identified several proteins which shared homology with characterised legume proteins such as mung bean seed albumin, pea α-fucosidase and soybean Kunitz-type trypsin inhibitor. The authors suggested that this shared homology might favour the crop in terms of becoming part of sustainable agricultural systems.

Velvet bean (*Mucuna* spp.) is a legume grown on a small scale by tribal groups in parts of Asia and Africa for their edible dried beans. Nearly 100 varieties of the crop exist, but only a few of them (including *M. gigantean*, *M. pruriens* and *M. solanei*) have been studied (Bhat and Karim, 2009). Although seed composition was found to vary among the varieties, *Mucuna* seeds are nutritionally comparable to common edible legumes, in terms of crude protein content (20g to 31.44g/ 100g), lipid content (2.8% to 14.39%) and lysine content (327 and 412mg/ g N) (Siddhuraju and Becker, 2005; Bhat and Karim, 2009). *Mucuna* seeds are, however, deficient in sulphur-containing amino acids, with levels ranging between 116 to 132 mg/g N (Josephine and Janardhanan, 1992). Added to these nutritional qualities, *Mucuna* seeds also carry antioxidant

properties (Tripathi and Upadhyay, 2001) and produce a therapeutic compound known as levodopa (L-DOPA), which has been investigated with moderate success in the treatment of Parkinson's disease (Nagashyana *et al.*, 2000; Katzenschlager *et al.*, 2004).

There are several factors which hamper any research undertaken to investigate the improvement of underutilised crops and their sustainable conservation. They include limited information, often available in rarely spoken local languages, on the basic developmental aspects of the crops, as well as scattered information on their genetic background and available germplasm. The application of biotechnology in the improvement of underutilised crops therefore holds potential benefits. Numerous positive outcomes can be achieved, including shortening the breeding cycle, finding genes with DNA markers which would allow for the design of better selection strategies, using genetic transformation technologies to produce disease resistant and nutritionally improved crops, as well as improving genebank reserves.

1.3. METHIONINE: AN ESSENTIAL AMINO ACID

The sulphur-containing amino acid methionine is a member of the aspartate-derived family of amino acids and is an essential metabolite in all living organisms (Rébeillé *et al.*, 2006). Methionine (Fig. 1.3.) is synthesised *de novo* in plants, fungi and microbes. It is a component of methionyl tRNA, which is required for the initiation of protein synthesis (Bartlem *et al.*, 2000). Methionine is also the precursor of *S*-Adenosyl-Methionine (AdoMet), which is the main methyl-group donor in transmethylation reactions as well as an intermediate in the biosyntheis of biotin, polyamines and the phytohormone ethylene (Hacham *et al.*, 2002). It is the only sulphur-containing amino acid that cannot be synthesised by human and monogastric livestock (Rébeillé *et al.*, 2006). Therefore, methionine can be supplied only from the diet, and is hence classified as an essential amino acid. In humans, methionine is metabolised through the reverse trans-sulfuration pathway where the γ-cleavage of the cystathionine intermediate allows the synthesis of cysteine (Cys). This reaction does not exist in plants.

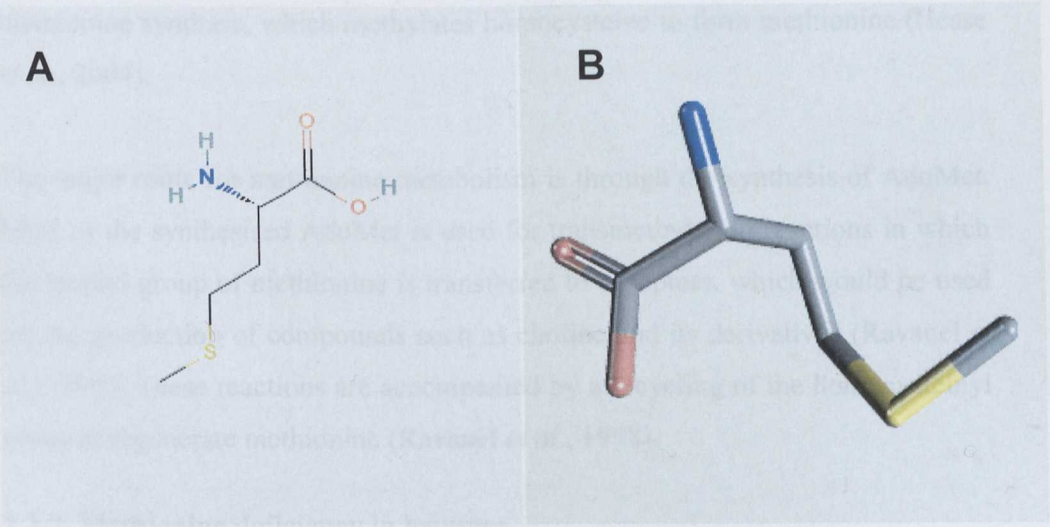


Fig. 1.3. Two-dimensional (A) and 3-dimensional (B) representation of the chemical structure of the amino acid methionine. **Source:** NCBI website

1.3.1. Methionine biosynthesis and metabolism in plants

The study of the metabolic pathways involved in the *de novo* synthesis of methionine in microorganisms, such as enteric bacteria and yeast, is extensive and well-documented (Choih *et al.*, 1977; Kadner, 1975; Belfaiza *et al.*, 1998). Similar pathways have been found to occur in plants (Ravanel *et al.*, 1998). The investigation of enzymatic reactions that lead to methionine (Met) synthesis in plants is important because it can help scientists better understand the molecular mechanisms that drive the homeostatic accumulations of amino acids in plants, as well as facilitate understanding of why the production of certain amino acids, such as lysine and methionine, is limited in crops such as cereals and legumes (Ravanel *et al.*, 1998).

In plants, the first committed step of *de novo* Met synthesis is the formation of the thioether cystathionine, catalysed by cystathionine γ-synthase (CgS) from the substrates cysteine and *O*-phosphohomoserine. This reaction involves a trans-sulphuration process *via* a γ-replacement reaction. Cystathionine β-lyase (CbL) then catalyses the conversion of cystathionine to homocysteine by a β-cleavage reaction. The last step of Met synthesis occurs in the cytosol, and is catalysed by

methionine synthase, which methylates homocysteine to form methionine (Hesse *et al.*, 2004).

The major route for methionine metabolism is through the synthesis of AdoMet. Most of the synthesised AdoMet is used for transmethylation reactions in which the methyl group of methionine is transferred to acceptors, which would be used for the production of compounds such as choline and its derivatives (Ravanel *et al.*, 1998). These reactions are accompanied by a recycling of the homocysteinyl group to regenerate methionine (Ravanel *et al.*, 1998).

1.3.2. Methionine deficiency in legumes

In developing countries where a meat-based diet is very rare, a deficiency in essential amino acids, such as methionine and lysine, can have severe negative effects on the physical and mental development of growing children. Protein-calorie malnutrition (PCM) is a nutritional syndrome which affects more than 170 million children and nursing mothers in developing African and Asian countries where legumes are a major source of protein (Iqbal *et al.*, 2006). These alarming figures suggest that scientific research directed towards improving the nutritional quality of legume crops may have a beneficial impact on agriculture in those countries. The important things to look for when assessing protein quality in legumes are the amino acid composition relative to the 1965 FAO/WHO reference pattern, the protein stability upon cooking, and the protein digestability after ingestion (Boulter *et al.*, 1976). Several economically important grain legumes, *e.g.* soybean (*Glycine max*), bean (*Phaseolus vulgaris* L.) and lupin (*Lupinus angustifolius* L.), despite being major sources of food and feed, produce seeds with a relatively low content of methionine (Aragão *et al.*, 1992; Molvig *et al.*, 1997; Nordlee *et al.*, 1996). Iqbal and co-workers (2006) determined the amino acid profile of 4 legumes (chickpea, cowpea, lentil and green pea) and concluded that there was no significant difference in the contents of total essential and non-essential amino acids among the 4 species. However, significant variations in individual amino acid contents, namely arginine, histidine and methionine, were reported. The methionine contents varied from 0.8% of protein in lentil to 2.2% of protein in cowpea (Iqbal *et al.*, 2006). The

methionine content in all of the assessed species was also relatively low when compared to other amino acids such as arginine and lysine. For example, chickpea was reported to contain 8.3% arginine, 7.2% lysine, but only 1.1% methionine (Iqbal *et al.*, 2006). The enhancement of the nutritional value of grain and forage legumes in terms of their methionine content is therefore of major importance for crop improvement.

1.3.3. Plant genetic engineering for enhanced methionine production

One strategy to improve the methionine composition of legume seeds involves the introduction of genes coding for methionine-rich proteins into target species *via* genetic transformation methods. Examples of such genes are the *ber e1* gene from Brazil nut (*Bertholletia excelsa*) (Lacorte *et al.*, 1997) and the *SFA8* gene from sunflower (*Helianthus annuus*) (Rafiqul *et al.*, 1996), which code for 18% and 16% methionine, respectively.

Previous studies which have adopted this strategy include the successful transformation of canola with the *ber e1* gene under the control of the phaseolin promoter, which resulted in an increased methionine content of up to 30-33% in the seed proteins of the recovered transgenic plants (Altenbach *et al.*, 1992). The grain legume lupin was also successfully transformed with the *SFA8* gene to yield transformants which showed a 5% accumulation of the SFA8 protein, corresponding to a 94% increase in the methionine content of the seeds as compared to the wildtype parent (Molvig *et al.*, 1997).

However, genetic engineering *via* DNA transformation has the potential to introduce new allergenic proteins into the target plants. Unfortunately, studies have confirmed that the ber e1 and SFA8 proteins have allergenic properties (Alcocer *et al.*, 2002; Pantoja-Uceda *et al.*, 2004). Food allergens typically stimulate the immune response by inducing the production of allergen-specific IgE. It has been shown that IgE fractions isolated from the sera of patients allergic to the Brazil nut react with proteins of the expected molecular mass in extracts of soybean genetically modified with *ber e1* (Shewry *et al.*, 2001). The

proven allergenicity of those genes therefore limits their use in plant genetic engineering to model transformation systems.

Alternative methionine-rich genes which have been identified are *β-zein* from maize (*Zea mays*) which codes for a 10kD protein with 30% methionine (Beauregard and Hefford, 2006), and *β-kafirin* from sorghum (*Sorghum bicolour*) which encodes for a protein with 172 amino acids, of which 16 are methionine residues (Chamba *et al.*, 2005). As there are, to date, no reports of allergenicity related to these genes, they would make excellent alternative genes of interest in research directed at improving the methionine content of legume seed crops *via* genetic engineering.

1.4. TARGET PLANTS

This higher degree programme investigated the underutilised food crops jicama (*Pachyrhizus erosus* L. Urban) and Bambara groundnut (*Vigna subterranea* (L.) Verdc.) for their genetic improvement.

1.4.1. Jicama [*Pachyrhizus erosus* (L.) Urban]

1.4.1.1. Origin and nomenclature

Pachyrhizus erosus (L.) Urban is one of the five species of the genus *Pachyrhizus* D.C., and is native to Mexico, where it is commonly known as jicama. Jicama belongs to the family Leguminosae, subfamily Papilionoideae (Mélo *et al.*, 2003). The exact nomenclature of the genus *Pachyrhizus* has remained undetermined for more than two centuries, since one of its first botanical references by Plukenet in 1696, who named the plant *Phaseolus nevisensis* (Sørensen, 1996). In 1753, Linnæus used Plukenet's description as a basis to rename the species *Dolichos erosus*, and stated that the plant originated from the Neotropics. However, in the second edition of Linnæus' Species Plantarum in 1763, the plant erroneously appeared under the name *Dolichos bulbosus* due to its similarity with the Indonesian species *Cacara bulbosa*, described by Rumphius in 1747 (Sørensen, 1996). In 1806, Du Petit-Thouars transferred the plant from the genus *Dolichos* L. to the genus *Cacara*. Over the

next century, the generic name *Pachyrhizus* was favoured over *Cacara* and is still used today (Sørensen, 1996).

1.4.1.2. Plant morphology

Jicama is a perennial climbing vine, usually cultivated as an annual crop. Seeds germinate approximately 6 days after sowing (DAS), producing a climbing stem which can reach up to 6m in height. The plant produces trifoliate palmate leaves, and purple flowers are borne on complex racemes as inflorescences. Petals of the jicama flowers are characterised by a lack of hairs on their surface. The flowers are self-pollinating and produce green pods which bear seeds. The seeds are square-shaped and the colour of the testa ranges from olive-green to brown (Fig. 1.4.). The plant has a tap root system which is characterised by the formation of turnip-shaped tuberous roots (Fuentes *et al.*, 2002). In association with appropriate *Rhizobiaceae* strains, *e.g. Rhizobium* strain NFB 747 (Stamford *et al.*, 2007), jicama roots will nodulate and fix atmospheric nitrogen.

Fig. 1.4. Jicama seeds. The seed colour ranges from olive-green to brown. Bar = 1cm.

1.4.1.3. Growth and development

The available literature on the growth and development patterns of jicama is limited and varied depending on the observations made at the respective areas of cultivation. Jicama is typically a short-day plant (Fernandez *et al.*, 1997), and is generally intercropped with maize and the common bean, *Phaseolus vulgaris*.

Growth is favoured in either light and sandy or loamy soils. The study by Fernandez *et al.* (1997) investigated the developmental patterns of jicama plants aged between 20 and 36 weeks, at weekly intervals. Seeds obtained from the Mexican State Nayarit were grown in the State of Sonora, which is to the north of Nayarit, and where the daylength ranged between 10.25 and 14.14 hours. Data collected between week 20 and week 35 showed an increase in fresh and dry weights of foliage, as well as increases in the number of leaves per plant and in the main stem length. From week 36 onwards, the number of leaves per plant dropped sharply, and as a consequence, so did the foliage fresh and dry weights. It was therefore suggested that the jicama plant reaches its maturity 36 weeks after sowing.

Few pests have been known to attack the plant, although common decay organisms found on the tubers after harvesting are *Penicillium*, *Rhizopus* and *Cladosporium* species (Cantwell *et al.*, 1992)

1.4.1.4. Nutritional and commercial properties

The only edible portion of the jicama plant is the tuberous root, which can be consumed raw (after peeling), or cooked. The flesh of the root is crisp and crunchy, and has a taste comparable to a cross between a water chestnut and an apple. The jicama tuber production capacity has been reported to be the highest among the tuber-bearing legumes (Morales-Arellano *et al.*, 2001). Starch is the main component of the jicama tuber and is made up of approximately 23% amylose (Mélo *et al.*, 2003). The tubers have a low carbohydrate content [32% as soluble sugars, and 15% as starch (Paull and Chen, 1988)] and have also been reported to have 3-5 times the protein content, (on a dry weight basis), of other tuberous roots, such as potato and cassava (Gomes *et al.*, 1997).

Industrial starches are generally extracted from cereal grains and tuberous roots (Mélo *et al.*, 2003). These two starches differ mainly in terms of gelatinising temperature, viscosity and paste clarity, and this is primarily due to factors such as the degree of chain branching, and the ratio of amylose to amylopectin. A study investigating the functional properties of starch extracted from jicama

tubers revealed that it was comparable to starch extracted from cassava, thus making the former a potential substitute for cassava starch in processed foods (Mélo *et al.*, 2003), and adding commercial value to the jicama crop.

Mature jicama seeds can contain up to 26% protein. The major protein components in jicama seeds are albumins (52.1 - 32.0%), followed by globulins (30.7 – 27.5%), and finally prolamins (0.8%) (Morales-Arellano *et al.*, 2001).

Aside from the tuber, the rest of the plant, including mature seeds, contains rotenone, a toxic isoflavonoid with insecticidal properties (Fuentes *et al.*, 2002). Mature seeds contain approximately 0.5% rotenone and are hence inedible. If the rotenone is removed, the seeds will contain up to 26% protein and 30% vegetable oil, a composition comparable to the ground nut and cotton seed oil (Sørensen, 1996). The current use of rotenone extracted from mature seeds as an insecticide (currently found mainly in flea powders) is limited, and the possibility of using the rotenone as a plant protective agent or to clean eutrophied water bodies is an attractive alternative with much improved commercial benefits (Sørensen, 1996).

1.4.1.5. Nitrogen fixation

Nitrogen fixation *via* symbiosis has been reported in jicama plants growing in the Philippines and countries of Latin America (Fuentes *et al.*, 2002). The formation of root nodules have been reported on lateral roots only and not on the tap root where the tubers develop. Isolates from the root nodules were analysed and 25 strains of bacteria were identified. Out of these, 10 strains were randomly selected for partial sequencing of their respective ribosomal RNA (rRNA) gene. Through sequence comparisons of the rRNA genes, it was possible to determine the phylogenetic relationships existing among the isolated strains of bacteria, and conclude that the analysed strains belonged to two genera of the *Rhizobiaceae*, namely the *Rhizobium* and *Bradyrhizobium* genera. The isolated strains YB4 and JEYF11 contained a high homology with *Rhizobium gallicum* and *Rhizobium tropici*, respectively, while isolated strains PAC24 and PAC29 exhibited a high homology with *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, respectively (Fuentes *et al.*, 2002).

1.4.2. Bambara groundnut [*Vigna subterranea* (L.) Verdc]

1.4.2.1. Origin and nomenclature

Bambara groundnut belongs to the family Leguminosae, subfamily Papilionoideae. Its scientific nomenclature has changed over the centuries since being described in *Species Plantarum* by Linnaeus in 1763, who named the plant *Glycine subterranea* (Heller *et al.*, 1997). In the 19th century, Du Petit-Thouars came across the crop in Madagascar, where it was locally known as 'voanjo'. He proposed to name the plant *Voandzeia subterranea* (L.) Thouars, and this nomenclature was kept for more than a century, until studies carried out in 1798 by Maréchal and co-workers showed significant similarities between Bambara groundnut and other plant species of the genus *Vigna* (Heller *et al.*, 1997). These studies were confirmed in 1980 by Verdcourt, who went on to suggest the name *Vigna subterranea* (L.) Verdc. (Verdcourt, 1980). The common name of the plant seems to have been derived from a tribe, the Bambara, who live in Mali (Heller *et al.*, 1997; Azam-Ali *et al.*, 2001).

Bambara groundnut was found in its wild form in the Yola province in Nigeria as well as near Garoua in Cameroon, in the early 20th century (Heller *et al.*, 1997). Begemann (1988) analysed Bambara groundnut samples collected less than 200 km from the putative centre of origin, and found that those samples consistently showed a greater seed-pattern diversity, as well as higher diversity indices for other characteristics such as pod length, internode length and the number of stems per plant, thus confirming the origin of Bambara groundnut to be in the region of north-eastern Nigeria and northern Cameroon (Begemann, 1988; Heller *et al.*, 1997).

1.4.2.2. Plant morphology

Bambara groundnut is an herbaceous, annual plant. Lateral stems begin to develop approximately 1 week after germination. Each lateral stem is made up of internodes, and differences in the length of the internodes have been shown to

vary between different Bambara groundnut plants, resulting in bunched, semi-bunched or spreading types (Heller *et al.*, 1997).

The development of leaf and flower buds occur alternately at each node. The plant has trifoliate leaves arising from stiff and grooved petioles. Each leaflet is oval, with the terminal leaflet being slightly larger than the lateral ones. Papilionaceous yellow flowers are borne in a raceme on hairy peduncles, and are usually self-pollinated in the bunched type, while cross-pollinated in the spreading type (Swanevelder, 1998). Once the flowers have been pollinated and fertilisation has occurred, the peduncle elongates so that the ovaries are on or just below the soil surface, and the pods develop. Mature Bambara groundnut pods are typically spherical and wrinkled, and mostly contain one or occasionally two seeds which are round, smooth and very hard when dried (Heller *et al.*, 1997; Swanevelder, 1998). The seeds are found in a variety of colours including white, cream, red, purple and black, with or without testa patterns such as mottled or blotched (Heller *et al.*, 1997).

The plant has a tap-root system, with an extensive network of geotropic lateral roots. In association with appropriate *Bradyrhizobium* strains, *e.g.* *Bradyrhizobium* strain MAO 113, Bambara groundnut roots will nodulate and fix atmospheric nitrogen (Gueye and Bordeleau, 1988; Gueye *et al.*, 1998).

1.4.2.3. Growth and development

Bambara groundnut is often intercropped with major food crops such as maize, cassava, peanut and cowpea. Grown in rotation, Bambara groundnut improves the nitrogen content of the soil. Growth is favoured in deep, well-drained soil with a light, friable seedbed. The plant grows well with 600-1200 mm of annual rainfall, and a soil pH between 5.0 and 6.5 (Baryeh, 2001). Bambara groundnut is a short-day plant, and grows best under a 10-12h photoperiod. Seeds germinate between 7 and 15 days after sowing (DAS), and flowering begins at around 60 – 65 DAS. Pod formation occurs approximately 30 days after fertilisation, and seed development takes place during the following 10 days.

Harvesting is done by pulling or lifting the plant. Most pods remain attached to the crown root, but those which get detached are then manually collected. Harvested pods are air-dried for several days before threshing. The plant is drought and pest-resistant, although it does become prone to fungal diseases such as Cercospora leafspot, Fusarium wilt and Sclerotium rot in humid environments.

1.4.2.4. Nutritional and commercial properties

Bambara groundnut is nowadays essentially cultivated on a small scale by subsistence farmers in several semi-arid regions of tropical and sub-tropical Africa, and can also be found growing in South and Central America, South-East Asia, and northern Australia (Linnemann and Azam-Ali, 1993). It is ranked the third most important grain legume in Africa, after groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* (L.) Walp), (Baryeh, 2001; Linnemann, 1992; Rachie and Silvestre, 1977).

Immature Bambara groundnut seeds can be eaten fresh or grilled, while mature seeds, being relatively harder, are usually boiled before being consumed (Adu-Dapaah and Sangwan, 2004). The seeds are of high nutritive value and investigations into the biochemical composition of the seeds have shown that they contain, on average, 63% carbohydrate, 19% protein and 6.5% oil (Heller *et al.*, 1997). The gross energy value of Bambara groundnut is more than that of other common pulses such as cowpea, lentil and pigeonpea (Azam-Ali *et al.*, 2001). Bambara groundnut seeds contain relatively low methionine and calcium contents, but still provide an important source of protein to people in Africa who cannot afford the more expensive animal protein, usually present in a meat-based diet (Baryeh, 2001).

1.4.2.5. Bambara groundnut landraces

To date, breeding programmes have not been established to create new, improved varieties of Bambara groundnut (Massawe *et al.*, 2005). As such, different Bambara groundnut genotypes are still referred to as landraces (Azam-

Ali *et al.*, 2001). Zeven (1998) defines a landrace as 'a variety with a high capacity to tolerate biotic and abiotic stress, resulting in high yield stability and an intermediate yield level under a low input agricultural system'. The present project investigated three Bambara groundnut landraces, namely Diphiri Cream (DipC) from Botswana, Uniswa-Red from Swaziland, and S19-3 from Namibia. The main phenotypic difference between the landraces was the colour of the testa, as shown in Fig. 1.5.

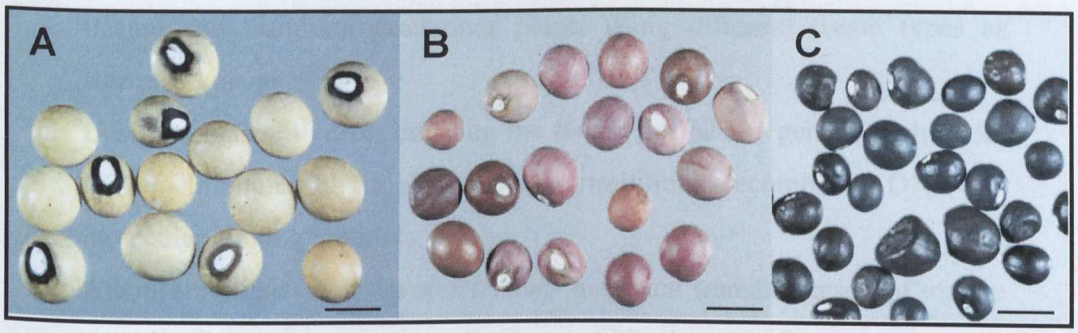


Fig. 1.5. Investigated Bambara groundnut landraces. **A:** DipC seeds have a cream coloured testa with a black hilum; **B:** Uniswa-Red seeds have a red testa; **C:** S19-3 seeds have seed coats which range from dark red to black. Bars = 1cm.

1.5. AIMS AND OBJECTIVES OF RESEARCH PROJECT

The aims of this project focused on designing and optimising tissue culture, molecular cloning and plant transformation strategies for the genetic engineering of target plants to enhance methionine production in the seeds. The objectives were to:

i. Establish optimised tissue culture systems for the *in vitro* regeneration of jicama and Bambara groundnut plants using different tissue types as starting explants.
ii. Design a plasmid vector carrying the Brazil nut *ber e1* gene, which codes for a methionine-rich 2S albumin, using traditional recombinant DNA and molecular cloning methods.
iii. Attempt biolistic- and *Agrobacterium*- mediated transformation of mature zygotic Bambara groundnut embryos with the transformation vectors pVDH65, pBI121 and pBI121-LeB4-Ber e1.
iv. Implement the corresponding established and optimised tissue culture systems for the *in vitro* regeneration of plants in order to recover putatively transformed shoots.
v. Perform molecular analyses on surviving selected shoots in order to confirm the integration and expression of the *npt*II, *uidA* and *ber e1* transgenes.
vi. Transfer recovered transgenic shoots onto root inducing media and successfully acclimatise plants *ex vitro*.

The success of the experiments would provide a model transformation and regeneration system for further research involving the introduction of novel traits in legume crops for the production of nutritionally-improved varieties.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1. PLANT MATERIALS

Jicama and Bambara groundnut seeds were obtained as starting materials for the generation of explants in tissue culture-based experiments. Dried, mature jicama seeds were purchased from Chiltern Seeds (Ulverston, Cumbria, UK). Dried, mature Bambara groundnut seeds for the three landraces investigated, DipC, Uniswa-Red and S19-3, were kindly donated by Dr. Sean Mayes (Tropical Crops Research Unit, University of Nottingham). Since the geographical origin of seed accessions for both jicama and Bambara groundnut were unknown, the potential for genetic variability to be introduced during *in vitro* culture was a possibility. All seeds were kept in sealed paper bags, and stored in darkness at 4°C. Seed stocks for jicama and Bambara groundnut were generated *via ex vitro* seed germination. The generation of explant material for tissue culture-based experiments was either *via* embryo excision or through *in vitro* seed germination.

2.2. *EX VITRO* PLANT GERMINATION AND MAINTENANCE

2.2.1. Compost mixes

Jicama plants were grown in 'Nottingham Traditional Mix' compost which consisted of Levington M3 compost (Scotts UK Professional, Ipswich, UK), John Innes No.3 (medium loam) compost (J. Bentley, Barton-Humber, UK), Perlite (Sinclair, Lincoln, UK) and Vermiculite (Sinclair) in the ratio 6:6:1:1 (v/v). Bambara groundnut plants were grown in a compost mix consisting of John Innes No. 3 compost (J. Bentley, Barton-Humber, UK) and sand in a 1:1 (v/v) ratio.

2.2.2. Glasshouse and growthroom conditions

Jicama plants were maintained under glasshouse conditions and were subjected to a day temperature of 28 ± 1°C, a night temperature of 24 ± 1°C and a 16h photoperiod. Photoperiods were maintained by natural daylight with light supplementation from 600 W pressure sodium lamps (Philips® Sun-t Pia Green Power, Philips Electricals UK Ltd., Guildford, UK), giving a mean photon flux density of 170 μmol quanta m^{-2} s^{-1} within the photosynthetically active radiation (PAR) wavelength. The plants were watered daily.

Bambara groundnut plants were maintained in growth rooms and were subjected to a 12 h photoperiod and a day and night temperature of 27±2°C. Photoperiods were maintained by light from 600 W pressure sodium lamps (Philips® Sun-t Pia Green Power), giving a mean photon flux density of 170 µmol quanta m^{-2} s^{-1} within the photosynthetically active radiation (PAR) wavelength. Watering was carried out on alternate days with 100ml tap water per plant.

Plant pest infestations were prevented through fortnightly applications of biological control agents comprising Swirskiline® (*Amblyseius swirskii*), Exhibitline® (*Steinernema feltiae*) and Phytoline® (*Phytoseiulus persimilis*) to control *Bemisia tabaci* (Whitefly) and *Frankliniella occidentalis* (Thrips), *Bradysia paupera* (Sciarid Fly) and *Tetranychus urticae* (Red Spider Mite) respectively (Syngenta Bioline, Little Clacton, Essex, UK).

2.3. *IN VITRO* PLANT REGENERATION AND CULTURE

2.3.1. Axenic technique

All tissue culture was carried out under axenic conditions in the laminar air-flow cabinet (Faster KBN, Cornaredo, Italy), to eliminate all possibility of contamination. The laminar air-flow cabinet was switched on and left running for 20 min, after which the working surface was sprayed on with 2% (v/v) Trigene (Medichem International, Sevenoaks, UK) solution and wiped using paper tissue. All surface-sterile and *in vitro* cultured plant material was handled on a sterile ceramic tile, using sterile forceps and scalpels.

Forcep tips and scalpel blades were sterilised by dipping in 100% (v/v) ethanol, and leaving the ethanol to evaporate for a few seconds before flaming the instrument tips. All the other equipment used, such as glassware, ceramic tiles, filter papers, micropestles and pipette tips were sterilised by autoclaving at 121°C under 104 kPa for 20 min.

Following explant culture and transfer to Petri dishes (Bibby Sterilin Ltd., Staffs, UK), Nesco film (Bando Chemical IND. Ltd., Osaka, Japan) was used to seal the Petri dishes prior to their storage in the culture room.

2.3.2. Tissue culture growth room conditions

The tissue culture room conditions were maintained at 24 ± 2°C, under a 16h photoperiod, at 50 – 80 µmol photons m^{-2} s^{-1} light intensity from 58 W white halophosphate fluorescent tubes (Cooper Lighting and Security, Doncaster, UK).

2.3.3. *In vitro* growth media and conditions

Unless stated otherwise, media used in tissue-culture based experiments was agar-based Murashige and Skoog (MS; 1962) medium (Appendix I). Media was autoclaved at 121°C and 104 kPa for 20 min, and allowed to cool to approx. 40°C before being supplemented with the appropriate nutrient additives, growth regulators and/or antibiotics. The medium was then poured either into sterile 9cm Petri dishes (20ml per dish) or into sterile 175ml screw-capped Powder Round jars (Beatson Clark & Co. Ltd, Rotherham, UK; 50ml per jar).

2.3.4. Preparation of stock solutions for media supplements

Unless stated otherwise, stock solutions of supplements such as nutrient additives (*e.g.* microsalts and vitamins), growth regulators (*e.g.* BAP and NAA) and antibiotics (*e.g.* kanamycin and cefotaxime) were prepared by dissolving the chemical salt into the appropriate solvent and passing the solution through a sterile 0.2µm disposable filter unit using a 10ml sterile disposable syringe. The solution was drawn into the syringe, and the syringe fitted into the filter unit. Both the syringe and the filter were then placed at the 'neck' of an open sterile 30ml universal tube, and the solution was slowly pumped through the filter, into the tube. Once all the solution had been filter sterilised, the tube was tightly capped and appropriately labelled. Stock solutions of nutrient additives and plant growth regulators were stored at 4°C, while antibiotic solutions were stored at -20°C.

2.3.5. Preparation of explant material

2.3.5.1. Surface sterilisation of seeds

Working in the laminar air-flow cabinet (Section 2.3.1.), seeds were surface-sterilised by immersing seeds of each species in 70% (v/v) ethanol (Fisher Scientific, Loughborough, UK) for 3 min, and rinsing away the ethanol in sterile reverse-osmosis (purified) water. The seeds were then immersed in 8% (v/v) 'Domestos' bleach (Weston Favell Centre, Northampton, UK) solution (20 min, with occasional swirling) followed by four rinses in sterile reverse-osmosis water. The seeds were then transferred into sterile 250ml Erlenmeyer flasks (20 seeds per flask) each containing 100ml of sterile deionised water, for imbibition in darkness. The flasks were placed onto a horizontal shaker set at 180 revolutions per minute (rpm), overnight, in a culture room set at a temperature of 21±2°C, during which time the seeds visibly swelled up, indicating that they were ready for embryo excision or germination. Seeds with a split testa were considered to be already germinating, and were not used in the experiments.

2.3.5.2. Embryonic axis excision procedures

Following an overnight imbibition in sterile reverse-osmosis water, each expanded seed was placed on a sterile tile. Using two pairs of sterile flamed forceps, and a sterile flamed scalpel blade, a cut was made through the testa, and the seed coat removed. Each seed cotyledon was held by a pair of forceps, and slightly pulled apart from each other. During this process, the embryonic axis broke off from one of the cotyledons, and this cotyledon was discarded. Holding the other cotyledon with a pair of forceps, the embryonic axis was gently excised using the scalpel blade, and transferred into a Petri dish containing the appropriate culture medium.

2.3.5.3. Generation of other explant types

To obtain cotyledon, hypocotyl, leaf, stem and root explants for tissue culture-based experiments, jicama and Bambara groundnut seeds were surface-sterilised and immersed in sterile water overnight (Section 2.3.2.1.). Following imbibition,

seeds were either cut in half on a sterile ceramic tile to obtain cotyledon explants, or cultured for 10 days in sterile 175ml powder jars, each containing 50ml of MS0.8 medium with no added growth regulators. Hypocotyl, leaf, stem and root tissue sections were then excised from jicama and Bambara groundnut plantlets and used as starting explants in subsequent experiments.

2.4. BACTERIAL TECHNIQUES

2.4.1. *Escherichia coli* strain DH5α

Escherichia coli strain DH5α was employed as the bacterial host for novel plasmid transformation. This strain was *endA1* thus, did not produce endonuclease I and so permitted cleaner plasmid preps by avoiding non-specific digestion of plasmid DNA. It also lacked the alpha portion of the *lacZ* gene and so was suitable for blue-white transformation screening.

2.4.2. *Agrobacterium tumefaciens* strains

Two *Agrobacterium tumefaciens* strains were employed in this research programme, namely *A. tumefaciens* strain 1065 (kindly donated by Dr. Paul Anthony, Plant and Crop Sciences Division, University of Nottingham) and *A. tumefaciens* strain GV3101 (kindly donated by Dr. Li Hong Yin, Plant and Crop Sciences Division, University of Nottingham).

The *A. tumefaciens* strain 1065 (Curtis *et al.*, 1994; Drake *et al.*, 1997) was derived from strain LBA4404 which contained the binary vector pMOG23 (Sijmons *et al.*, 1990) and the hypervirulent pTOK47 which contained the *vir*B, *vir*C and *vir*G operons from pTiBo542 (Jin *et al.*, 1987; Cancino *et al.*, 2004). Strain 1065 carried the binary vector pVDH65 with the β-glucuronidase (*uidA*) reporter gene and the neomycin phosphotransferase (*npt*II) selectable marker gene.

A. tumefaciens strain GV3101 (Koncz and Schell, 1986) was selected as host strain for the methionine-rich *LeB4::Ber e1* gene construct used during transformation studies. Strain GV3101 carried chromosomal resistance to

rifampicin and a Ti-plasmid which coded for virulence genes and gentamycin resistance. It was sensitive to kanamycin and therefore suitable for transformations with constructs conferring bacterial kanamycin resistance. To maintain selection for this strain, rifampicin and gentamycin were added to growth media at a concentration of 50 $\mu g\ ml^{-1}$.

2.4.3. Growth of bacterial strains

Liquid cultures of *E. coli* strain DH5α were maintained in Luria-Bertani (LB) broth (Appendix II, Section AII.1) which was autoclaved prior to appropriate antibiotic supplementation. *E. coli* cultures were grown in sterile 250ml Erlenmeyer flasks by inoculating antibiotic supplemented LB medium with a single colony and incubating for 16 h at 37°C in the dark at 180 rpm in a Stuart Scientific SI50 orbital incubator (Bibby Sterilin Ltd., Stone, UK).

Liquid *A. tumefaciens* cultures were maintained in autoclaved APM medium (Appendix II, Section AII.3) supplemented with the appropriate antibiotics. The antibiotic supplemented APM inoculated with *Agrobacterium tumefaciens* was incubated at 28°C for 12 - 16 h in darkened conditions at 250rpm in a Stuart Scientific SI50 orbital incubator (Bibby Sterilin Ltd).

2.4.4. Storage of bacterial cultures

Bacterial cells were stored for interim periods as semi-solid cultures at 4°C in 9cm Petri dishes each containing 20ml of agar-based LB medium (Appendix II, Section AII.2) supplemented with the appropriate antibiotics. Long-term storage of bacteria was achieved by making glycerol stocks of *E. coli* and *A. tumefaciens* cultures. In sterile screw-capped cryovials (Simport, Beloeil, Canada), 1ml of an overnight culture (initiated from a single bacterial colony) was mixed with 500µl of 60% (v/v) glycerol. The bacterial suspension was in a final concentration of 20% (v/v) glycerol. Each tube was quickly inverted 1 – 2 times to mix the suspension, and immediately dropped into a Dewar flask containing liquid nitrogen for 5 min. The cryotubes were then removed from the liquid nitrogen

using a pair of forceps, and transferred into labeled cryoboxes. Long term storage of *E. coli* and *A. tumefaciens* was at -80°C.

2.4.5. Plasmid transformation into competent *E. coli* cells

Plasmids were transformed into chemically competent *E. coli* DH5α cells using the following method. One microcentrifuge tube containing 50µl of competent cells was removed from the freezer at -80°C, thawed on ice, before adding approximately 10ng of plasmid DNA. The tube was gently tapped to mix the components and the mixture incubated on ice for 30 min to allow the DNA to associate to the bacteria. Following incubation, the tube was transferred into a water bath set at 42°C for 90 s to heat shock the mix, and then immediately placed back on ice for 2 min. A volume of 950µl of liquid LB medium (Appendix II, Section AII.1.) was added to the cell suspension, and the mixture incubated for 1 h in the dark on a horizontal shaker maintained at 37°C and at 225 rpm, to allow the cells to recover and to produce an initial growth culture of unselected transformed *E. coli* DH5α cells. The bacterial suspension was then plated onto semi-solid LB medium supplemented with antibiotics to select for cells which had incorporated the plasmid.

2.4.6. Transformation of *Agrobacterium tumefaciens* GV3101 by electroporation

To produce a stock of competent *A. tumefaciens* GV3101 cells, a single colony of *A. tumefaciens* GV3101 was grown at 28°C for approximately 30 hours, in 100ml of liquid LB with 50 µg ml^{-1} rifampicin as a selective agent, to an optical density $(OD)_{600nm}$ of 0.5 – 0.7. The culture was cooled on ice and centrifuged at 3000 g for 2 min. The pellet was washed by resuspension in 1 culture volume of cold 10% (v/v) sterile glycerol. Cells were finally resuspended in a 0.01 culture volume of 10% glycerol to give $10^{11} – 10^{12}$ cells ml^{-1}. Aliquots of 100 µl were pipetted into sterile microcentrifuge tubes, frozen in liquid nitrogen and stored at -80°C.

When required for transformation, cells were thawed on ice and a 40µl aliquot was transferred to a pre-cooled, sterile 0.2cm electroporation cuvette. Plasmid DNA (2 – 10 µg) was mixed with the cell suspension and an electric pulse immediately applied (2.5V, 400Ω, 25 µFD) using a gene pulser (BioRad, Hemel, Hempstead, UK). Cells were then transferred to 1ml LB medium without antibiotics and incubated at 28°C for 3h in an orbital shaker at 250 rpm, to allow the cells to recover. The bacterial suspension was then plated onto semi-solid LB medium supplemented with the appropriate antibiotics and incubated for 3 days at 28°C to select for cells which had successfully incorporated the plasmid.

2.4.7. Screening of transformed bacteria by colony PCR

Bacterial colonies growing on selective medium were picked using a sterile 200µl pipette tip. Each tested colony was streaked onto a fresh plate containing semi-solid LB medium supplemented with the appropriate antibiotics, and incubated at 37°C overnight for *E. coli*, and at 28°C for 3 days in the case of *A. tumefaciens* (this would allow for the preparation of glycerol stocks of the colonies if they tested positive for the gene insert of interest). The pipette tip was then swirled into a labeled PCR tube containing 15µl of PCR Master Mix (Appendix VI, Section AVI.2). Control reaction tubes contained only the Master Mix and no bacteria. PCR amplification was performed in a 96-well GeneAmp® PCR System 9700 machine (Applied Biosystems; CA, USA) using primers specific to the fragment of interest. Amplicons were separated on agarose gels by electrophoresis. Amplified PCR products of the expected size indicated bacterial colonies containing DNA inserts.

2.4.8. Plasmid isolation from *E. coli*

A single *E. coli* colony containing the fragment of interest was grown for 16 h in 10 ml liquid LB supplemented with the appropriate antibiotics, shaking at 250 rpm at 37°C. According to the manufacturer's instructions (Appendix III, Section AIII.3), the plasmid was isolated from these overnight cultures using the Qiaprep Miniprep kit (Qiagen, Crawley, UK). DNA concentrations were measured by

spectrophotometry using NanoDrop software at default settings (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Wilmington, DE 19810, USA).

2.5. PLANT TRANSFORMATION TECHNOLOGIES

Bambara groundnut zygotic embryo explants were transformed using biolistic-mediated and *Agrobacterium*-mediated transformation methods. This section briefly describes the operating instructions for using the biolistic particle delivery system, and the general steps followed to prepare *Agrobacterium* cultures prior to explant inoculation. A more detailed account of the methods employed, which includes plasmid DNA preparation for biolistics, as well as explant manipulation prior to and after transformation, is given in Chapters 5 and 6.

2.5.1. Gene transfer by particle delivery

Biolistic-mediated transformation was carried out using the Biolistic® PDS-1000/He particle delivery device (Bio-Rad Laboratories, Hemel Hempstead, UK) Safety spectacles were always worn when operating the PDS-1000/He device. Before using the PDS-1000/He device, the helium cylinder regulator was opened and the helium delivery pressure adjusted to the appropriate setting before the device was powered on. The operating instructions described below were adapted from the Bio-Rad instruction manual.

The rupture disc retaining cap was unscrewed from the end of the gas acceleration tube. A rupture disc was dipped in isopropanol to remove static charge and placed into the retaining cap, ensuring that it was correctly seated at the base of the cap. The rupture disc retaining cap was screwed onto the gas acceleration tube and tightened using the tightening device.

The macrocarrier launch assembly was removed from the sample chamber, and the cover was unscrewed from the assembly. A sterile stopping screen was placed on the stopping screen support. The macrocarrier holder containing the macrocarrier was installed on the top rim of the fixed nest, with the DNA-coated side of the macrocarrier facing downwards. The macrocarrier cover lid was

placed on the assembly and tightened until snug. The macrocarrier launch assembly was fitted on the appropriate shelf in the sample chamber.

A 9cm Petri dish containing the target material was placed on the Petri dish holder. This was inserted on the desired shelf within the sample chamber. The door of the chamber was closed and latched, and the helium tank was checked to confirm that it was regulated to the correct pressure. The vacuum switch was set to the VAC position and the vacuum pump was switched on using the remote switch. The vacuum chamber gauge was observed and once the desired vacuum is reached, the vacuum switch was set to the 'HOLD' position, and the vacuum pump was switched off.

At this stage, the red light was illuminated in the 'FIRE' switch. The FIRE switch was pressed and held to allow the pressure to build up in the gas acceleration tube. It took 12 -15 s for the rupture pressure to be reached. The rupture disc burst within 10% of the indicated rupture pressure, and once this occurred, the 'FIRE' switch was immediately released.

The vacuum pressure was released by setting the vacuum switch to the 'VENT' position. Once the vacuum had been released, the red light on the 'FIRE' switch went off, indicating that it was safe to open the chamber door. The Petri dish containing the bombarded target tissue was removed from the chamber and treated appropriately.

The macrocarrier launch assembly was removed and the now distorted macrocarrier and the rupture disc were discarded. The rupture disc retaining cap was unscrewed from the gas acceleration tube. The remains of the rupture disc were removed and discarded. The above steps were repeated for each 'shot'. After use, the biolistics device was switched off and the chamber was cleaned with 70% (v/v) ethanol. Once the work was completed, the residual pressure from the helium supply was released and all the equipment were switched off and unplugged.

2.5.2. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation was performed using *A. tumefaciens* strain 1065 and *A. tumefaciens* strain GV3101. Bacterial cultures were initiated from -80°C glycerol stocks by streaking loops of the bacteria into 9cm Petri dishes containing agar-solidified Luria broth (L.B) medium (Appendix II, Section AII.2.) supplemented with appropriate antibiotics The addition of antibiotics was to select for the *Agrobacterium* colonies that had taken up the plasmid vector during bacterial transformation. The Petri dishes were incubated upside down at 28°C for 4-5 days to allow the bacteria to grow, and then stored upside down at 4°C. The plates were stored upside down so that any moisture would collect on the inner side of the lid and not flood the bacteria.

Liquid cultures of *A. tumefaciens* were initiated by transferring loopfuls of the bacteria into a sterile 100ml capacity Erlenmeyer flask containing 50ml of APM medium (Appendix II, Section AII.3.) supplemented with appropriate antibiotics. The Erlenmeyer flasks were then incubated in the dark on an orbital incubator maintained at 28 ± 2°C (180 revolutions per min; rpm), for a period of 12 – 16 h, during which time a cloudy bacterial suspension was formed.

To monitor the growth of the bacteria, the optical density (OD) of the bacterial culture was measured at a wavelength of 600nm (OD_{600}) using a LibraS4 spectrophotometer (Biochrom, Cambridge, UK). The OD of 1.0ml of bacterial culture was measured against a blank volume of APM medium containing the same antibiotic concentrations in which the bacteria were cultured. The bacterial cultures were used to inoculate the explants when the OD_{600} was between 1.0 and 1.1. The bacterial suspension was mixed with full-strength liquid MS medium (Appendix I, Section AI.1) in a 1:9 (v:v) ratio for explant inoculation. Target explants were superficially wounded with a sterile scalpel blade before being immersed in the diluted bacterial suspension for 20min. Following inoculation, explants were blotted onto sterile 55mm filter paper (Whatman, UK) and transferred onto co-culture medium, and subsequently onto selective medium.

2.6. NUCLEIC ACID ISOLATION AND MANIPULATION

2.6.1. Plant genomic DNA and total RNA isolation

Plant genomic DNA was extracted using the GenElute® Plant Genomic DNA Miniprep kit according to the manufacturer's instructions (Sigma-Aldrich, Poole, Dorset, UK). Total RNA was extracted using the RNeasy Plant Mini kit according to the manufacturer's instructions (Qiagen, Crawley, UK). DNA and RNA samples were quantified using NanoDrop software (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Wilmington, DE 19810, USA).

2.6.2. Agarose gel electrophoresis of DNA

DNA samples were mixed with 6X loading buffer and separated by gel electrophoresis at 100 V in 1% (w/v) agarose dissolved in 1X Tris-acetate-EDTA (TAE) buffer. The gel was supplemented with 5µl of 10 mg mL^{-1} ethidium bromide. Gels were run in 1X TAE buffer. TAE buffer was composed of 240g of Tris base, 57.1ml of glacial acetic acid, 100ml of 0.5M ethylene diamine tetraacetic acid ($C_{10}H_{12}N_2Na_4O_8$; EDTA) and sterile reverse-osmosis water to a final volume of 1L.

2.6.3. DNA purification from agarose gels

Gel electrophoresed bands were visualised under a short wavelength UV transilluminator. Bands corresponding to the correct size or molecular weight *i.e.* containing the correct DNA fragment of interest were excised from the gel using a sterile scalpel, placed into 1.5 ml microfuge tubes and flash frozen in liquid nitrogen. DNA extraction was performed using a MinElute Qiagen Gel Extraction Kit, following the manufacturer's protocol (Qiagen, Crawley, UK).

2.6.4. Endonuclease digestion and ligation of DNA

Digestion of DNA using restriction endonucleases for both DNA PCR fragments of interest and for plasmids was performed with enzymes and corresponding buffers according to manufacturers instructions (Promega, Southampton, UK). For all analytical restriction enzyme digestions, reactions were performed in a volume of 20µl on 0.2 - 1.5µg of substrate DNA using a 2- to 10-fold excess of enzyme over DNA, based on unit definition. Unless otherwise stated the following protocol was employed. Under sterile conditions, 2µl of restriction enzyme 10X buffer, 2µl of 1mg/ml acetylated Bovine Serum Albumin (BSA), and 0.5 – 1µg of DNA sample were added to a microcentrifuge tube. To this, 1µl of each restriction endonuclease (2 – 10 units) was added, depending on whether the reaction was a single or a double restriction digestion. Finally, sterile water was added to a final volume of 20µl. The reaction was gently mixed by pipetting and incubated for 4 h at 37°C. To the digested products, 4µl of 6X Blue/Orange loading dye was added (Promega), and the mixture was electrophoresed on a 1% (w/v) agarose gel to separate the fragments according to size.

Ligation reactions were usually performed after restriction digestion and gel purification of DNA fragments. T4 DNA ligase (Promega) was used to ligate DNA fragments (usually an insert and a linearised vector backbone) in order to produce novel circular plasmids, following the manufacturer's instructions.

2.7. POLYMERASE CHAIN REACTION (PCR) TECHNIQUES

2.7.1. Oligonucleotide primers

For the amplification of genomic DNA fragments, oligonucleotide primers were designed based on gene sequences of interest recorded in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). The internet package Primer 3 Version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was employed in designing primers to anneal to specific regions of interest. Unless otherwise stated default settings were employed but with both "Max Self Complementarity" and "Max 3′ Self Complementarity" settings were adjusted to 2, to reduce the likelihood of hairpin loops and dimerisation. A BLAST search

was performed for each primer against 'Short Nearly Exact Matches' in the NCBI (National Centre for Biotechnology Information) GenBank database (Bethesda, USA) to confirm specificity for the gene of interest.

For the amplification of plasmid DNA fragments, the plasmid DNA sequence was entered into the VectorNTI Version (v.) 11 software package (Invitrogen, Paisley, UK) which generated a map of the plasmid. Using VectorNTI v.11, oligonucleotide primers for gene sequences of interest were generated.

All oligonucleotide primers were synthesised by MWG Biotech, Ebersberg, Germany (http://www.mwg-biotech.com/html/s_synthesis/s_overview.shtml). Stock solutions were made by resuspension in sterile reverse-osmosis water to a concentration of 100 pmol μl^{-1}. Working solutions were made by dilution with sterile water to 10 pmol μl^{-1}. Oligonucleotide primers were stored in solution form at -20°C.

2.7.2. PCR amplification of DNA using Phusion™ high-fidelity DNA polymerase

Phusion™ high fidelity DNA polymerase (New England Biolabs, Hitchin, UK), was employed for cloning and sequencing reactions since it was more robust and higher yielding than Taq polymerase, with enhanced extension times of 1 Kbp per 15 sec and low error rates at 4.4 X 10^{-7} in Phusion HF buffer and 9.5 X 10^{-7} in GC buffer. Reaction volumes comprised of 10.0µl of Phusion HF or GC buffer (5X), 1µl of dNTPs (10mM), 5µl of both forward and reverse primers (10 pmol μl^{-1}), DNA (1 - 50 ng), 1.5µl of DMSO, 0.5µl of Phusion DNA polymerase and SDW to a total volume of 50µl. PCR conditions required 30 cycles of denaturation at 98°C (5 – 10 sec), annealing at the T_m temperature assigned to the primer sequence and extension at 72°C for 15 sec per 1 Kbp amplicon.

2.7.3. PCR amplification of DNA using RedTaq DNA polymerase

For all Taq polymerase PCR reactions, the hotstart technique was employed to reduce the likelihood of amplifying spurious DNA. RedTaq® polymerase (Sigma) was employed in reaction volumes containing 7.0 µl of ready-mixed RedTaq® polymerase mixture, 1µl of both forward and reverse primers (10 pmol μl^{-1}), DNA (1 - 50 ng), and sterile water to a total volume of 15 µl. PCR conditions required denaturation at 94°C, annealing at the T_m temperature assigned to the primer sequence and extension at 72°C for 1 min per 1 Kbp amplicon.

2.8. SEQUENCING AND COMPUTATIONAL ANALYSIS OF DNA SEQUENCES

DNA fragments from gel extracted PCR reactions and plasmid extractions from *E. coli* were initially size resolved to a molecular marker run along side the amplified samples including 100 bp (Promega) and 1 Kbp ladders (New England Biolabs) for appropriate fragment sizes. Once fragments appeared to be the desired size, DNA was gel extracted (Section 2.6.3.) and sequenced.

2.8.1. Dideoxy sequencing of DNA

Plasmid DNA extracted from transformed *E. coli* DH5α bacterial cultures was sequenced in both directions using the Value Read, Single Read Service based on Sanger *et al.* (1977) sequencing methods by Eurofins MWG (Ebersberg, Germany) (http://www.mwg-biotech.com/html/i_custom/i_valueread.shtml).

Sequencing was performed using the ABI 3730 XL capillary sequencer with BigDye v.3.1 dye-terminator chemistry as per manufacturer's instructions (Applied Biosystems) and typically returned sequencing reads up to 1 kbp. Each of the four dideoxy terminators was tagged with a different fluorescent dye which fluoresced upon illumination at specific wavelengths and produced a chromatogram from which sequences were deduced.

2.8.2. Comparative sequence analyses

Two software types were employed to align both nucleotide and amino acid sequences. ClustalW version 1.83 (http://www.ebi.ac.uk/Tools/clustalw2/index .html) was employed to align nucleotide and amino acid sequences at default settings with 'gap open penalty' set to 15.0 and 'gap extension penalty' set to 6.66 for nucleotides and 0.2 for amino acids. AlignX software from VectorNTI version 11 (Invitrogen) was similarly employed using identical parameters. Sequence identities were calculated using the Dot Matrix algorithm, VectorNTI version 11 at default settings with the window size set to 5 and stringeny set to 30% (the minimal number of matches in the window to cause a dot to be set in the matrix).

2.8.3. Sequence profiling

All genomic, PCR and plasmid sequence data were characterised and annotated using vector map software from the Vector NTI v.11 package (Invitrogen). Gene prediction software was employed to detect putative novel genes from *de novo* sequence data (http://genes.mit.edu/GENSCAN.html). Secondary structure and thermodynamic profiling of all sequences was performed using the relevant functions within the Vector NTI v.11 package.

2.9. DATA ANALYSIS

Unless stated otherwise, data collected from experiments were processed in Microsoft Excel 2003 for statistical analysis. Regression, descriptive statistics and analysis of variance were conducted correspondingly with appropriate parameters in Microsoft Excel 2003. In all analyses, a probability of $P = 0.05$ or less was taken to be significant.

CHAPTER 3. TISSUE CULTURE OF JICAMA [*PACHYRHIZUS EROSUS* (L.) URBAN] AND BAMBARA GROUNDNUT [*VIGNA SUBTERRANEA* (L.) VERDC.]

3.1. INTRODUCTION

During recent years, tissue culture techniques have been recognised as valuable tools in crop improvement programmes. Legume cultures were first established in the 1950s by Nickell (1956). The published literature on the tissue culture of legumes shows that regeneration is achievable, but occurs at low frequency. A major limitation to increasing genetic variability through tissue culture in legumes is the poor ability of isolated tissues to regenerate into whole plants (Mehta and Ram, 1980).

Forage legumes (*e.g.* clovers) have been found to be more amenable to *in vitro* regeneration than seed legumes (Phillips and Collins, 1984). The culture of seed legumes often requires highly specific phytohormone concentrations, and in some cases, unique additives are necessary. The establishment of optimised tissue culture systems for underutilised seed legumes is therefore an integral part of crop improvement programmes which aim to produce disease-resistant and nutritionally-improved crops.

3.1.1. Advances in the culture of jicama

Most of the published scientific studies on jicama have focused mainly on the morphological development of the plant *ex vitro*, and on the characterisation of the nutritive components of the tuber (Gomes *et al.*, 1997) and seeds (Moralles-Arellano *et al.*, 2001). There is, however, relatively less documentation of attempts at studying the development of the plant *in vitro*. The only available published study is by Cadiz and co-workers (2000), who investigated the nodulation process in jicama roots by establishing an *in vitro* hairy root-Rhizobia co-culture between jicama and *Rhizobium* spp.

The successful micropropagation of legume species such as common bean (*Phaseolus vulgaris*) (Zambre *et al.*, 1998), cowpea (*Vigna unguiculata*) (Brar *et al.*, 1999) and peanut (*Arachis hypogea*) (Ponsamuel *et al.*, 1998) suggest that the

same techniques could potentially be applied to jicama. The establishment of an optimised tissue culture system for jicama will allow for the study of the developmental stages of the plant at the cellular and gene levels, and will be a fundamental prerequisite in future genetic transformation studies.

3.1.2. Advances in the culture of Bambara groundnut

Over the past decade, Bambara groundnut has generated some interest among scientific communities and has come across as a crop with a huge potential to be integrated into sustainable agricultural systems (Azam-Ali *et al.*, 2001; Baryeh, 2001; Heller *et al.*, 1997). The improvement of the crop by traditional breeding practices is a lengthy and often unproductive process, and recently, a few scientific studies have investigated the regeneration potential of Bambara groundnut explants *in vitro*, with the aim of establishing a faster regeneration system which would be used in genetic improvement studies (Lacroix *et al.*, 2003; Ochatt *et al.*, 2002, Koné *et al.*, 2007). These studies, however, have been focused on only a few Bambara groundnut landraces, mainly from Mali, Ghana, and the Ivory Coast (Lacroix *et al.*, 2003; Koné *et al.*, 2007), and the responses of the crop *in vitro* have been found to be landrace-dependent (Koné *et al.*, 2007). It is therefore essential that similar studies be carried out on other Bambara groundnut landraces from other countries, to ensure that established techniques can be applied to a maximum number of landraces with minimal variations in terms of landrace-dependent responses.

3.2. AIMS AND OBJECTIVES

The aim of the work carried out in this chapter was to determine the optimum *in vitro* conditions necessary for the explant-to-plant regeneration of jicama and Bambara groundnut. It was intended that the work carried out on Bambara groundnut would include and expand on previously published methods, namely Lacroix *et al.* (2003) and Koné *et al.* (2007). The successful establishment of the protocols determined which of the species were to be investigated further in terms of genetic transformation, and these protocols would serve as baselines for the selection and micropropagation of transgenic lines of these target species.

The objectives of the experiments carried out were:

i. To investigate the *in vitro* regenerative response of zygotic embryo, cotyledon, hypocotyl, stem, leaf and root tip explants for jicama and Bambara groundnut on MS-based medium supplemented with different PGR combinations.
ii. To establish explant-to-plant regeneration systems for jicama and Bambara groundnut based on the results obtained from (i).
iii. To investigate the pathways of *in vitro* regeneration in jicama and Bambara groundnut using histological approaches, and determine whether regeneration occurred *via* organogenesis and/or somatic embryogenesis.

3.3. MATERIALS AND METHODS

3.3.1. *Ex vitro* germination and maturation of jicama and Bambara groundnut

Mature jicama seeds were sown in 24-cell trays containing 'Nottingham Traditional Mix' compost (Section 2.2.1.). The trays were placed in a propagator tray with the vents closed, in the glasshouse (Section 2.2.2.). A week after germination, the seedlings were transplanted into 9cm pots (1 seedling per pot) for two weeks, during which time the seedlings developed into plants. The plants were then transferred into 32L pots containing the same compost mix, and allowed to grow into mature plants in the glasshouse (Section 2.2.2.) where they were maintained and allowed to reach maturity, flower and set seed (Results, Section 3.4.1.1.) in order to build up a seed stock.

To build up a seed stock for Bambara groundnut, mature seeds from landraces DipC, Uniswa-Red and S19-3 were sown in PVC columns (3 seeds per column) containing John Innes No.3 compost (J. Bentley, Barton-Humber, UK) and sand in a 1:1 (v/v) ratio. The columns were set up in a growth room maintained at the conditions described in Section 2.2.2. Thinning was carried out 7 days after sowing (DAS), and only 1 plant per column was allowed to grow, flower and set seed (Results, Section 3.4.2.1.). Watering was carried out on alternate days, with 100ml of tap water per column.

Harvested jicama and Bambara groundnut seeds were stored in paper bags at 4°C and used as donors to obtain explants for tissue-culture based experiments.

3.3.2. Jicama tissue culture

3.3.2.1. Preparation of explants for tissue culture

To determine the best explant type and optimum *in vitro* growth conditions for jicama regeneration, zygotic embryos and cotyledons, as well as hypocotyl, leaf, stem, and root tissues were used as starting explants in tissue culture experiments. Cotyledon and zygotic embryo explants were obtained from

surface-sterilised seeds following an overnight imbibition in sterile reverse-osmosis water, while hypocotyl, stem, leaf and root explants were collected from *in vitro*-grown 10-day old plantlets (Section 2.3.4.).

3.3.2.2. Investigations into the *in vitro* regenerative response of different jicama explant types

This experiment aimed at determining the concentrations of the cytokinin N^6-benzylaminopurine (BAP) and the auxin α-naphthaleneacetic acid (NAA) in Murashige and Skoog (1962) (MS)-based medium (Appendix I, Sections AI.1 and AI.2) that would be optimum for the *in vitro* regeneration of jicama shoots and roots, respectively.

Jicama explants [cotyledons, excised zygotic embryos (whole and transversely sliced), hypocotyls, leaves, stems and roots] were cultured on MS-based medium (Appendix I, Section AI.2) containing 35 different combinations of BAP and NAA as shown in Fig. 3.1. Control explants were also cultured on MS-based medium without any plant growth regulators (PGRs), designated MS0 (Fig. 3.1.).

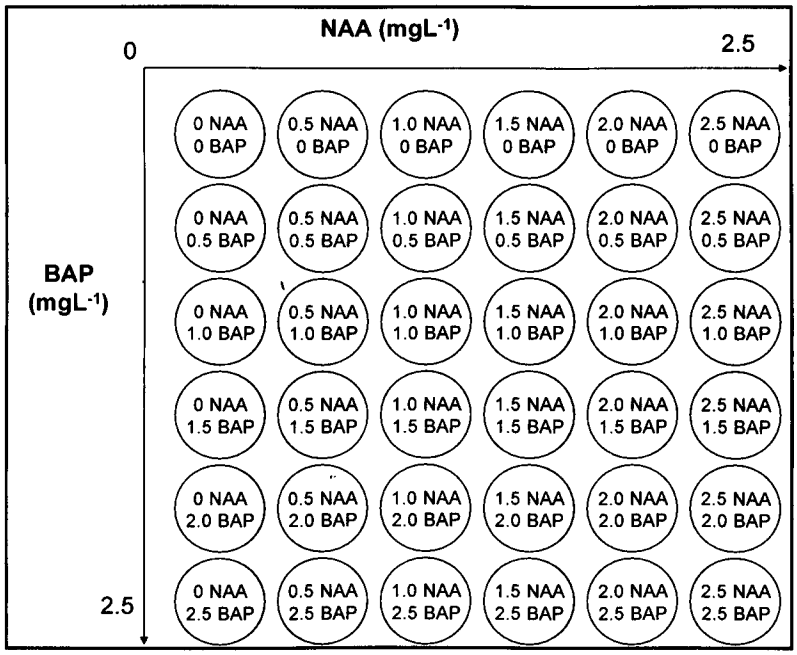


Fig. 3.1. The different combinations of BAP and NAA concentrations (in mgL^{-1}) used in the culture of jicama explants.

Three independent experiments were carried out for each explant type. In each experiment, three explants were cultured in 9cm Petri dishes each containing 20ml of each medium condition and 3 replicates of each condition were set up. The Petri dishes were kept in the tissue culture room (Section 2.3.2.), and the explants were subcultured onto their respective new medium every 2 wk.

The response of jicama explants to the different PGR concentrations was monitored on a weekly basis, and observations were recorded over a period of 12 wk to determine the best explant type and optimum concentrations of PGRs for regenerating shoots and roots *in vitro* (Results, Sections 3.4.1.2 and 3.4.1.3.). The establishment of an explant-to-plant regeneration system for jicama was based subsequently on the results obtained from this experiment, using the explant type which showed the best response to *in vitro* manipulation and the PGR concentrations which produced the highest shoot and root regeneration.

3.3.2.3. Establishment of an explant-to-plant tissue culture system for jicama

This experiment was set up to establish an *in vitro* explant-to-plant regeneration system for jicama, using whole mature zygotic embryos as starting explants, and the BAP and NAA concentrations found optimum for shoot and root regeneration (Results, Section 3.4.1.3.). At least 6 independent experiments were carried out. Each experiment contained 6 replicates, with each replicate containing 5 experimental units (*i.e.* 5 embryos).

Zygotic embryos of jicama were excised from surface-sterilised seeds (Sections 2.3.5.1. and 2.3.5.2.), and cultured in 9cm Petri dishes each containing 20ml of MS-based medium (Appendix I, Section AI.2.) supplemented with 2.5mgL^{-1} BAP, hereafter referred to as Jicama Shoot Medium (JSM) (5 embryos per dish). Embryos were subcultured onto new JSM every 2 wk, for a period of 10 wk, and the stages of shoot regeneration were monitored and recorded (Results, Section 3.4.1.4.).

Elongated jicama shoots (each $\geq$ 5cm in height) were excised from the parent explant and transferred to sterile 175ml powder round jars (3 shoots per jar), the

latter each containing 50ml of Jicama root medium (JRM). JRM was MS0 medium supplemented with 0.5mgL^{-1} NAA. The shoots were subcultured onto fresh JRM every two weeks, and monitored for signs of root formation (Results, Section 3.4.1.4.).

3.3.3. Bambara groundnut tissue culture

3.3.3.1. Investigations into the *in vitro* regenerative response of different Bambara groundnut explant types

This experiment aimed at determining the best explant type and optimum *in vitro* growth conditions for regeneration of Bambara groundnut DipC, Uniswa-Red and S19-3 landraces. Zygotic embryos, as well as cotyledon, hypocotyl, leaf, stem, and root tip sections were used as starting explants in tissue culture experiments. Cotyledon and zygotic embryo explants were obtained from surface-sterilised seeds following an overnight imbibition in sterile reverse-osmosis water, while hypocotyl, stem, leaf and root explants were collected from *in vitro*-grown 10-day old plantlets (Section 2.3.5.). Unless stated otherwise, three independent experiments per landrace were carried out for each tested protocol. In each experiment, three replicates of each treatment were set up, with 6 explants per replicate. The explants were cultured in 9cm Petri dishes (3 explants per dish) and the dishes each contained 20ml of medium with the appropriate PGRs.

3.3.3.1.1. *In vitro* culture of zygotic embryos

Following a modified protocol of the regeneration system reported in Lacroix *et al.* (2003), excised whole zygotic embryos were cultured for 6 d in 9cm Petri dishes, the latter each containing semi-solid MS medium with Nitsch and Nitsch (1969) (NN) vitamins, hereafter referred to as 'basal medium' (BM) (Appendix I, Section AI.3.), supplemented with the auxin NAA and the cytokinin BAP, both at a concentration of 1mgL^{-1}. Following this, the embryos were either kept whole, or transversely bisected before being transferred onto basal medium supplemented with combinations of BAP, NAA and triiodobenzoic acid (TIBA) at different concentrations (Table 3.1). The Petri dishes were incubated in the

tissue culture room (Section 2.3.2.), and the explants were subcultured onto their respective new medium every 2 wk.

Table 3.1. Combinations and concentrations of BAP, NAA and TIBA used for the *in vitro* culture of whole and transversely bisected zygotic embryo explants.

Media designation	BAP (mgL^{-1})	NAA (mgL^{-1})	TIBA (mgL^{-1})
E1	1	-	-
E2	1.5	-	-
E3	3	-	-
E4	0.5	-	0.5
E5	1	-	0.5
E6	1	-	1
E7	1.5	0.2	-
E8	3	0.2	-
E9	-	0.5	-
E10	-	1	-

3.3.3.1.2. *In vitro* culture of cotyledon explants

The regenerative potential of cotyledon explants was investigated for DipC, Uniswa-Red and S19-3 based on the work published by Koné *et al.* (2007). Under aseptic conditions, cotyledons from turgid seeds were cut into cubes (5mm x 5mm x 5mm) and cultured in 9cm Petri dishes, the latter each containing 20 ml of MS0 medium with the PGR combinations listed in Table 3.2. The Petri dishes were incubated in the tissue culture room (Section 2.3.2.), and the explants were subcultured onto their respective new medium every 2 wk.

Table 3.2. Concentrations and combinations of BAP and NAA used for the *in vitro* culture of Bambara groundnut cotyledon explants.

Media designation	BAP (mgL^{-1})	NAA (mgL^{-1})
C1	1	-
C2	1	0.05
C3	5	0.01
C4	5	0.5

3.3.3.1.3. *In vitro* culture of hypocotyl, leaf, stem and root explants

The regenerative response of hypocotyl, leaf, stem and root explants of Bambara groundnut (DipC, Uniswa-Red and S19-3) were also tested by culturing explants on MS0 medium (Appendix I, Section AI.2) supplemented with 25 different combinations of BAP and NAA as shown in Figure 3.2. Control explants were cultured on MS0 without any plant growth regulators (PGRs). Three independent experiments were carried out for each explant type. In each experiment, 3 explants were cultured in 9cm Petri dishes each containing 20ml of each medium condition and 3 replicates of each condition were set up. The Petri dishes were kept in the tissue culture room (Section 2.3.2.), and the explants were subcultured onto their respective new medium every 2 wk.

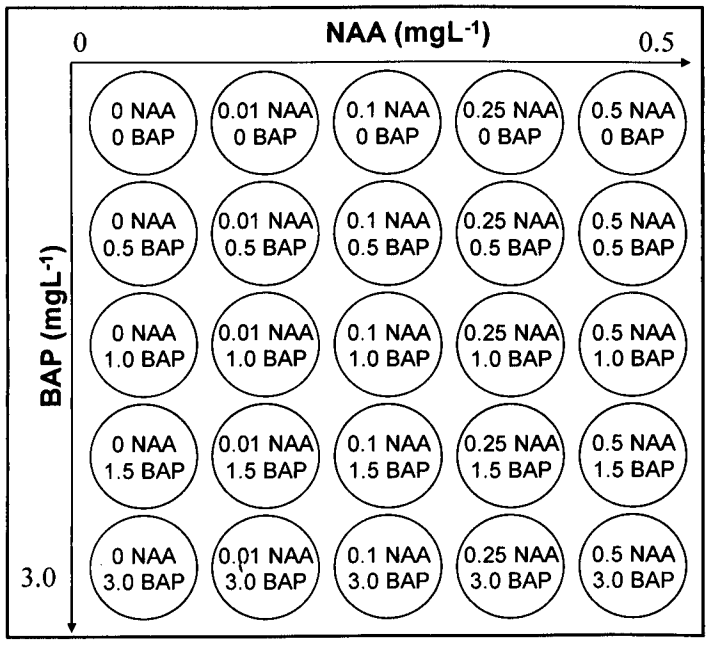


Fig. 3.2. The different combinations of BAP and NAA concentrations (in mgL^{-1}) used in the *in vitro* culture of Bambara groundnut hypocotyl, leaf, stem and root explants.

For each of the 3 experiments described above, the response of Bambara groundnut explants to the different media compositions was monitored on a weekly basis, and observations were recorded over a period of 12 wk to determine the best explant type and optimum media composition for *in vitro* shoot and root regeneration (Results, Section 3.4.2.2.). The establishment of an explant-to-plant regeneration system for Bambara groundnut was subsequently

based on the results obtained from this experiment, using the explant type which showed the best response to *in vitro* manipulation and the media composition which resulted in the highest level of shoot and root regeneration.

3.3.3.2. Establishment of an explant-to-plant tissue culture system for Bambara groundnut

Whole mature, zygotic embryos of Bambara groundnut were used as starting explants in this experiment. Seeds were surface-sterilised and prepared for embryo excision following the procedure outlined in Sections 2.3.5.1. and 2.3.5.2. At least 6 independent experiments were carried out for each landrace. Each experiment contained 6 replicates of 5 experimental units (*i.e.* 5 embryos) each. Throughout the experiments, embryos were cultured in 9cm Petri dishes each containing 20ml of relevant medium, unless stated otherwise.

Whole, excised embryos were transferred into 9cm Petri dishes containing basal medium (Appendix I, Section AI.3), supplemented with $1mgL^{-1}$ BAP and $1mgL^{-1}$ NAA, hereafter known as 'initiation medium', and incubated for 6 d in the culture room (Section 2.3.2). Embryos were then transferred into sterile 175ml powder round jars (5 explants per jar) each containing 50ml of Bambara shoot regeneration (BSR) medium. The latter consisted of basal medium supplemented with $1.5mgL^{-1}$ BAP. The embryo explants were subcultured onto new BSR medium (5 explants per jar) every 2 wk, and regenerated shoots (≥3cm in height) were regularly excised from the parent explants and subcultured into jars containing new BSR (50ml per jar) for elongation.

Following shoot regeneration, root formation was induced by transferring the elongated shoots of Bambara groundnut into sterile 175ml powder round jars (1 explant per jar) containing Bambara root regeneration (BRR) medium. BRR medium was basal medium (Appendix I, Section AI.3.) supplemented with $1mgL^{-1}$ NAA. The shoots were subcultured onto fresh BRR medium every 2 wk, until the regenerated roots began to elongate. Subculturing was terminated at that point, since initial attempts at introducing a rooted plantlet into solidified medium resulted in most of the root system to break.

Once a healthy root system was obtained, the regenerated plantlets were transferred to 9cm pots containing the compost mix described in Section 2.3.1.1., and covered with a transparent plastic sleeve. The sleeves were used to provide a simulation of the high moisture content of the *in vitro* culture conditions, as well as to minimise the probability of pathogen attacks. The sleeves were kept closed for 2 d, and a hole was punched in each sleeve everyday for 7 d. Sleeves were removed completely on day 8. The regenerated plantlets were acclimatised under growthroom conditions (Section 2.2.2.), to be morphologically assessed against seed-grown Bambara groundnut plants (Results, Section 3.4.2.5.).

3.3.4. Histological studies

To investigate the regeneration pathways in jicama and Bambara groundnut embryos cultured *in vitro*, histological techniques were employed to determine whether adventitious shoot formation from cultured embryos occurred *via* organogenesis and/or somatic embryogenesis.

3.3.4.1. Preparation of tissue material

Jicama embryos surviving on MS0 medium supplemented with 2.5mgL^{-1} BAP, and Bambara groundnut (DipC, Uniswa-Red and S19-3) embryos surviving on basal medium supplemented with 1.5mgL^{-1} BAP were processed for microscopy. In each case, 5 *in vitro*-cultured, 6-week old embryos were selected randomly, and were cut transversely into thin sections (not more than 1mm thin). From each embryo, the two middle-most sections were used for histology analyses, as the regeneration of adventitious buds was found to occur at a higher frequency at the site where the zygotic embryo was attached to the cotyledon, hereafter referred to as the 'embryonic-cotyledonary node'.

3.3.4.2. Fixation

The tissue sections were fixed in a solution of FAA. FAA was 50% (v/v) ethanol, 3.7% (v/v) formaldehyde and 5% (v/v) acetic acid. Each tissue section was placed in a 1.5ml microfuge tube containing 500µl of FAA solution, and

vacuum-infiltrated for 15 min. This was to remove any air present in the plant cells, and to allow for an easier penetration of the fixative into the tissue. The sections were fixed for 2.5 h at 4°C, with the time of vacuum infiltration counting towards the total.

3.3.4.3. Dehydration

Following fixation, the tissue samples were dehydrated in a series of ethanol solutions of increasing concentrations, to gently remove any water present in the cells, without irreversibly affecting the cellular structure and assembly in the tissue. The FAA solution in each microfuge tube was replaced with solutions of 30%, 50%, 60%, 70%, 80%, 90% and 100% (v/v) ethanol, separately, for 30min each. The dehydration process was carried out at room temperature.

3.3.4.4. Infiltration

The ethanol (E) in the dehydrated samples was gradually replaced with Histoclear (H) [National Diagnostics, Atlanta, Georgia] by incubating the samples in the following solutions for 0.5 h each, at room temperature: 25%H + 75%E; 50%H + 50%E; 75%H + 25%E. The last mixture of Histoclear and ethanol solution was then replaced by 3 changes of 100% (v/v) Histoclear (0.5 h for each concentration).

Following removal of ethanol, the tissue sections were ready for infiltration with the support medium. Paraplast (Sigma-Aldrich Co., Steinheim, Germany) was the support medium used in this protocol, and since it had a T_m of 58°C, the remaining infiltration steps in this section were carried out at a temperature of 58°C - 60°C, unless otherwise stated. The tissue samples were transferred into a mixture of 50% (v/v) Histoclear and 50% (v/v) molten Paraplast, and incubated overnight at 58°C. This mixture was replaced by 100% (v/v) molten Paraplast for 4 h. Six changes of molten Paraplast were made, each at 4 h intervals. The tissue sections were ready for embedding.

3.3.4.5. Embedding

Working on a hotplate at 58°C, molten Paraplast was poured into embedding boats, and the tissue sections were positioned at the bottom of each boat using a pair of fine, hot forceps. An embedding ring (Simport, Beloeil, Canada) was then positioned on the top of the boat, and a small volume of molten paraplast was poured into the boat to just cover the base of the embedding ring. The embedding boat and ring were transferred into a sterile casserole dish containing a mixture of ice and water, and the paraplast allowed to harden at the low temperature for at least 20 min.

Once hardened, the Paraplast blocks were removed from the embedding boats (which acted as moulds) with the embedding rings still attached to them. The paraplast blocks containing the embedded Bambara groundnut tissue were stored in a cool and dry place at room temperature, until required for sectioning.

3.3.4.6. Tissue sectioning and microscopy analysis

The blocks of embedded tissue were sectioned to 8μm using a rotary microtome. Ribbons of Paraplast sections were floated onto drops of water placed on polylysine coated slides (VWR International, Lutterworth, UK), and the slides were placed on a slide warmer at 42°C until the paraplast sections were no longer wrinkled. The excess water was drained out of the slides using a piece of clean tissue paper, and the slide was left to dry overnight at room temperature.

Once dried, the slides were cleared by immersion in a solution of Histoclear for 10 min. This removed the dried Paraplast from the slides, leaving only the tissue section. The tissue sections were then rehydrated by immersing the slides in ethanol solutions of decreasing concentrations [90%, 70%, 50% and 30% (v/v)] for 2 min each, followed by immersion in sterile reverse-osmosis water for 5 min. This step also removed any traces of Histoclear from the slides. The tissue sections were ready to be stained.

The tissue sections were stained by immersing the slides in a 0.1% (v/v) methylene blue solution for 30 min. The slides were dried at room temperature and coverslips were mounted onto the stained tissue using a few drops of Histomount (National Diagnostics, Atlanta, Georgia). The Histomount was allowed to dry for several hr. The mounted tissue sections were then ready to be examined under the stereomicroscope (Results, Sections 3.1.4.5. and 3.4.2.6.).

3.4. RESULTS

3.4.1. Jicama tissue culture

3.4.1.1. *Ex vitro* development of seed-derived jicama plants

Seed-derived jicama plants were cultivated *ex vitro* in 32L pots containing 'Nottingham Traditional Mix' compost (Section 2.2) in order to build up a seed stock for subsequent tissue culture-based experiments (MM, Section 3.3.1). Seeds germinated within 7 d after sowing (DAS), and the germination rate was 82%. Mature plants grew to approximately 2m in height (Fig. 3.3, A). Flowering (Fig. 3.3, B) occurred between 8 – 10 mth after sowing, and seed set (Fig. 3.3. C) was observed at 9 – 11 mth after sowing. Harvested seeds ranged from brown to olive green in colour and an average of 0.2kg of seeds per plant was obtained.

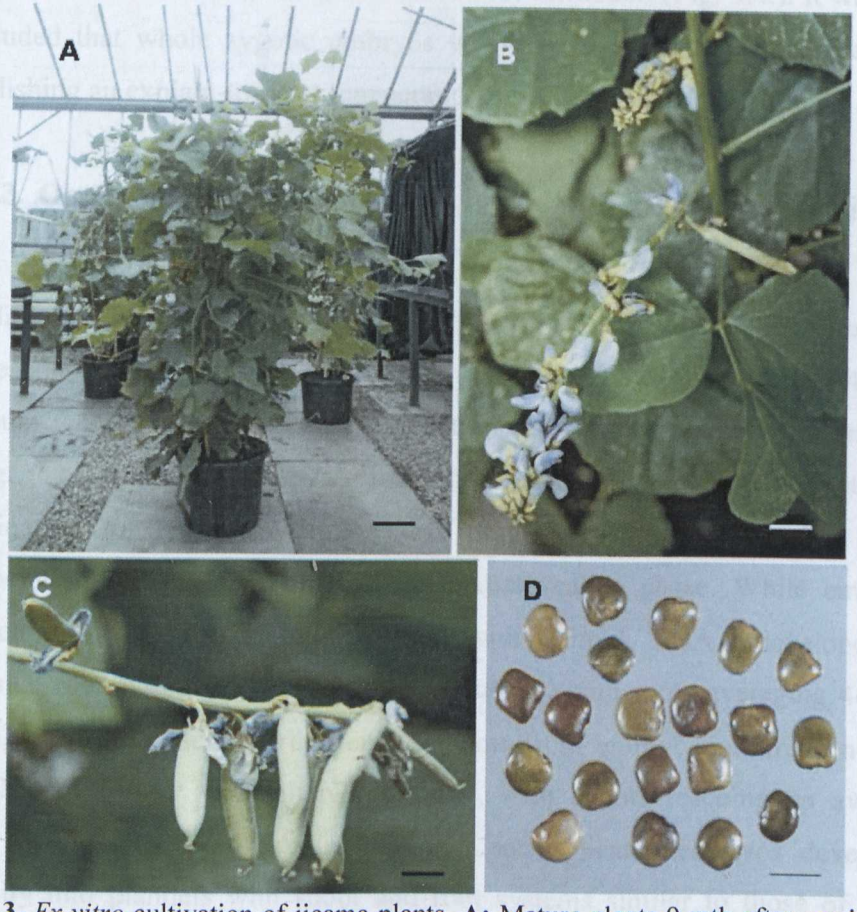


Fig. 3.3. *Ex vitro* cultivation of jicama plants. **A:** Mature plants 9 mth after sowing; **B:** Onset of flowering; **C:** Seed pod formation; **D:** Harvested seeds.
Bars: A=15cm, B=2cm, C=1cm, D=1cm

3.4.1.2. Regenerative response of different explant types to *in vitro* manipulation

Six explant types (cotyledons, whole and transversely sliced zygotic embryos, hypocotyls, leaves, stems and roots) were cultured on MS0.8 medium with different BAP and NAA concentrations over a 12-week period to test for their potential to regenerate *in vitro* (Section 3.3.2.2.). Cultured leaf, stem and root material turned necrotic within 2 wk and did not show any signs of regeneration. Cotyledon explants turned green as a result of chlorophyll production, while hypocotyl and transversely cut embryo explants visibly expanded. However, none showed any sign of callus or adventitious organ formation during the tested period. Whole zygotic embryos were the only explant type that exhibited signs of regeneration out of all the explant types tested, either through callus formation, adventitious bud formation or adventitious root formation (Fig. 3.4.). It was thus concluded that whole zygotic embryos would be used as starting explants in establishing an explant-to-plant regeneration system for jicama.

3.4.1.3. Optimum PGR concentrations for *in vitro* regeneration of jicama zygotic embryos

The different combinations of BAP and NAA (Fig. 3.1.) had different effects on the response of the jicama embryos *in vitro*, as shown in Figure 3.4. The optimum combination of PGR concentrations for shoot regeneration was $2.5mgL^{-1}$ of BAP without NAA for shoot regeneration, while the optimum combination for root regeneration was $0mgL^{-1}$ BAP and $0.5mgL^{-1}$ NAA. Regeneration occurred without an intermediate callus phase. While embryos cultured on MS medium supplemented with $0.5mgL^{-1}$ NAA developed an extensive root system after only 2 wk in culture, with roots averaging 4cm in length, the development of regenerated shoots from embryos cultured on MS0 with $2.5mgL^{-1}$ BAP was relatively slower, with shoots reaching an average height of 1.5cm after 4 wk in culture. Control jicama embryos developed normally into plantlets with shoot and root systems similar to those of seed-grown jicama plants.

In the remaining conditions, the cultured embryos showed 3 main responses to their respective combinations of PGR concentrations. Either the embryos developed a single shoot and root, or the embryos expanded, and pale brown callus formed around the embryonic-cotyledonary node of each embryo. The third response was an expansion of the embryos for the first 2 wk in culture, followed by an arrested development in the subsequent weeks. Those embryos exhibited neither shoot nor root formation.

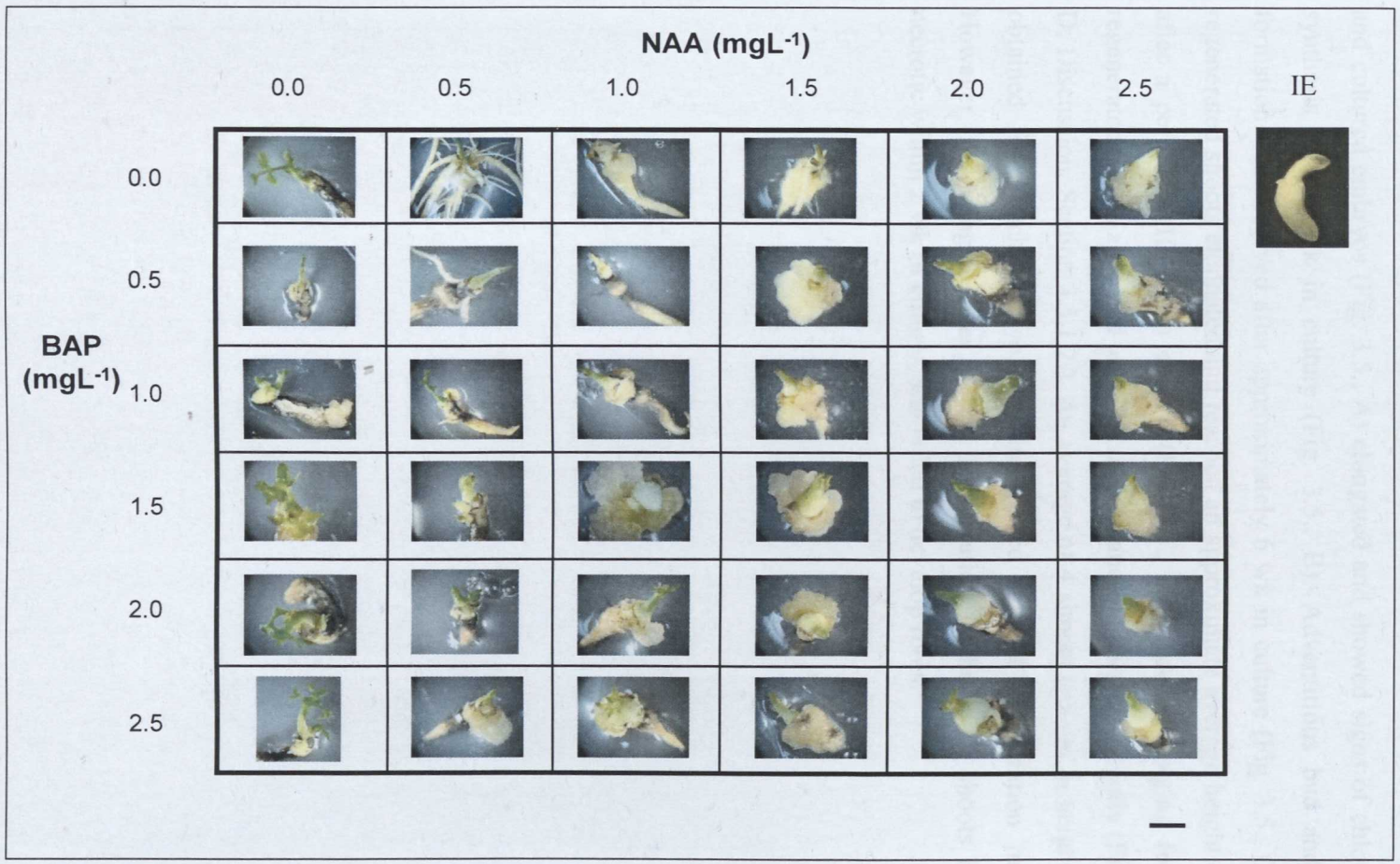


Fig. 3.4. The response of jicama embryos to different combinations of BAP and NAA concentrations after 4 wk in culture, with reference to a freshly excised initial explant (IE). (Bar = 5mm)

3.4.1.4. Establishment and maintenance of jicama tissue culture system

The stages of shoot regeneration in jicama are shown in Plate 2.2. The excised and cultured embryos (Fig. 3.5., A) elongated and showed signs of chlorophyll synthesis after 2 wk in culture (Fig. 3.5., B). Adventitious bud and shoot formation was observed after approximately 6 wk in culture (Fig. 3.5., C). The regenerated shoots elongated and reached an approximate average height of 5cm after a period of 10 wk in culture (Fig. 3.5., D). Leaves emerging from the regenerated shoots appeared under developed and did not expand fully (Fig. 3.5., D; Discussion, Section 3.5.1.2.). An average of 4 shoots (>5 cm in height) were obtained from each embryo, and transferred to root regeneration medium. However, following transfer to root regeneration medium, all shoots became necrotic within 2 wk of culture, and failed to develop roots.

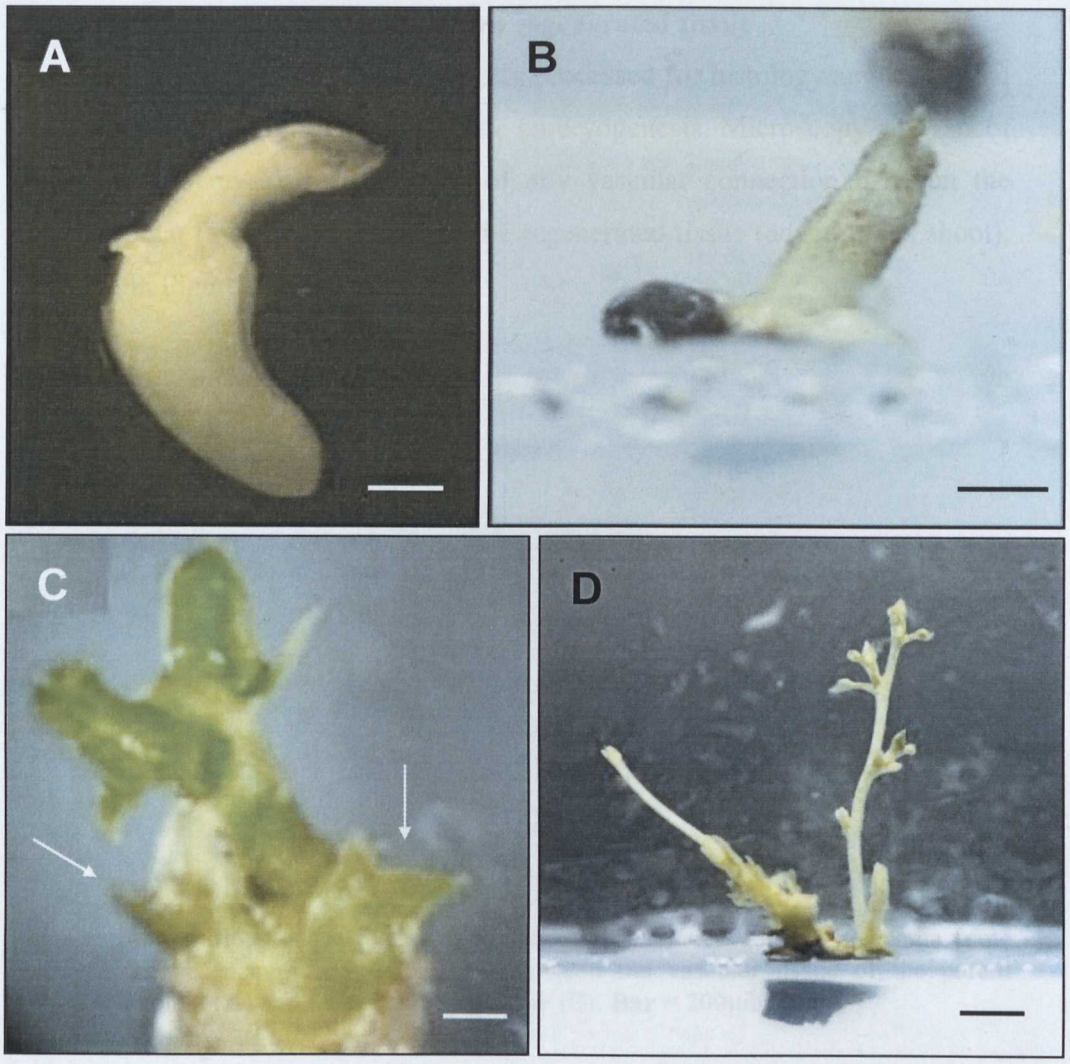


Fig. 3.5. *In vitro* shoot regeneration of jicama from zygotic embryo. **A:** Freshly excised zygotic embryo of jicama; **B:** Elongation of excised embryo within 6 days of culture on JSM (MS0 suplemented with 2.5mgL^{-1} BAP); **C:** Adventitious shoot formation (arrows) from zygotic embryo after 6 wk of culture on JSM; **D:** Adventitious shoot elongation after 10 wk of culture on JSM with underdeveloped leaf structures. **Bars:** A = 0.5mm; B-C = 1mm; D = 1cm

3.4.1.5. Histological analysis of *in vitro* regenerated tissue

A preliminary analysis of jicama samples processed for histology suggested that shoot regeneration occurred *via* somatic embryogenesis. Microscopy analysis of tissue sections revealed an absence of any vascular connection between the parent explant (zygotic embryo) and the regenerated tissue (adventitious shoot), as shown in Figure 3.6.

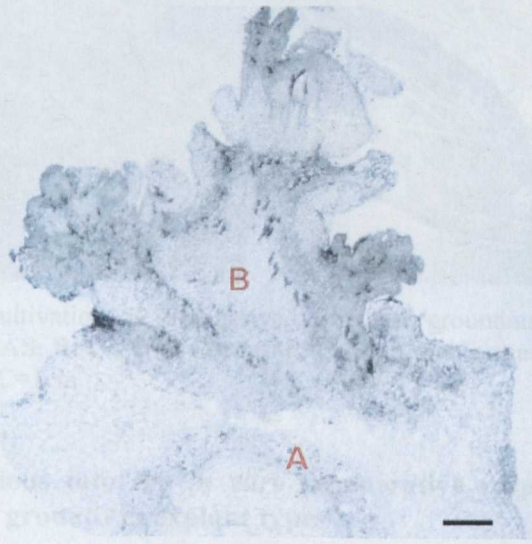


Fig. 3.6. Adventitious tissue regeneration *via* somatic embryogenesis in jicama [note the absence of any connection between the vascular tissue of the parent explant (A) and the regenerated tissue (B). **Bar** = 200μm.

3.4.2. Bambara groundnut tissue culture

3.4.2.1. *Ex vitro* development of seed-derived Bambara groundnut plants

Seed-derived Bambara groundnut plants (landraces DipC, Uniswa-Red and S19-3) were cultivated *ex vitro* under growthroom conditions (Section 2.2) to obtain a seed stock for future tissue culture-based experiments (MM, Section 3.3.3). The stages of development were recorded, as illustrated in Figure 3.7. Bambara groundnut plants reached maturity around 30 DAS (Fig. 3.7., A). The onset of flowering (Fig. 3.7., B) was at 42 DAS, followed by seed set (Fig. 3.7., C), which occurred between 60 – 80 DAS. The seed pods were left to dry before being harvested. The average number of seeds harvested per plant was 43 for DipC, 38 for Uniswa-Red and 47 for S19-3.

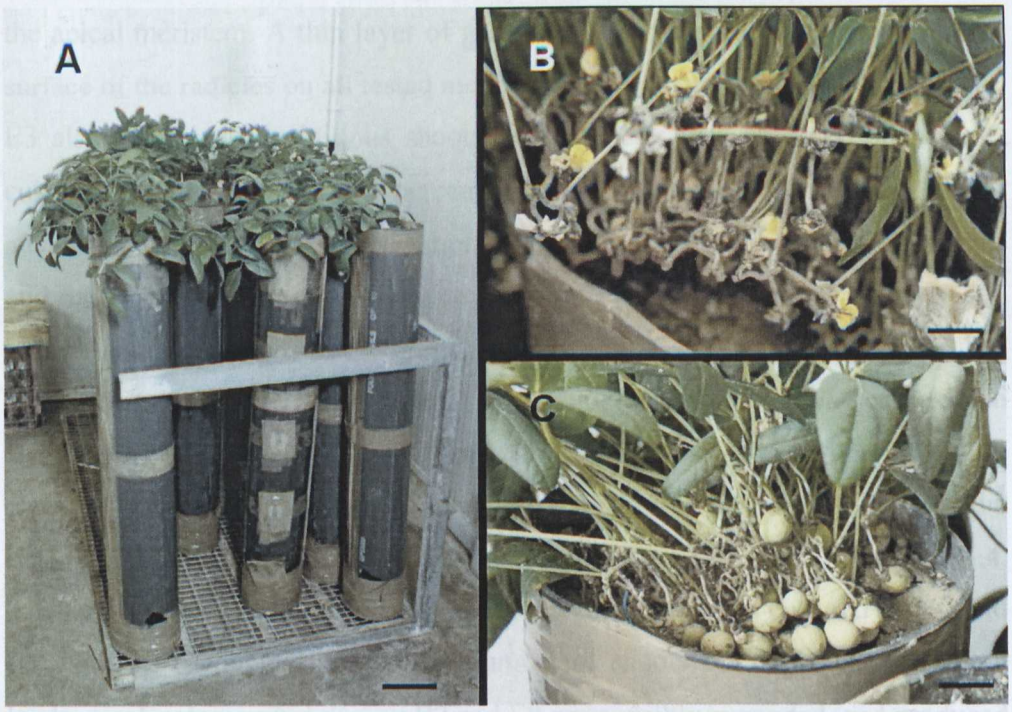


Fig. 3.7. *Ex vitro* cultivation of seed-derived Bambara groundnut plants. **A:** Mature plants 30DAS; **B:** Onset of flowering; **C:** Seed pod formation. **Bars:** A=20cm, B=1.5cm, C=1cm.

3.4.2.2. Investigations into the *in vitro* regenerative response of different Bambara groundnut explant types

Six explant types (cotyledons, whole and transversely sliced zygotic embryos, hypocotyls, leaves, stems and roots) from landraces DipC, Uniswa-Red and S19-3 were cultured on media of different compositions over a 12-wk period to test for their potential to regenerate *in vitro* (Section 3.3.3.1.).

3.4.2.2.1. Regenerative response of zygotic embryos

This experiment was modified from a published protocol by Lacroix and co-workers (2003) [MM, Section 3.3.3.1.1.]. Whole and transversely bisected zygotic embryos (DipC, Uniswa-Red and S19-3) were cultured on basal medium supplemented with BAP and NAA at different concentrations (Table 3.1.). Bisected embryos showed limited regeneration in all 3 landraces. Plumule sections did not exhibit signs of callogenesis, adventitious shoot organogenesis or rhizogenesis on any of the tested media. They turned green as a result of chlorophyll production, and only produced an average of 1 shoot per explant, at

the apical meristem. A thin layer of green callus tissue was observed on the cut surface of the radicles on all tested media; moreover, radicles cultured on E2 and E3 also produced adventitious shoots. Rhizogenesis was observed on radicles cultured on E9 and E10. The percentage of cultured explants which exhibited adventitious shoot and root formation, as well as the average number of shoots and roots produced per explant for each landrace is given in Table 3.3.

Pale yellow callus developed around the radicle of whole embryos cultured on E1, with no subsequent differentiation. This phenomenon was also observed on embryos cultured on E4, E5 and E6. Embryos cultured on the remaining media types exhibited shoot and root organogenesis, as detailed in Table 3.4. Adventitious shoot organogenesis was observed on embryos cultured on E2 and E3, with E2 producing the greatest number of shoots per explant across the 3 landraces. Explants surviving on E7 and E8 were visibly expanded, and produced on average 1 adventitious shoot per explant, without an intermediate callus phase. Embryos cultured on E9 and E10 exhibited rhizogenesis with no intermediate callus phase.

Table 3.3. Shoot organogenesis and rhizogenesis in Bambara groundnut radicle explants after 12 wk in culture

Medium	DipC				Uniswa-Red				S19-3			
	Adventitious shoot organogenesis (%)	Average no. of shoots formed	Rhizogenesis (%)	Average no. of roots formed	Adventitious shoot organogenesis (%)	Average no. of shoots formed	Rhizogenesis (%)	Average no. of roots formed	Adventitious shoot organogenesis (%)	Average no. of shoots formed	Rhizogenesis (%)	Average no. of roots formed
E2	85.2	1.8 ± 0.8	0	0.0 ± 0.0	100	1.5 ± 0.9	0	0.0 ± 0.0	100	2.1 ± 0.7	0	0.0 ± 0.0
E3	75.9	1.9 ± 0.9	0	0.0 ± 0.0	100	1.8 ± 0.7	0	0.0 ± 0.0	100	2.0 ± 0.8	0	0.0 ± 0.0
E9	0	0.0 ± 0.0	96.3	3.5 ± 1.2	0	0.0 ± 0.0	79.6	2.6 ± 1.4	0	0.0 ± 0.0	94.4	4.2 ± 1.3
E10	0	0.0 ± 0.0	90.7	3.8 ±1.2	0	0.0 ± 0.0	85.1	3.9 ± 1.7	0	0.0 ± 0.0	92.6	4.4 ± 1.4

Table 3.4. Shoot organogenesis and rhizogenesis in whole Bambara groundnut embryos after 12 wk in culture

Medium	DipC				Uniswa-Red				S19-3			
	Adventitious shoot organogenesis (%)	Average no. of shoots formed	Rhizogenesis (%)	Average no. of roots formed	Adventitious shoot organogenesis (%)	Average no. of shoots formed	Rhizogenesis (%)	Average no. of roots formed	Adventitious shoot organogenesis (%)	Average no. of shoots formed	Rhizogenesis (%)	Average no. of roots formed
E2	96.3	5.2 ±1.2	0	0.0 ± 0.0	100	5.5 ± 0.6	0	0.0 ± 0.0	100	5.1 ± 0.9	0	0.0 ± 0.0
E3	92.6	5.0 ± 1.5	0	0.0 ± 0.0	96.3	4.7 ± 1.1	0	0.0 ± 0.0	98.1	4.4 ± 0.8	0	0.0 ± 0.0
E7	90.7	1.0 ± 0.4	0	0.0 ± 0.0	90.7	1.0 ± 0.5	0	0.0 ± 0.0	88.9	1.0 ± 0.5	0	0.0 ± 0.0
E8	92.6	1.4 ± 0.6	0	0.0 ± 0.0	88.9	1.1 ± 0.6	0	0.0 ± 0.0	94.4	1.1 ± 0.4	0	0.0 ± 0.0
E9	0	0.0 ± 0.0	88.9	3.1 ± 1.2	0	0.0 ± 0.0	98.1	3.6 ± 0.8	0	0.0 ± 0.0	96.3	3.5 ± 0.9
E10	0	0.0 ± 0.0	87	4.8 ± 1.9	0	0.0 ± 0.0	92.6	5.1 ± 1.5	0	0.0 ± 0.0	90.7	5.0 ± 1.7

3.4.2.2.2. Regenerative response of cotyledon explants

Cotyledon explants from Bambara groundnut DipC, Uniswa-Red and S19-3 were cultured following a modified protocol by Koné and co-workers (2007) [MM, Section 3.3.3.1.2.]. Cotyledons that responded to culture turned from white to green, as a result of chlorophyll production, within 7 – 14 d. Formation of callus, which remained green throughout the 12 wk testing period, was observed on the cut edges of the explants. Low BAP concentrations appeared to promote rhizogenesis, as explants cultured on media C1 and C2 exhibited adventitious root formation (1 – 2 roots per explant). There was, however, no occurrence of shoot organogenesis from the cotyledons on any of the tested culture media.

3.4.2.2.3. Regenerative response of hypocotyl, leaf, stem and root explants

Hypocotyl, leaf, stem and root tip explants were cultured on MS0 supplemented with 25 combinations of BAP and NAA at different concentrations (Fig. 3.2.). Leaf and stem explants showed no sign of regeneration on any of the tested culture media, and exhibited necrosis within 2 wk of culture. Elongation was observed in all root explants during the first week in culture and within 3 wk of culture, limited formation of pale yellow callus was observed at the cut end of the explants at the BAP/NAA combinations presented in Table 3.5. However, proliferation of the callus mass failed to occur, and necrosis set in within the following 7 - 10 d. Limited callogenesis was also observed at the cut ends of hypocotyls within 5 wk of culture. The callus tissue remained green until the end of the 12-week testing period, but did not proliferate. The percentage of hypocotyl explants showing occurrence of callus formation for each landrace is detailed in Table 3.6. None of the explant types displayed organogenesis or rhizogenesis on the tested media compositions.

Table 3.5. Regenerative response of root tip explants of Bambara groundnut on MS0 medium supplemented with BAP and NAA at varying concentrations.

BAP conc. (mgL^{-1})	NAA conc. (mgL^{-1})	Root tip explants exhibiting callogenesis (%)		
		DipC	Uniswa-Red	S19-3
1.5	0	61.1	55.6	61.1
3	0	77.8	9.3	68.5
3	0.5	74.1	0	61.1

Table 3.6. Regenerative response of hypocotyl explants of Bambara groundnut on MS0 medium supplemented with BAP and NAA at varying concentrations.

BAP conc. (mgL^{-1})	NAA conc. (mgL^{-1})	Hypocotyl explants exhibiting callogenesis (%)		
		DipC	Uniswa-Red	S19-3
1	0	42.6	22.2	44.4
1.5	0	50	18.5	64.8
3	0	72.2	18.5	75.9
0	0.1	31.5	-	-
1.5	0.1	40.7	-	51.9
0	0.25	-	-	5.6
1.5	0.25	57.4	-	29.6
0	0.5	20.4	-	-
3	0.5	68.5	-	72.2

3.4.2.3. Establishment of an explant-to-plant regeneration system for Bambara groundnut

An explant-to-plant regeneration system for Bambara groundnut was established based on a modified protocol of the method published by Lacroix and co-workers (2003) [MM, Section 3.3.3.2.]. Preliminary investigations demonstrated that, for each tested landrace (DipC, Uniswa-Red and S19-3), the best regenerative response was achieved through the culture of whole zygotic embryos (Section 3.4.2.2.1.), and as such, they were used as starting explants. Following *in vitro* embryo excision and culture, the stages of shoot and root regeneration and development in Bambara groundnut (DipC, Uniswa-Red and S19-3) were monitored and assessed morphologically.

Figure 3.8. shows the different stages of shoot and root development from cultured Bambara groundnut DipC embryos. This sequence of stages has also been observed in landraces Uniswa-Red and S19-3. Excised embryos of Bambara groundnut (Fig. 3.8., A) had green pigmentation, brought about by chlorophyll synthesis, at the shoot apical meristem within the first 6 days of culture on basal medium (Appendix I, Section AI.3.) supplemented with $1mgL^{-1}$ BAP and $1mgL^{-1}$ NAA.

Within 2 wk of their transfer onto BSR medium (MM, Section 3.3.3.2.) all the zygotic embryos had increased in girth, and structures which bore a strong resemblance to somatic embryos (Fig. 3.8., B), as well as adventitious buds (Fig. 3.8., C), were observed around the base of the upper half of the embryos, more specifically at the embryonic-cotyledonary node.

Elongated shoots (Fig. 3.8., D) were rooted (Fig. 3.8., E) in BRR medium (MM, Section 3.3.3.2.), and regenerated 12-week old Bambara groundnut plantlets were acclimatised *ex vitro* (Fig. 3.8., F) in compost, under growth room conditions (Section 2.2.).

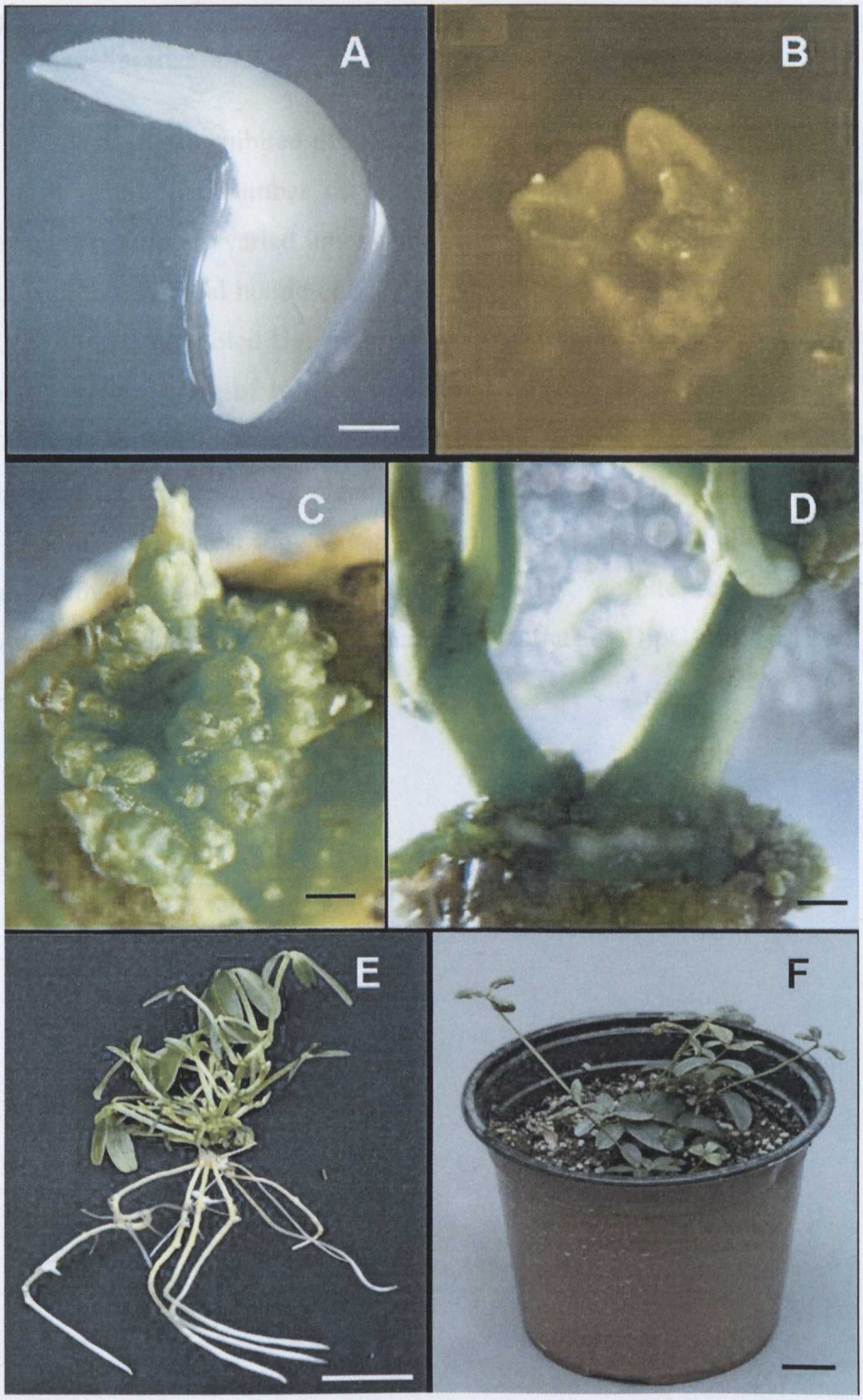


Fig. 3.8. Bambara groundnut plant regeneration *via* tissue culture of whole zygotic embryos. **A:** Freshly excised Bambara groundnut zygotic embryo; **B:** Embryo-like structures regenerated at the embryonic-cotyledonary node; **C:** Multiple adventitious buds regenerated from embryo explant, suggesting organogenesis; **D:** Adventitious shoots elongating on shoot regeneration medium (BSR); **E:** *In vitro* root regeneration from a cultured Bambara groundnut shoot; **F:** Regenerated Bambara groundnut plantlet acclimatised *ex vitro*. **Bars:** A, C-D = 0.5mm; B = 200µm; E-F = 1cm.

3.4.2.4. Landrace-dependent differences in the response to *ex vitro* acclimatisation

The three landraces exhibited different stress levels when acclimatised *ex vitro*, and as a result, the number of regenerated plantlets which survived under glasshouse conditions varied among the landraces. The plants did not seem to adapt well to the initial humid conditions produced by the sleeves (MM, Section 3.3.3.2.). All of the rooted Uniswa-Red plantlets were attacked by a white fungal pathogen within 1 wk of being acclimatised *ex vitro*, and none survived. The same fungal pathogen destroyed 57% of the DipC plantlets, and 63% of the S19-3 plantlets. Of the remaining plantlets, only 3 out of 38 DipC, and 4 out of 26 S19-3 survived. The rest showed signs of wilting as early as 2 d after the sleeves were removed, and were dead by the 10^{th} day, despite regular watering. Flowering was observed in the 7 remaining plantlets (3 DipC and 4 S19-3), but seed set did not occur.

3.4.2.5. Morphological comparison of *in vitro* generated plants to seed-derived plants

The observations made for the surviving regenerated plants of DipC and S19-3 landraces were similar for both landraces. The leaves and flowers of *in vitro* regenerated Bambara groundnut plants were morphologically similar to those of seed-grown plants, *i.e.* leaves were trifoliate, and pea-shaped yellow flowers were produced approximately 8 wk following acclimatisation. The main difference between *in vitro* regenerated plants and plants grown from seeds *ex vitro* was a significant reduction in the overall size of *in vitro* regenerated plants (Fig. 3.9.). The plant height and leaf length of *in vitro* generated plants was approximately one third that of seed-grown plants (Discussion, Section 3.5.2.3.).

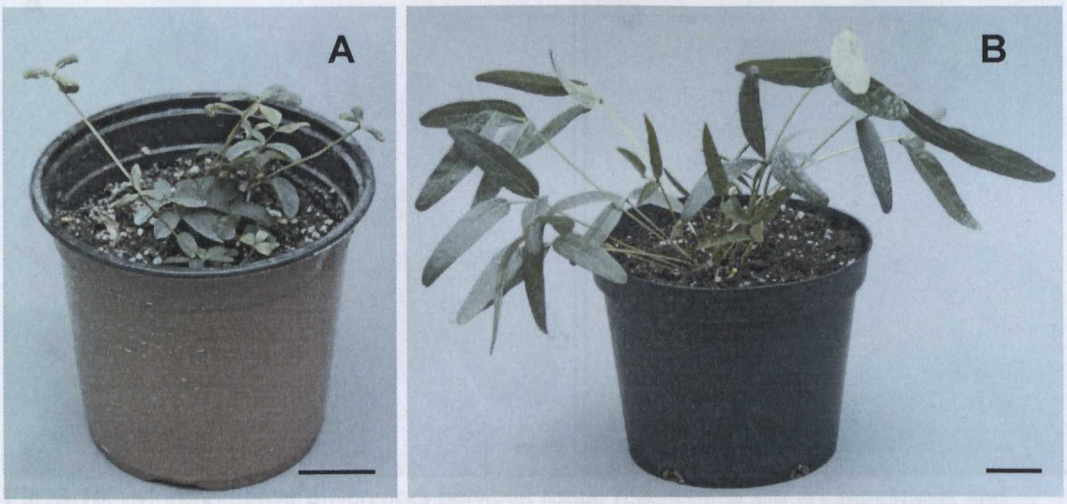


Fig. 3.9. Morphological comparison of an *in vitro* generated plant to an *ex vitro* seed-grown plant of Bambara groundnut DipC. **A:** A DipC plant, regenerated *in vitro* and acclimatised under growthroom conditions, 8 wk after root initiation *in vitro*; **B:** A DipC plant grown *ex vitro* from seed, 60 DAS. **Bars** = 1cm.

3.4.2.6. Histological analysis of *in vitro* regenerated tissue

A preliminary analysis of Bambara groundnut samples processed for histology suggested that shoot regeneration occurred both *via* organogenesis and somatic embryogenesis in landrace DipC, while the examination of tissue sections for landraces Uniswa-Red and S19-3 suggested that regeneration occurred *via* somatic embryogenesis only. Figure 3.10. (A and B) shows the difference between regeneration *via* organogenesis and regeneration *via* somatic embryogenesis in a DipC embryo. Similar observations to Figure 3.10 (B) were made from tissue sections of Uniswa-Red and S19-3 embryos.

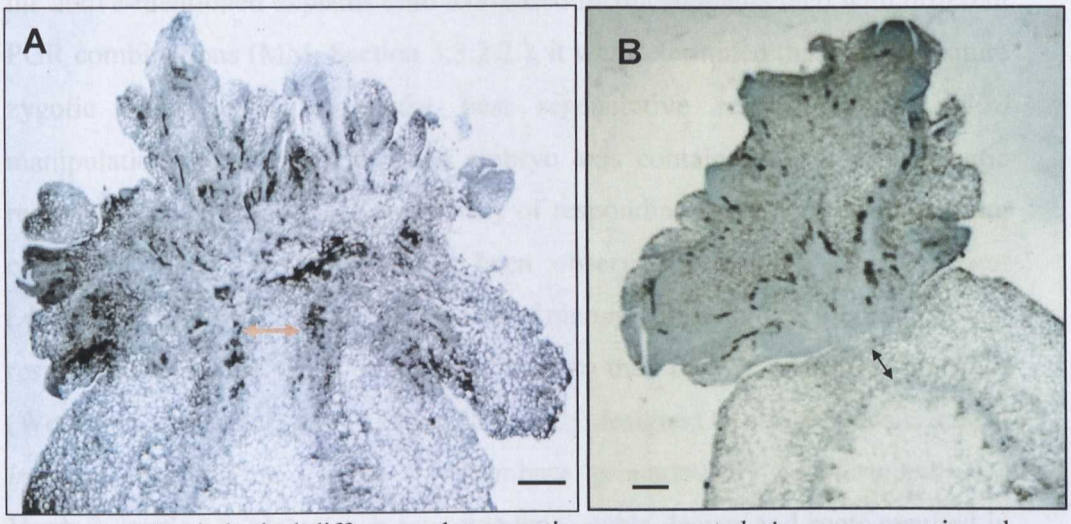


Fig. 3.10. Morphological differences between tissue regenerated *via* organogenesis and somatic embryogenesis in 6 wk-old Bambara groundnut explants. **A:** Tissue regeneration *via* organogenesis [note the vascular connection between the parent explant and the regenerated tissue (arrow)]; **B:** Tissue regeneration *via* somatic embryogenesis [note the absence of any vascular connection (arrow) between the parent explant and the regenerated tissue. **Bars** = 200μm.

3.5. DISCUSSION

3.5.1. Jicama tissue culture

3.5.1.1. Choice of starting material

The type of explant tissue used as starting material is essential towards establishing a stable and optimised tissue culture system. Since there was, at the time of reporting, no published literature on the culture and regeneration *in vitro* of jicama, a preliminary experiment was set up, whereby several tissue types, namely zygotic embryo, cotyledon, hypocotyl, stem, leaf and root material, were tested for their regenerative response to *in vitro* culture. Following the culture of the above-mentioned explants onto MS-based media supplemented with different PGR combinations (MM, Section 3.3.2.2.), it was determined that whole, mature zygotic embryos displayed the best regenerative response to *in vitro* manipulation, possibly because the embryo axis contains young, meristematic regions which offer a higher probability of responding favourably to the culture conditions. This phenomenon has been observed in the cultivated peanut (*Arachis hypogaea* L.), whereby the use of mature cotyledons as starting explant resulted in a relatively poorer response *in vitro* than the use of zygotic embryos (Wetzstein and Baker, 1999). In the first study designed to establish a successful *in vitro* regeneration system for Bambara groundnut [*V. subterannea* (L.) Verdc.], starting explants such as cotyledons, stems, leaves and roots resulted in limited callus proliferation without shoot formation, while the use of zygotic embryos resulted in a high incidence of shoot formation by direct organogenesis (Lacroix *et al.*, 2003).

3.5.1.2. *In vitro* shoot and root regeneration of jicama

Plant growth regulators (PGRs) are critical components in determining the developmental responses of explants in culture, and general paradigms now exist on the effects of different combinations of PGRs at varying concentrations on cultured explants. Some of those responses have, effectively, been observed from the jicama embryos cultured on media supplemented with different

concentrations of BAP and NAA (Results, Section 3.4.1.3.). For example, a higher cytokinin to auxin ratio favoured shoot regeneration. In this experiment, shoot regeneration was maximum in media with high cytokinin concentrations but no auxin. The average number of regenerated shoots per explant was highest at a BAP concentration of $2.5mgL^{-1}$, and no NAA. Similar observations have been recorded in the *in vitro* propagation of black gram (*Vigna mungo*) where a relatively high BAP concentration of $5.0mgL^{-1}$ in the culture medium correlated with an increase in the number of regenerated shoots (Geetha *et al.*, 1997). Other general observations which have been recorded in this experiment were that, while an intermediate auxin to cytokinin ratio resulted in callus formation without any organogenesis, a higher auxin to cytokinin ratio favoured root formation.

Leaves produced by regenerating shoots remained under developed after 10 wk in culture (Results, Section 3.4.1.4.) This may be because the concentration of BAP in the culture medium was high enough to encourage shoot bud formation, but too high to generate normal shoot differentiation. Similar observations have been made in studies carried out on the propagation of chickpea (Polisetty *et al.*, 1997) and adzuki bean (*Vigna angularis*) (Avenido and Hattori, 2000). The full-strength MS-based medium used in the experiment (Appendix I, Section AI.1) could also be responsible for limited leaf development, as it could be providing an excessive amount of nutrients to the shoots, thus creating a nutrient stress. Halving the strength of the medium is an alternative that could reduce the stress and contribute to improved shoot development.

3.5.2. Bambara groundnut tissue culture

3.5.2.1. Regenerative responses of different explant types to *in vitro* culture

In this study, several tissue types of Bambara groundnut, namely zygotic embryo, cotyledon, hypocotyl, stem, leaf and root material, were tested for their regenerative response to culture. Firstly, a modified protocol published by Lacroix and co-workers (2003) was employed here to investigate the response of whole and transversely bisected embryos *in vitro*. The levels of shoot organogenesis reported in Lacroix *et al.* (2003) from whole and bisected embryo

explants were much higher than obtained in the present study (Results, Section 3.4.2.2.1.). Cultured on the optimal hormone combination, Lacroix *et al.* (2003) reported a 100% morphogenetic response of embryo explants, with 7.0 ± 0.7 shoots forming per explant. Here, signs of regeneration were observed from the radicle sections of bisected embryos only, with the highest number of shoots per explant being 2.1 ± 0.7 for S19-3. The use of whole zygotic embryos as starting explants provided a relatively higher incidence of shoot regeneration (5.2 ± 1.2 for DipC, 5.5 ± 0.6 for Uniswa-Red and 5.1 ± 0.9 for S19-3) at an optimum PGR concentration of 1.5mgL^{-1} BAP for all 3 landraces. Adventitious shoot regeneration was generally observed at the site where the zygotic embryos were previously attached to the cotyledonary node, suggesting that the presence of meristematic tissue at these sites has acted as a source of totipotent cells which were amenable to axillary bud formation.

Koné *et al.* (2007) reported shoot organogenesis from cotyledon explants but this was not reproduced in the present study (Results, Section 3.4.2.2.2). Here, the cultured cotyledon sections were devoid of the meristematic cotyledonary node, and only exhibited callogenesis, as well as limited rhizogenesis, on media supplemented with low BAP concentrations. Furthermore, the use of hypocotyl, stem, leaf and root tip tissues as starting explants failed to exhibit any shoot or root formation *in vitro* (Results, Section 3.4.2.2.3.). From these preliminary experiments, it was determined that the use of whole zygotic embryos as starting explants provided the most attractive regeneration outcomes, since the presence of young meristematic regions at the embryonic-cotyledonary nodes seemed to respond and adapt relatively better to culture conditions. This was the case for all 3 investigated landraces (DipC, Uniswa-Red and S19-3). The use of embryo axes in legume *in vitro* regeneration systems has been well documented, and remains one of the favoured starting explant types as they contain exposed pre-existing meristematic tissues that have a high regeneration potential to rapidly form adventitious shoots in a genotype-independent fashion (Anuradha *et al.*, 2008).

3.5.2.2. *In vitro* explant-to-plant regeneration of Bambara groundnut

The *in vitro* regeneration of Bambara groundnut plants *via* somatic embryogenesis and organogenesis from mature zygotic embryos has been achieved by following the modified protocol of Lacroix *et al.* (2003). Stages of regeneration for the 3 landraces, including initial chlorophyll production, and the formation of the first organ primordia (Results, Section 3.4.2.3.) were observed within a similar time frame of 6 days and 15 days respectively, as reported by Lacroix *et al.* (2003).

The successful acclimatisation of plants generated *in vitro* is especially desirable in transgenic studies. In previous studies, rates of successful acclimatisation of regenerated Bambara groundnut plants have been low. For example, Koné *et al.* (2007) reported the establishment of only 40% of plantlets in compost. In this present study, *ex vitro* acclimatisation resulted in the establishment of 43% of DipC and 37% of S19-3 plantlets. The high percentage of lost plants prevents the regeneration system from being fully exploited. During *in vitro* culture, plantlets survive in a very different environment compared to plants grown *ex vitro*. Cultivation vessels are generally relatively air-tight, giving rise to increased levels of air humidity, and lower levels of irradiation than observed in *ex vitro* conditions. Also, the cultivation medium is usually supplemented with saccharides which decrease the water potential of the medium. All these conditions often result in regenerated plantlets which are morphologically and physiologically abnormal. For example, root systems generated *in vitro* may have low hydraulic conductivity and poor root-stem connections. During the acclimatisation from *in vitro* to *ex vitro* conditions, the plantlets are subjected to lower air humidity and higher irradiance, and because their abnormalities are sometimes not corrected in time, wilting may occur relatively quickly, resulting in a heavy loss of regenerated plants. A possible way of preventing a high number of lost plants during the acclimatisation process is to harden the regenerated plants *in vitro* prior to their transfer to glasshouse conditions. This can be achieved by using culture vessels with permeable lids which will decrease air humidity, by increasing irradiance, and by increasing CO_2 concentrations in

the vessel by forced ventilation (Roberts *et al.*, 1990; Kanechi *et al.*, 1998). The use of permeable lids might cause the culture medium to dry out, and the addition of abscisic acid (ABA) to the medium was found to help reduce the water loss from leaves of *in vitro* grown plantlets (Colón-Guasp *et al.*, 1996).

3.5.2.3. Morphological comparison of *in vitro* generated plants to seed-derived plants

In vitro regenerated Bambara groundnut plants were comparatively smaller in size than seed-grown plants (Results, Section 3.4.2.5.). One possible explanation for this occurrence could be that since plants cultured *in vitro* have a ready supply of nutrients and survive on limited light and gaseous exchange, they often display a highly reduced capacity for photosynthesis (Kane, 1999). Once transferred to *ex vitro* conditions, the plants have to adapt rapidly to the external environment, and to produce new leaves which will be able to photosynthesise. Photosynthetic activity is associated with ribulose bisphosphate carboxylase (RuBPcase) activity, and it is possible that the surviving acclimatised Bambara groundnut plants have developed *in vitro* with a genetic malfunction which, when expressed *ex vitro*, resulted in reduced RuBPcase activity in the new leaves. The reduced photosynthetic activity could be responsible for preventing the plants to reach their normal size. Another possibility would be that the plants have a low endogenous gibberellin production, resulting in a dwarfed phenotype.

3.5.2.4. Histological analysis of *in vitro* regenerated tissue

This preliminary histological study of the *in vitro* regeneration of adventitious tissue in cultured Bambara groundnut explants suggested that regeneration in DipC embryos occurred by both organogenesis and somatic embryogenesis, while in Uniswa-Red and S19-3 embryos, regeneration was by somatic embryogenesis. Histological studies conducted by Lacroix *et al.* (2003) on landraces Nkorenza and Yoroba from Ghana, showed regeneration *via* direct organogenesis only. This experiment needs to be further investigated by processing tissue sections from explants of all three landraces collected at several

stages of their development, and would result in a more extensive understanding of the developmental pathways of the plant *in vitro*.

3.5.3 Summary: *In vitro* tissue culture of jicama and Bambara groundnut

The culture of jicama explants on MS-based medium with combinations of BAP and NAA allowed for the optimum phytohormone conditions for shoot and root regeneration to be determined. Different tissue types, namely zygotic embryos, cotyledons, hypocotyls, stems, leaves and root tips, were tested for their regenerative potential *in vitro*, and whole, mature zygotic embryos were found to be the best explant type for *in vitro* regeneration. However, the establishment of an explant-to-plant regeneration system for jicama was unsuccessful, as regenerated shoots failed to elongate and leaf development was arrested after 10 wk in culture, resulting in the leaves never fully expanding. Root regeneration failed to occur when these shoots were transferred onto root regeneration medium, and the shoots became necrotic.

In vitro tissue culture and plant regeneration of Bambara groundnut from whole, mature zygotic embryos was successful. Adventitious shoot regeneration occurred *via* somatic embryogenesis and organogenesis, and elongated shoots were successfully rooted. Acclimatisation of regenerated plantlets *ex vitro* resulted in dwarfed plants which reached maturity and flowered, but seed set did not occur.

3.5.4. Future strategies based on tissue culture results

The investigation into the tissue culture and genetic manipulation of jicama was not pursued based on the data obtained and analysed in this chapter. The successful *in vitro* establishment of Bambara groundnut explant-to-plant cultures provided a baseline for subsequent genetic transformation studies for the production, selection and micropropagation of putative transgenic plants potentially carrying a methionine-rich 2S albumin gene.

CHAPTER 4. PLASMID DESIGN AND MOLECULAR CLONING

4.1. INTRODUCTION

The genetic engineering of Bambara groundnut to enhance seed methionine production was one of the main aims of this project. The strategy employed to alter the seed storage protein content in this way was by introducing a candidate gene of interest encoding for a methionine rich protein into the plant. Traditional recombinant DNA (rDNA) technology and molecular cloning methods have been employed to design recombinant plasmids to carry such a gene and which could be easily manipulated for plant transformation methodologies.

The *ber e1* gene codes for 18% methionine (Lacorte *et al.*, 1997), and was the chosen candidate gene in this experiment as a model gene, to investigate its potential in the enhancement of methionine content in Bambara groundnut seed storage proteins. The reason for choosing *ber e1* as the candidate gene was because several studies have reported the successful use of the gene to transform a range of plant species such as canola (Altenbach *et al.*, 1992), *Vicia narbonensis* (Pickardt *et al.*, 1995) and potato (Tu *et al.*, 1998), and to generate transformants which produced greater methionine concentrations than their non-transformed counterparts. However, the confirmed allergenicity of the Ber e1 protein (Nordlee *et al.*, 1996; Alcocer *et al.*, 2002) limited the use of the *ber e1* gene in Bambara groundnut transformation to a model study only. The gene was therefore used in this study only to demonstrate the potential for the design and optimisation of a tissue-culture based transformation system for the improvement of the methionine content in Bambara groundnut seeds.

All DNA manipulations reported in this chapter were performed based on standard procedures described by Sambrook and Russell (2001), unless stated otherwise. A detailed account is given of the methods employed to generate an expression cassette which contained the coding region of the *ber e1* 2S albumin gene from Brazil nut placed under the control of the seed specific Legumin B4 (LeB4) promoter and CaMV 35S terminator sequences. The methods employed

to introduce this expression cassette into an appropriate plasmid vector as well as the transformation of the vector into bacterial hosts are also described. Figure 4.1. outlined the general strategy employed to generate a recombinant vector carrying the LeB4-Ber e1-35S gene construct.

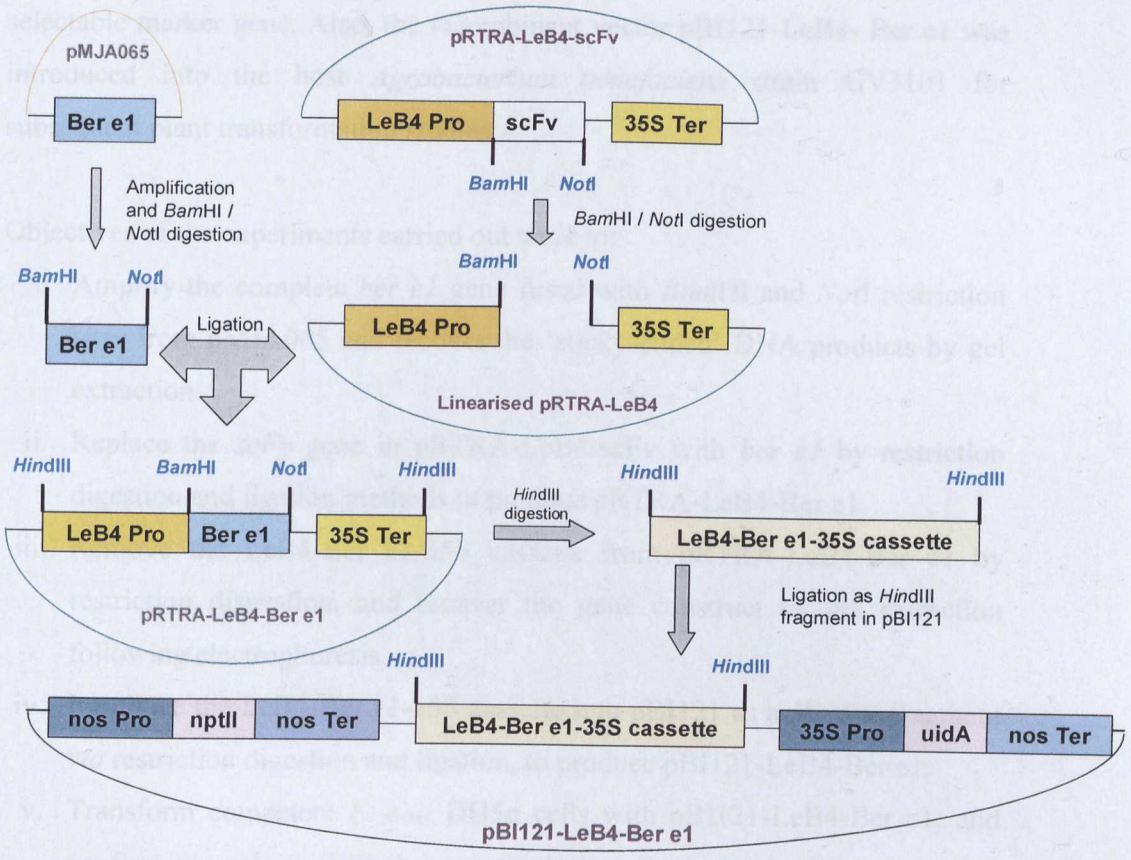


Fig. 4.1. General strategy employed for the generation of the recombinant vector pBI121-LeB4-Ber e1. Briefly, the LeB4-Ber e1-35S expression cassette was produced following amplification of the coding region of Ber e1 from pMJA065, restriction digestion of the amplicon with *Bam*HI and *Not*I, and ligation of the gene insert with the linearised pRTRA-LeB4 vector backbone. From this recombinant vector, the LeB4-Ber e1-35S cassette was digested and ligated into linearised pBI121 as a *Hin*dIII fragment, to produce pBI121-LeB4-Ber e1.

4.2. AIMS AND OBJECTIVES

The aim of the work carried out in this chapter was to use traditional recombinant DNA technologies to construct a gene cassette containing the *ber e1* gene placed under the control of the seed-specific promoter LeB4, and to introduce this cassette into vector pBI121 which carried the *uidA* reporter gene and the *npt*II selectable marker gene. Also, the recombinant vector pBI121-LeB4- Ber e1 was introduced into the host *Agrobacterium tumefaciens* strain GV3101 for subsequent plant transformation studies.

Objectives of the experiments carried out were to:

i. Amplify the complete *ber e1* gene fused with *Bam*HI and *Not*I restriction sites from pMJA065 and recover the 'sticky-ended' DNA products by gel extraction.
ii. Replace the *scFv* gene in pRTRA-LeB4-scFv with *ber e1* by restriction digestion and ligation methods to produce pRTRA-LeB4-Ber e1.
iii. Remove the LeB4-Ber e1-35S cassette from pRTRA-LeB4-Ber e1 by restriction disgestion, and recover the gene construct by gel extraction following electrophoresis.
iv. Introduce the LeB4-Ber e1-35S cassette into pBI121 as a *Hin*dIII fragment *via* restriction digestion and ligation, to produce pBI121-LeB4-Ber e1.
v. Transform competent *E. coli* DH5α cells with pBI121-LeB4-Ber e1, and confirm *via* colony PCR that putatively transformed colonies growing on selective medium carried the recombinant vector in the correct orientation.
vi. Initiate liquid cultures of *E. coli* DH5α carrying pBI121-LeB4-Ber e1 from glycerol stocks and recover large amounts of plasmid to be used in (vii).
vii. Sequence the LeB4-Ber e1-35S cassette in pBI121-LeB4-Ber e1 using the Sanger sequencing method. This would also confirm the orientation of the cassette in the plasmid, and highlight any mis-matches that might have occurred during PCR amplification of the *ber e1* gene.
viii. Introduce the recombinant vector pBI121-LeB4-Ber e1 into *A. tumefaciens* strain GV3101 by electroporation, and make glycerol stocks of transformed colonies.

4.3. Materials and Methods

4.3.1. Starting materials

Two expression vectors, pMJA065 (Fig. 4.2.) and pRTRA LeB4-scFv (Fig. 4.3.) as well as the binary vector pBI121 (Chen *et al.*, 2002; Fig. 4.4.), were used as starting materials for the construction of the recombinant DNA plasmid used in the molecular cloning experiments of this project. pMJA065 and pRTRA LeB4-scFv were kindly provided as lyophilized DNA pellets, by Dr. Marcos Alcocer (Division of Nutritional Sciences, University of Nottingham) and Dr. Udo Conrad (Institute of Plant Genetics, Gatersleben, Germany), respectively. The vector pBI121 carried in the *E. coli* host strain JM109 was purchased as a bacterial agar stab from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus, USA). The gene sequences of all 3 plasmids were provided by their donors, and were entered in VectorNTI v.11 software package (Invitrogen, Paisley, UK) to generate the corresponding plasmid maps.

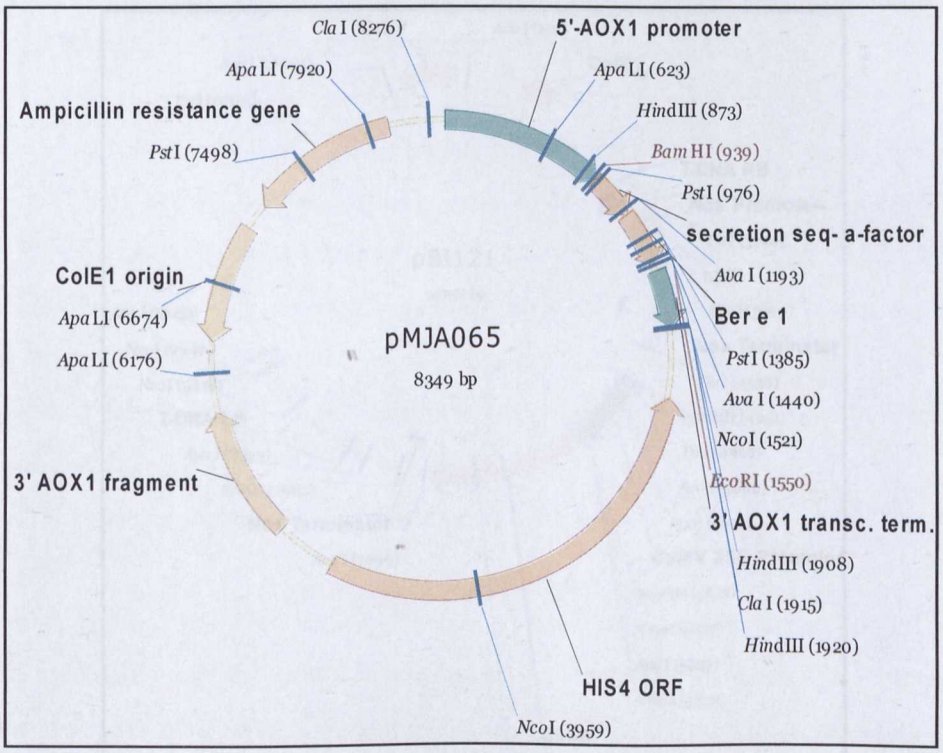


Fig. 4.2. pMJA065 carrying the coding region of the candidate gene *ber e1*.

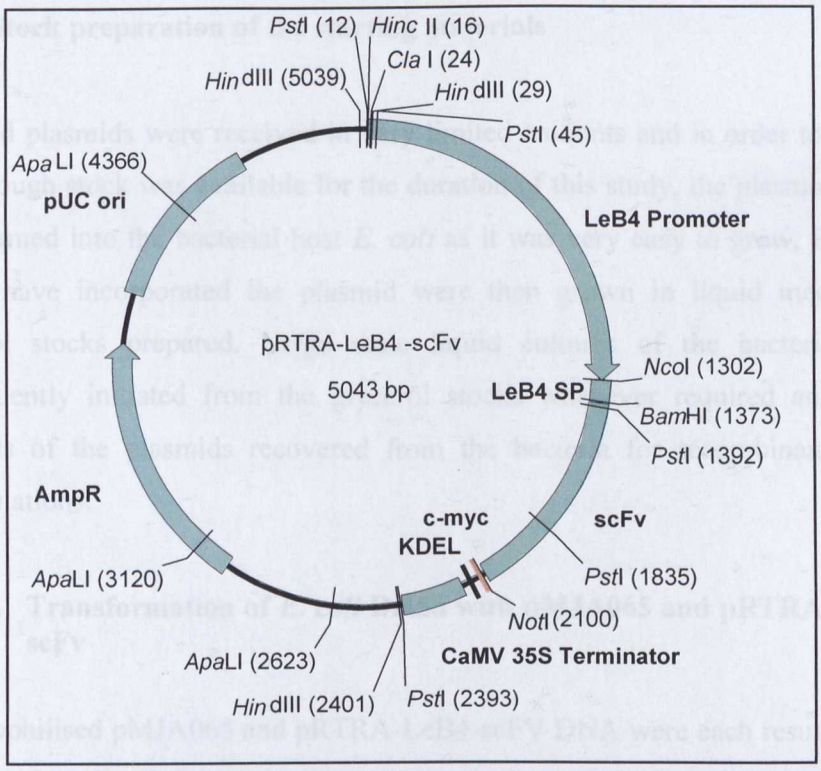


Fig. 4.3. pRTRA-LeB4-scFv carrying the LeB4 seed-specific promoter and CaMV 35S terminator sequences.

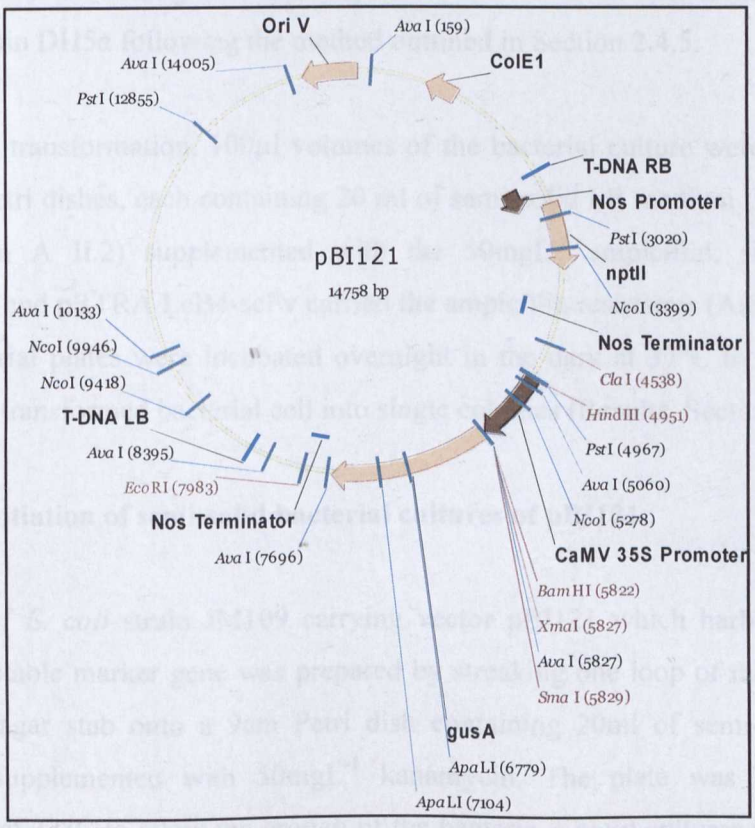


Fig. 4.4. pBI121 harbouring the *npt*II selectable marker and *uidA* (*gusA*) reporter genes.

4.3.2. Stock preparation of the starting materials

Donated plasmids were received in very limited amounts and in order to ensure that enough stock was available for the duration of this study, the plasmids were transformed into the bacterial host *E. coli* as it was very easy to grow. Bacteria which have incorporated the plasmid were then grown in liquid media and glycerol stocks prepared. Large scale liquid cultures of the bacteria were subsequently initiated from the glycerol stocks whenever required and large amounts of the plasmids recovered from the bacteria for recombinant DNA manipulations.

4.3.2.1. Transformation of *E. coli* DH5α with pMJA065 and pRTRA-LeB4-scFv

The lyophilised pMJA065 and pRTRA-LeB4-scFV DNA were each resuspended in 20µl of sterile H_2O and the DNA concentrations quantified using NanoDrop software (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Wilmington, DE 19810, USA). Ten nanograms of each plasmid were used for transformation into *E. coli* strain DH5α following the method outlined in Section 2.4.5.

Following transformation, 100µl volumes of the bacterial culture were cultured in 9 cm Petri dishes, each containing 20 ml of semi-solid LB medium (Appendix II, Section A II.2) supplemented with the $50mgL^{-1}$ ampicillin, since both pMJA065 and pRTRA LeB4-scFv carried the ampicillin resistance (Amp^R) gene. The bacterial plates were incubated overnight in the dark at 37°C to allow the growth of transformed bacterial cell into single colonies (Results, Section 4.4.1.).

4.3.2.2. Initiation of semi-solid bacterial cultures of pBI121

A stock of *E. coli* strain JM109 carrying vector pBI121 which harboured the *npt*II selectable marker gene was prepared by streaking one loop of the bacteria from the agar stab onto a 9cm Petri dish containing 20ml of semi-solid LB medium supplemented with $50mgL^{-1}$ kanamycin. The plate was incubated overnight at 37°C to allow the growth of the bacteria. Liquid cultures were then

initiated from single bacterial colonies growing on this plate and glycerol stocks were also prepared.

4.3.2.3. Initiation of liquid bacterial cultures of pMJA065, pRTRA-LeB4-scFV and pBI121

Ten individual colonies were selected randomly for each plasmid and each colony transferred to a 50ml screw-capped centrifuge tube containing 5ml of liquid LB medium (Appendix II, Section A II.1) supplemented with antibiotics ($50mgL^{-1}$ ampicillin for pMJA065 and pRTRA-LeB4-scFV; $50mgL^{-1}$ kanamycin for pBI121). The tubes were incubated overnight in a Stuart Scientific SI50 orbital incubator (Bibby Sterilin Ltd., Stone, UK) maintained at 37°C and 150 rpm.

4.3.2.4. Preparation of glycerol stocks of bacterial culture

Following the method detailed in Section 2.4.4., bacterial glycerol stocks of pMJA065, pRTRA-LeB4-scFv and pBI121 were made from liquid cultures initiated from individual colonies of each plasmid (Section 4.3.2.3.). Cryotubes (Scientific Laboratory Supplies Ltd., Hessle, UK) were labeled accordingly with the plasmid name and colony number. Long term storage of the bacteria was at -80°C

4.3.2.5. Recovery of plasmid DNA from liquid bacterial cultures

Plasmid DNA extraction was carried out from the liquid bacterial cultures initiated in Section 4.3.2.3., using the QIAprep Spin Miniprep kit (Qiagen GmBH, Hilden, Germany) following the manufacturer's instructions (Appendix III, Section AIII.1). The plasmid extraction from individual liquid cultures of pMJA065, pRTRA-LeB4-scFv and pBI121 was carried out separately, and the concentration of the recovered plasmid DNA determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Wilmington, DE 19810, USA). Plasmid DNA was stored at -20°C.

4.3.3. Construction of the LeB4-Ber e1-35S expression cassette

4.3.3.1. Amplifcation and restriction digestion of *ber e1* gene from pMJA065

Primers were designed using the VectorNTI software (Invitrogen; Section 2.7.1.) for the amplification of the complete *ber e1* sequence fused with the restriction sites *Bam*HI and *Not*I, from pMJA065. The forward and reverse primers were designed with their 5′ termini fused with the restriction cloning sites *Bam*HI and *Not*I respectively. Eight arbitrarily chosen bases were then added next to each restriction site sequence to optimise restriction enzyme activity during restriction digestion. The designed primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany) and are listed as follows:

	Extra bases	*Bam*HI	primer sequence
Forward primer: 5′	GATCAAGT	GGATCC	AGGAGGAGTGTCGCGAGCA 3′

	Extra bases	*Not*I	primer sequence
Reverse primer: 5′	GAATCAAT	GCGGCCGC	TCAGAACCCGGCAATGGAGC 3′

The amplification of the *ber e1* gene was carried out *via* PCR using Phusion™ Hot Start High-Fidelity DNA polymerase (New England Biolabs; Section 2.7.2.). The reaction mixture was set up using pMJA065 as plasmid DNA. A negative control was also set up, which used 1µl of sterile H_2O instead of DNA. The total reaction volume was 20µl.

PCR amplification was performed using the 96-well GeneAmp® PCR System 9700 machine (Applied Biosystems, Warrington, UK) under the following cycling conditions:

1 cycle at 98°C for 30 s
35 cycles at 98°C each for 10 s
35 cycles at 71°C each for 30 s
35 cycles at 72°C each for 30 s
1 cycle at 72°C for 10 min
Hold at 4°C

The PCR products were electrophoresed on 1% (w/v) SeaKem® LE agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA.) against a 100bp ladder (New England Biolabs) following the protocol detailed in Appendix VII. The visible band corresponding to the amplified *ber e1* gene (Results, Section 4.4.2.1.) was cut from the gel and subjected to gel extraction to recover the *ber e1* DNA fragment.

Gel extraction was carried out using the Qiaquick Gel Extraction Kit (as detailed in Appendix III, Section AIII.2.) from Qiagen, Crawley, UK. The recovered *ber e1* DNA fragment was stored at -20°C until ready for restriction digestion.

The next step in this cloning strategy was to digest the *ber e1* DNA fragment with the restriction enzymes *Bam*HI and *Not*I (Promega), to create sticky ends at the two extremities of the DNA fragment. A double restriction digestion reaction was set up with *Bam*HI and *Not*I according to the manufacturer's instructions (Section 2.6.4). The total reaction volume was 20µl. The reaction mix was incubated at 37°C in an incubator oven for 3 h followed by incubation at 65°C for 10 min. The last step stopped the digestion reaction by inactivating the restriction enzymes.

4.3.3.2. Double restriction digestion of pRTRA-LeB4-scFv to produce the linearised pRTRA-LeB4 vector backbone

A double restriction digest of pRTRA-LeB4-scFv was set up with the restriction enzymes *Bam*HI and *Not*I. The aim of this enzymatic digestion was to generate two linear fragments, namely the scFv DNA fragment which was discarded, and the pRTRA-LeB4 vector backbone carrying *Bam*HI and *Not*I sticky ends. The reaction mix was prepared as detailed in Section 2.6.4. The digested products were run on a 1% (w/v) agarose gel (Results, Section 4.4.2.2.) against a 1kb ladder (New England Biolabs). The corresponding band was cut and extracted from the gel (Appendix III, Section AIII.2.) for the recovery of the pRTRA-LeB4 vector backbone.

4.3.3.3. Ligation of the *ber e1* insert to the linearised pRTRA-LeB4 vector and transformation into *E. coli* DH5α

At this stage, the *ber e1* insert and the pRTRA-LeB4 vector (both recovered by gel extraction) contained *Bam*HI and *Not*I cohesive ends. T4 DNA ligase (Promega) was used to ligate the insert to the vector following the manufacturer's instructions, to produce the circular pRTRA-LeB4-Ber e1. A vector to insert molar ratio of 1:3 was used in the reaction mix. The following formula was used to calculate the amount of insert DNA to be added to 100ng of vector DNA:

$$\frac{\text{ng of vector} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The reaction mix was prepared as detailed in Table 4.1 below.

Table 4.1. Composition of ligation reaction mix

Component	Volume used (μl) in 1 reaction tube
Vector DNA (100ng)	x (to be calculated)
Insert DNA	y (to be calculated)
10x Ligase Buffer	1μl
T4 DNA ligase (1 unit)	0.33μl
Sterile H_2O	$8.67 - (x + y)$ μl

The total reaction volume was 10μl and the reaction mix was incubated at room temperature for 3 h. There was no need to heat-treat the mix to halt the reaction. After incubation, *E. coli* DH5α competent cells were transformed with the ligation reaction mix following the method outlined in Section 2.4.5. The transformed cells (100μl volumes) were cultured overnight at 37°C in 9cm Petri dishes, each containing 20ml of semi-solid LB medium, supplemented with 50mgL^{-1} ampicillin.

4.3.3.4. Colony PCR of *E. coli* DH5α transformed with pRTRA-LeB4-Ber e1

Colony PCR was performed on ampicillin resistant *E. coli* DH5α colonies to confirm their transformation with pRTRA-LeB4-Ber e1, following the method outlined in Section 2.4.7. A master mix using RedTaq™ ReadyMix™ PCR Reaction Mix (Sigma-Aldrich Co.) was prepared (Appendix VI., Section AVI.2.) using a forward primer which spanned the LeB4 promoter region and a reverse

primer which spanned the *ber e1* region. Details of the primer sequences are given below:

Forward primer: 5′ GACGGTATCGATAAGCTTGC 3′
Reverse primer: 5′ GCCTCATCATCCTTCGCATC 3′

PCR amplification was performed using the 96-well GeneAmp® PCR System 9700 machine (Applied Biosystems) under the following cycling conditions:

1 cycle at 94°C for 3 min
35 cycles at 94°C each for 1 min
35 cycles at 56°C each for 1 min
35 cycles at 72°C each for 1.5 min
1 cycle at 72°C for 10 min
Hold at 4°C

The PCR products were electrophoresed on 1% (w/v) SeaKem® LE agarose gels (BioWhittaker Molecular Applications) against a 1kb ladder (New England Biolabs) following the protocol detailed in Appendix VII. The gel was viewed under UV light (Results, Section 4.4.2.3.).

To prepare bacterial stocks of colonies which tested positive for LeB4-Ber e1 insert, liquid cultures were initiated (Section 2.4.3.) in LB medium supplemented with 50mgL^{-1} ampicillin using their corresponding bacterial streak. Glycerol stocks were prepared as outlined in Section 2.4.3. The recovery of the plasmid vector pRTRA-LeB4-Ber e1 (Fig. 4.5.) was performed using the QIAprep Spin Miniprep kit (Qiagen) following the manufacturer's instructions (Appendix III, Section AIII.1). The recovered plasmid DNA was quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer, and stored at -20°C.

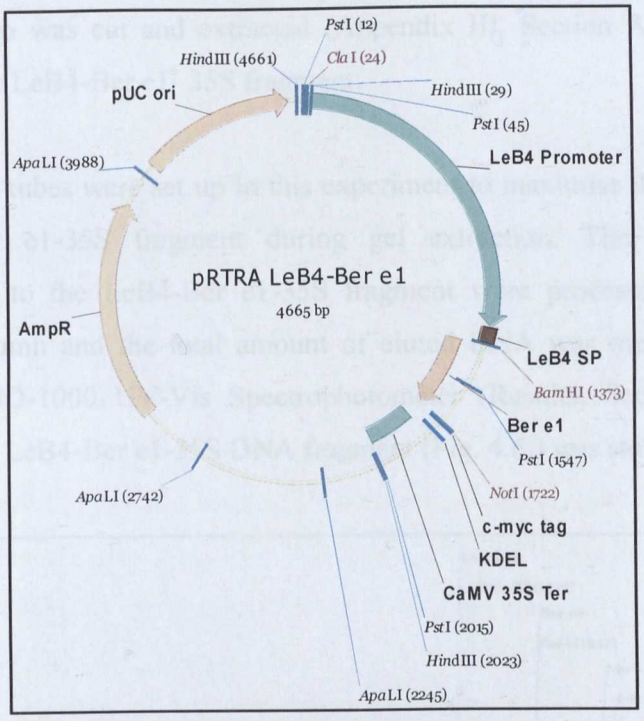


Fig. 4.5. Vector pRTRA-LeB4-Ber e1 carrying the LeB4-Ber e1-35S expression cassette.

4.3.3.5. Restriction digestion of pRTRA-LeB4-Ber e1 to isolate the LeB4-Ber e1-35S cassette

The circular pRTRA-LeB4-Ber e1 was digested with the restriction enzyme *Hin*dIII (Promega) to produce two linear fragments, namely the LeB4-Ber e1-35S cassette (Fig. 4.5.), and the pRTRA vector backbone. The single digestion reaction was performed according to the manufacturer's instructions and the reaction mix was prepared as detailed in Table 4.2.

Table 4.2. Composition of reaction mix for restriction digest with *Hin*dIII

Component	Volume used (µl) in 1 reaction tube
Sterile H_2O	14
Restriction Enzyme 10X Buffer E	2
Acetylated BSA (1mg mL^{-1})	2
DNA (0.5 – 1µg)	1
*Hin*dIII (10 units/µl)	1

The digestion reaction was performed as described in Section 2.6.4. The digested products were run on a 1% (w/v) agarose gel (Results, Section 4.4.2.4.) against a 1kb ladder (New England Biolabs) and the band corresponding to the predicted

size of 1.99 kb was cut and extracted (Appendix III, Section AIII.2.) for the recovery of the LeB4-Ber e1- 35S fragment.

Three reaction tubes were set up in this experiment to maximise the recovery of the LeB4-Ber e1-35S fragment during gel extraction. The 3 gel bands corresponding to the LeB4-Ber e1-35S fragment were processed onto 1 gel extraction column and the total amount of eluted DNA was measured on the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Results, Section 4.4.2.4.). The recovered LeB4-Ber e1-35S DNA fragment (Fig. 4.6.) was stored at 4°C.

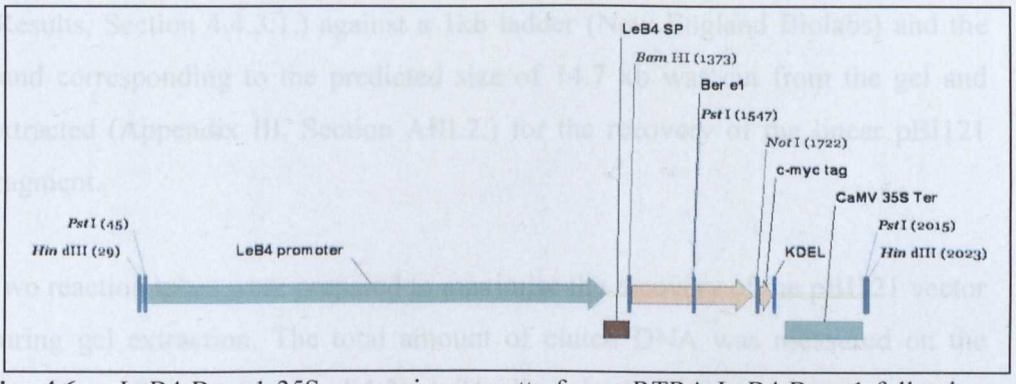


Fig. 4.6. LeB4-Ber e1-35S expression cassette from pRTRA-LeB4-Ber e1 following restriction digest with *Hin*dIII

4.3.4. Introduction of LeB4-Ber e1-35S cassette into pBI121

The next stage of this cloning strategy was to introduce the LeB4-Ber e1-35S cassette into a plasmid vector which carried a selectable marker gene as well as a reporter gene. pBI121 is an ideal candidate for this DNA recombination step as it harbours the *npt*II selectable marker and *uidA* reporter genes, both important to confirm transgene integration in plant transformation systems.

4.3.4.1. Linearisation of pBI121 by restriction digestion with *Hin*dIII

The circular pBI121 was digested with the restriction enzyme *Hin*dIII (Promega). The enzyme cuts the vector at the unique *Hin*dIII restriction site to produce a single linear fragment. The single digest was performed according to the

manufacturer's instructions and the reaction mix was prepared as detailed in Table 4.2.

The digestion reaction was performed as described in Section 2.6.4. To eliminate the risk of vector self-ligation at the *Hin*dIII site during subsequent ligation experiments, the linearised pBI121 vector in each reaction tube was further treated with 0.5µl of Calf Intestinal Phosphatase (CIP; New England Biolabs), at 37°C for 1h. CIP removes phosphate groups from the 5′ ends of the double stranded DNA, making it impossible for the vector to fuse back to its own ends. The digested and CIP-treated products were then run on a 1% (w/v) agarose gel (Results, Section 4.4.3.1.) against a 1kb ladder (New England Biolabs) and the band corresponding to the predicted size of 14.7 kb was cut from the gel and extracted (Appendix III, Section AIII.2.) for the recovery of the linear pBI121 fragment.

Two reaction tubes were prepared to maximise the recovery of the pBI121 vector during gel extraction. The total amount of eluted DNA was measured on the NanoDrop (Results, Section 4.4.3.1.). The linearised pBI121 vector was stored at 4°C.

4.3.4.2. Ligation of LeB4-Ber e1-35S insert to linearised pBI121 vector and transformation into *E. coli* DH5α

T4 DNA ligase (Promega) was used to ligate the LeB4-Ber e1-35S insert to the linearised pBI121 vector following the manufacturer's instructions to produce the circular pBI121-LeB4-Ber e1. The methods employed were as outlined in Section 4.3.3.3. A molar vector:insert ratio of 1:3 was used in a total reaction volume of 10µl. *E. coli* DH5α competent cells were transformed with the ligation reaction mixture (Section 2.4.5.). The transformed cells were cultured overnight at 37°C on semi-solid LB medium supplemented with 50mgL^{-1} kanamycin.

4.3.4.3. Colony PCR analysis to test for correct orientation of insert in transformed *E. coli* DH5α colonies

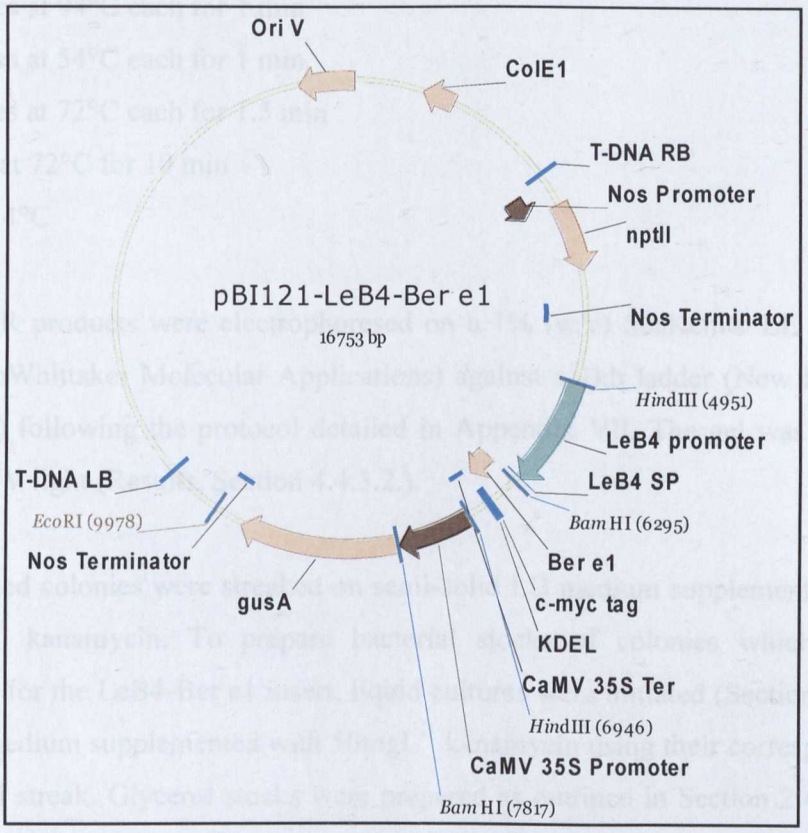


Fig. 4.7. Recombinant vector pBI121-LeB4-Ber e1 carrying the LeB4-Ber e1-35S cassette in the correct orientation

Colony PCR (Section 2.4.5.) was performed on 10 randomly selected kanamycin resistant *E. coli* DH5α colonies to confirm their transformation with pBI121-LeB4-Ber e1. To test which colonies carried the LeB4-Ber e1-35S insert in the correct orientation, *i.e.* downstream of the *npt*II marker gene (Fig. 4.7), a forward primer which spanned the *npt*II selectable marker gene region and a reverse primer which spanned the *ber e1* region were used.

Details of the primer sequences are given below:

Forward primer: 5′ ATGATTGAACAAGATGGATTGC 3′
Reverse primer: 5′ GCCTCATCATCCTTCGCATC 3′

PCR amplification was carried out under the following cycling conditions:

1 cycle at 94°C for 3 min
35 cycles at 94°C each for 1 min
35 cycles at 54°C each for 1 min
35 cycles at 72°C each for 1.5 min
1 cycle at 72°C for 10 min
Hold at 4°C

The PCR products were electrophoresed on a 1% (w/v) SeaKem® LE agarose gel (BioWhittaker Molecular Applications) against a 1kb ladder (New England Biolabs) following the protocol detailed in Appendix VII. The gel was viewed under UV light (Results, Section 4.4.3.2.).

The tested colonies were streaked on semi-solid LB medium supplemented with 50mgL^{-1} kanamycin. To prepare bacterial stocks of colonies which tested positive for the LeB4-Ber e1 insert, liquid cultures were initiated (Section 2.4.3.) in LB medium supplemented with 50mgL^{-1} kanamycin using their corresponding bacterial streak. Glycerol stocks were prepared as outlined in Section 2.4.4. and stored at -80°C.

4.3.4.4. Recovery of pBI121-LeB4-Ber e1 DNA from liquid *E. coli* cultures

Liquid cultures were initiated from glycerol stocks of *E. coli* DH5α carrying pBI121-LeB4-Ber e1 in LB medium supplemented with 50mgL^{-1} kanamycin. Plasmid DNA extraction was carried out from the liquid bacterial cultures following the method outlined in Section 2.4.8. Recovered DNA was stored at -20°C. The recombinant pBI121-LeB4-Ber e1 was subsequently sequenced using the Sanger sequencing method (Section 4.3.6.) and employed in biolistic-mediated transformation of Bambara groundnut (Chapter 5).

4.3.5. Transformation of *A. tumefaciens* strain GV3101 with pBI121-LeB4-Ber e1

Competent cells of *A. tumefaciens* strain GV3101 which are resistant to rifampicin, gentamycin and tetracycline, but sensitive to kanamycin were used as host strain for transformation with plasmid DNA.

4.3.5.1. Transformation of competent *A. tumefaciens* GV3101 by electroporation

A. tumefaciens strain GV3101 was transformed with pBI121-LeB4-Ber e1 by electroporation following the method outlined in Section 2.4.6. Using a sterile spreader, 100µl volumes of the transformed bacterial culture were spread in 9 cm Petri dishes, each containing 20ml of semi-solid LB medium (Appendix II, Section AII.2) supplemented with 50mgL^{-1} rifampicin, 50mgL^{-1} gentamycin and 5mgL^{-1} tetracycline, as well as 50mgL^{-1} kanamycin to select for transformed bacteria. The plates were sealed with Nesco film (Bando Chemical IND. Ltd., Osaka, Japan) and incubated upside down in the dark at 28°C for 2-3 d to allow the transformed bacteria to grow.

4.3.5.2. Colony PCR analysis to test for presence of pBI121-LeB4-Ber e1 in transformed *A. tumefaciens* GV3101 colonies

To test whether the *A. tumefaciens* colonies growing on selective medium were transformed with pBI121-LeB4-Ber e1, colony PCR analysis was performed on 4 randomly selected individual colonies. The method outlined in Section 2.4.7. was followed, but using semi-solid LB medium supplemented with 50mgL^{-1} kanamycin, 50mgL^{-1} rifampicin, 50mgL^{-1} gentamycin and 5mgL^{-1} tetracycline to culture the tested colonies. In the master mix, a forward primer which spanned the *npt*II selectable marker gene region and a reverse primer which spanned the *ber e1* region were used. Details of the primer sequences are given below:

Forward primer: 5′ ATGATTGAACAAGATGGATTGC 3′
Reverse primer: 5′ GCCTCATCATCCTTCGCATC 3′

PCR amplification was carried out under the cycling conditions described in Section 3.2.4.3. The PCR products were electrophoresed on a 1% (w/v)

SeaKem® LE agarose gel (BioWhittaker Molecular Applications) against a 1kb ladder (New England Biolabs) following the protocol detailed in Appendix VII. The gel was viewed under UV light (Results, Section4.4.4.).

To prepare bacterial stocks of colonies which tested positive for the *npt*II-LeB4-Ber e1 fragment (Results, Section 4.4.4.), liquid cultures were initiated (Section 2.4.3.) in LB medium supplemented with 50mgL^{-1} kanamycin, rifampicin and gentamycin, and with 5mgL^{-1} tetracycline, using their corresponding bacterial streak. Glycerol stocks were prepared as outlined in Section 2.4.4. and stored at -80°C. The transformed *A. tumefaciens* GV3101 was used in *Agrobacterium*-mediated transformation of Bambara groundnut, as reported in Chapter 6.

4.3.6. Sanger sequencing of the LeB4-Ber e1 gene construct

Plasmid BI121-LeB4-ber e1 was isolated from *E. coli* DH5α liquid bacterial cultures using the Qiaprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions (Appendix III, Section AIII.1). The LeB4-Ber e1 insert (1682bp) was sequenced by Sanger technology (Sanger *et al.*, 1977) using the ABI 3730 XL capillary sequencer using BigDye v.3.1 dye-terminator chemistry (Value Read, Single Read Service, Eurofins MWG GmbH, Ebersberg, Germany; Section 2.8.). The primer sequences used in the forward and reverse sequencing reactions were as follows:

Forward primer: 5′ - ACGCCAAGCTTGCATGCCT – 3′
Reverse primer: 5′ – GTTTTTGTTCTGCGGCCG – 3′

Forward and reverse Sanger-generated nucleotide sequences were assembled to form one consensus sequence (1602 bp) using ContigExpress software at default settings from Vector NTI v.11 (Invitrogen). This data was then aligned to the reference insert sequence, kindly donated by Dr. Udo Conrad (Institute of Plant Genetics, Gatersleben, Germany), and isolated from the construct map of pBI121-LeB4-Ber e1 in VectorNTI v.11 (Invitrogen) using ClustalW v.1.83 at default settings (EBI-EMBL). The alignments were employed to identify base pair mismatches introduced artificially, as a result of the PCR amplification of the *ber e1* gene from pMJA065 (Section 4.3.3.1.), while also confirming

sequence fidelity between pBI121-LeB4 Sanger sequencing results and the donated reference sequence data (Results, Section 4.4.5.).

4.3.7. Comparative sequence analysis of the LeB4 promoter region using BLASTn and ClustalW

The locations of mismatches identified between the Sanger sequenced LeB4 promoter region and the reference sequence used in the VectorNTI-generated LeB4-Ber e1 construct were identified using both the Basic Local Alignment Search Tool (BLAST) (NCBI, BLASTn algorithm) and ClustalW alignment software. Sequences from both reference and Sanger sequenced LeB4 promoter regions were compared to all nucleotide data deposited in NCBI using BLASTn. For both, results showing the highest sequence identity were aligned to their corresponding original query sequence, using Pairwise alignment from ClustalW, before being examined to locate all nucleotide mismatches (Results, Section 4.4.6.).

4.4. RESULTS

4.4.1. Transformation of *E. coli* DH5α with pMJA065 and pRTRA LeB4-scFv

An average of 109 colonies per plate were obtained for *E. coli* transformed with pMJA065, while an average of 68 colonies were obtained for *E. coli* transformed with pRTRA-LeB4-scFv.

4.4.2. Construction of the LeB4-Ber e1 cassette

4.4.2.1. PCR amplification of *ber e1* gene from pMJA065

The amplification of the *ber e1* gene with the attached restriction sites *Bam*HI and *Not*I from pMJA065 was successful, as shown by the band in lane 6 of Fig. 4.8. The size of the *ber e1* gene on its own is 332bp long, but the added restriction sites and extra bases (MM, Section 4.3.3.1.) increased the length of the PCR product to 362bp, which corresponded to the size of the amplified band, *i.e.* between 300bp and 400bp.

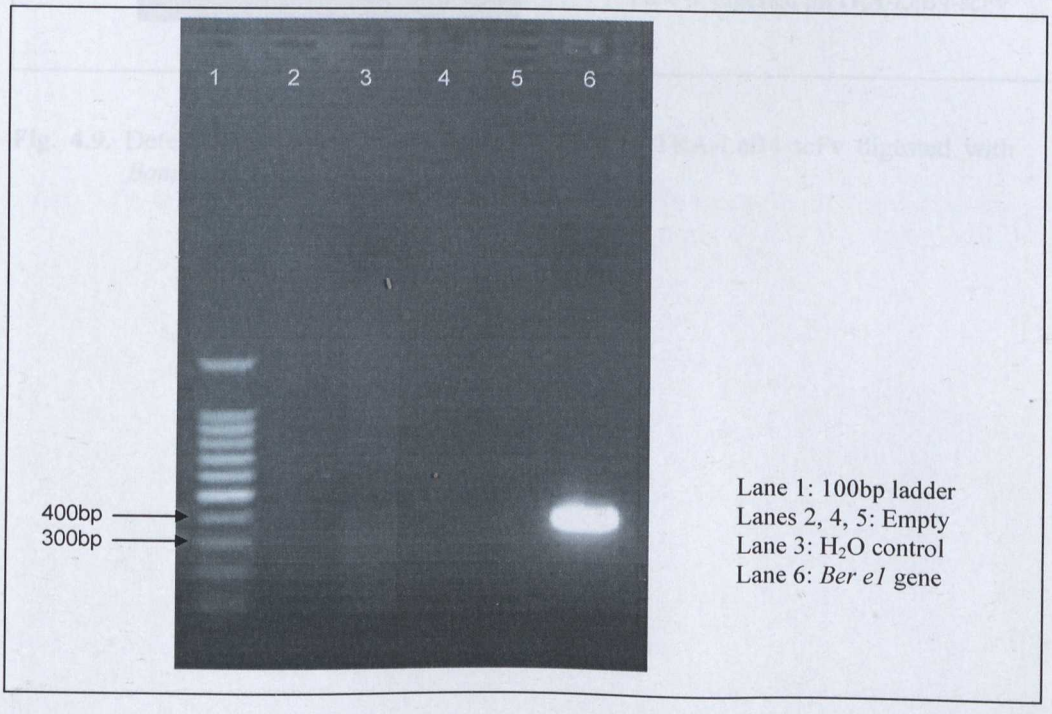


Fig. 4.8. Detection of amplified *ber e1* gene with attached *Bam*HI and *Not*I restriction sites.

4.4.2.2. Double restriction digestion of pRTRA-LeB4-scFv

A double restriction digest of pRTRA-LeB4-scFv was set up with the restriction enzymes *Bam*HI and *Not*I. This reaction yielded 2 distinct bands following the electrophoresis of the reaction mixture on a 1% (w/v) agarose gel (Fig. 4.9.). The larger band corresponded to the linearised 4.31 kb pRTRA-LeB4 vector backbone, while the smaller band corresponded to the 0.72 kb scFv fragment .

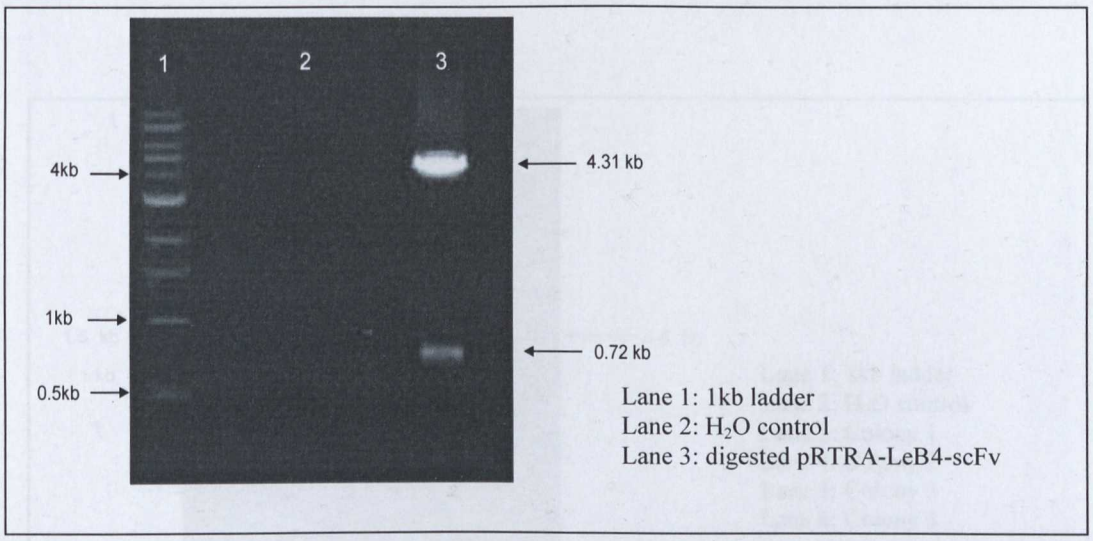


Fig. 4.9. Detection of 2 linearised fragments from pRTRA-LeB4-scFv digested with *Bam*HI and *Not*I.

4.4.2.3. Colony PCR of *E. coli* DH5α transformed with pRTRA-LeB4-Ber e1

E. coli DH5α competent cells were transformed with pRTRA-LeB4-Ber and 4 bacterial colonies, which grew on selective medium, were tested for the presence of the LeB4-Ber e1 fragment *via* colony PCR. The results of the amplification reaction showed that out of the 4 colonies tested, Colony 3 was the only one positive for the presence of the fragment (Fig. 4.9.). The primer pair used in this PCR reaction (MM, Section 4.3.3.4) was expected to amplify a 1.6kb region of the plasmid, and this was confirmed by the position of the amplified band relative to the 1.5kb band of the DNA ladder (Fig. 4.10).

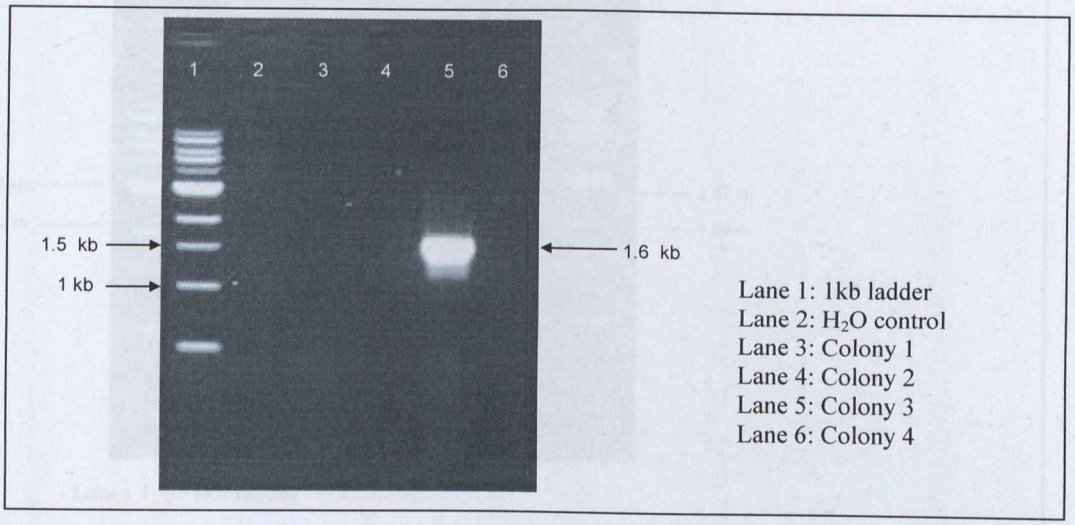


Fig. 4.10. Colony PCR on *E. coli* DH5α colonies transformed with pRTRA-LeB4-Ber e1. Amplification of the LeB4-Ber fragment was detected in Colony 3 only, out of 4 tested colonies.

4.4.2.4. Isolation and recovery of the LeB4-Ber e1-35S cassette *via* restriction digestion of pRTRA-LeB4-Ber e1

The restriction digest of pRTRA-LeB4-Ber e1 with *Hin*dIII produced 2 fragments which appeared as 2 distinct bands under UV light following the electrophoresis of the reaction mixture on a 1% (w/v) agarose gel (Fig. 4.11.). The presence of bands between the 2kb and 3kb mark corresponded to the 2.67 kb linearised pRTRA vector backbone, while the bands just below the 2kb mark corresponded to the 1.99 kb LeB4-Ber e1-35S fragment.

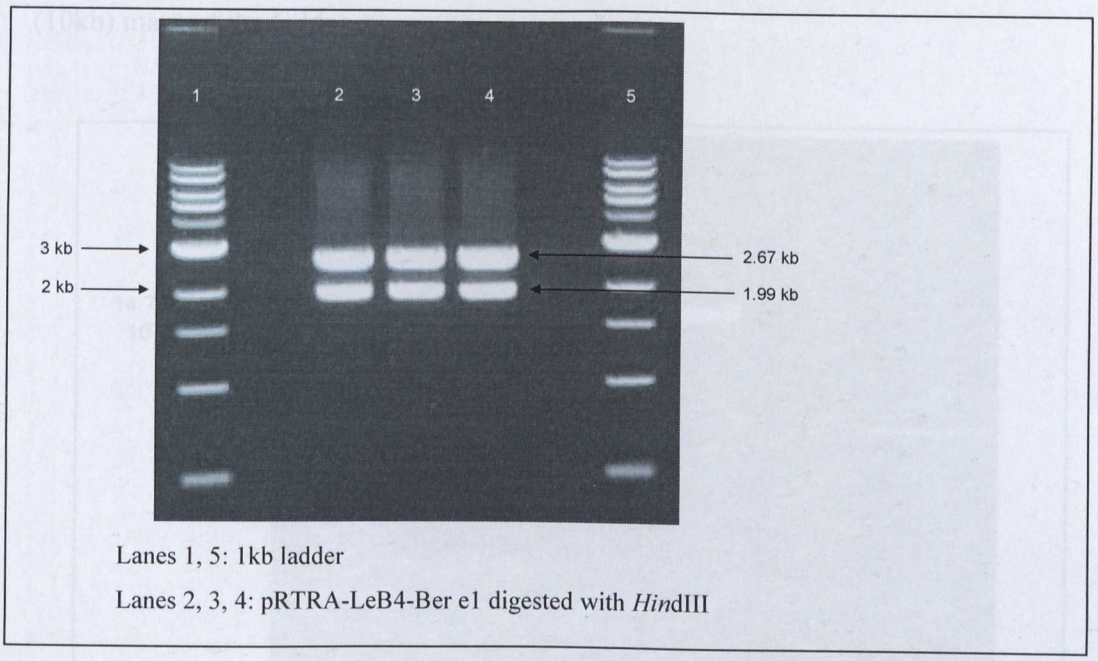


Fig. 4.11. Detection of 2 linearised fragments from pRTRA-LeB4-Ber e1 digested with *Hin*dIII. The smaller fragment is the LeB4-Ber e1-35S expression cassette.

The quantity of LeB4-Ber e1-35S DNA recovered from gel extraction of the excised bands (MM, Section 4.3.3.5.) was 12.5ng μl^{-1}.

4.4.3. Introduction of LeB4-Ber e1-35S cassette into pBI121

4.4.3.1. Linearisation of pBI121 by restriction digestion with *Hin*dIII

The circular pBI121 was digested with the restriction enzyme *Hin*dIII (Promega) following the manufacturer's instructions. The enzyme cut the vector at the unique *Hin*dIII restriction site to produce a single linear fragment, which appeared as a distinct band under UV light following the electrophoresis of the reaction mixture on a 1% (w/v)agarose gel (Fig. 4.12.). The expected size of the fragment was 14.7 kb, as confirmed by the position of the band above the highest (10kb) mark of the ladder.

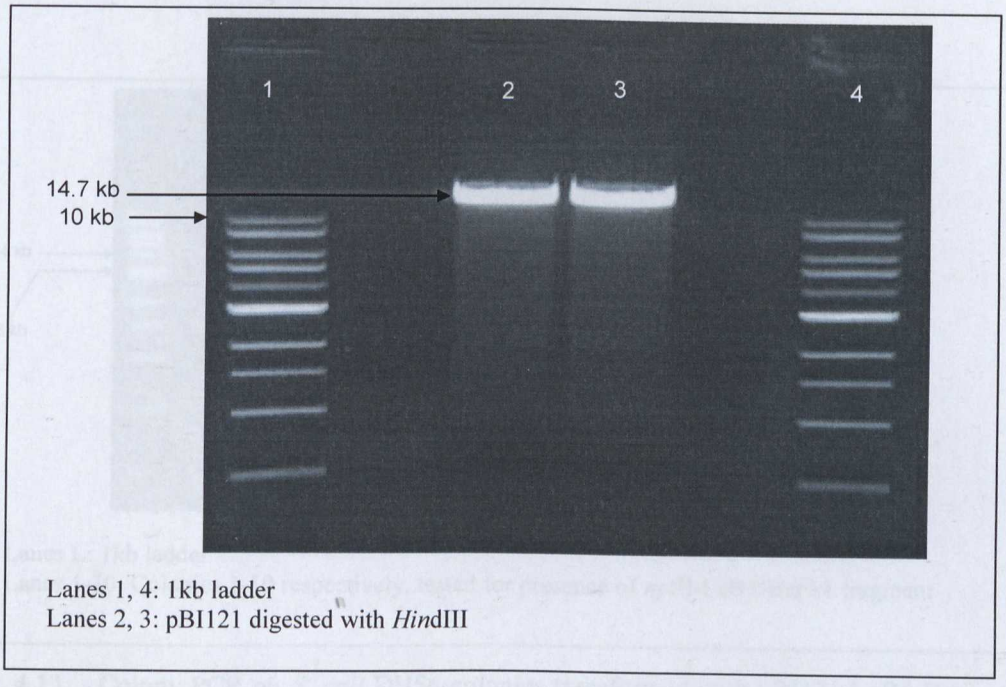
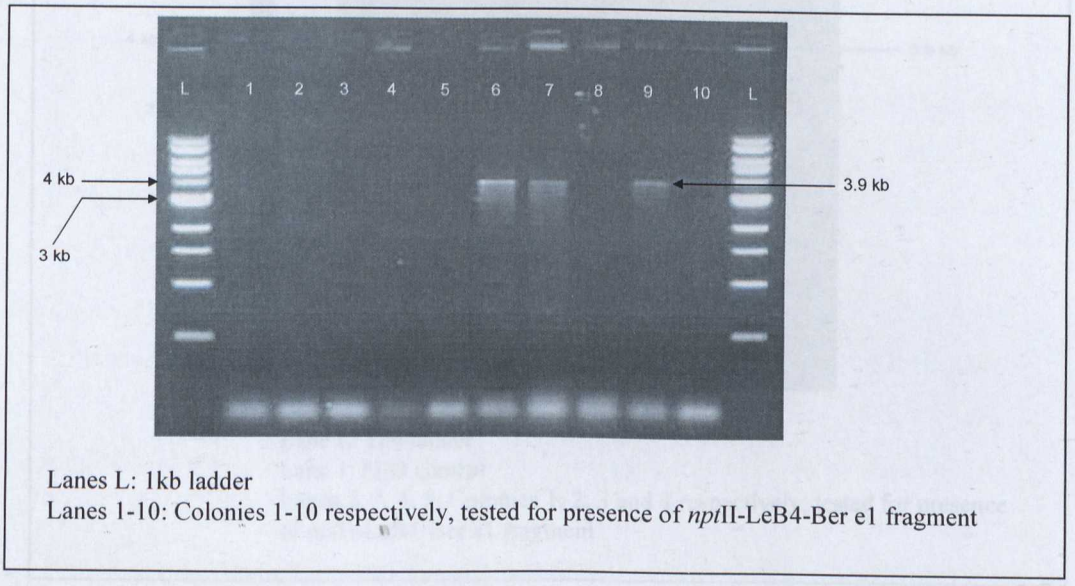


Fig. 4.12. Restriction digestion of pBI121 with *Hin*dIII. A single linearised fragment was obtained from pBI121 following gel electrophoresis of the digestion reaction. The size of the linear fragment corresponds to the size of the circular plasmid which is 14.7 kb in length.

The quantity of linear pBI121 DNA recovered from gel extraction of the excised bands (MM, Section 4.3.4.1.) was 19.6ng μl^{-1}.

4.4.3.2. Colony PCR analysis to test for correct orientation of LeB4-Ber e1-35S insert in transformed *E. coli* DH5α colonies

The circular transformation vector pBI121-LeB4-Ber e1 was constructed *via* a ligation reaction between the linearised pBI121 and the LeB4-Ber e1 cassette (MM, Section 4.3.4.2.). *E. coli* DH5α competent cells were then transformed with pBI121-LeB4-Ber e1 and 10 bacterial colonies which grew on selective medium were tested for the presence of the *npt*II-LeB4-*Ber e1* 3.9kb fragment *via* colony PCR. The results of the amplification reaction (Fig. 4.13.) showed that of 10 colonies tested, Colonies 6, 7 and 9 were positive for the presence of the desired fragment, as confirmed by the position of the bands just below the 4kb mark of the DNA ladder.

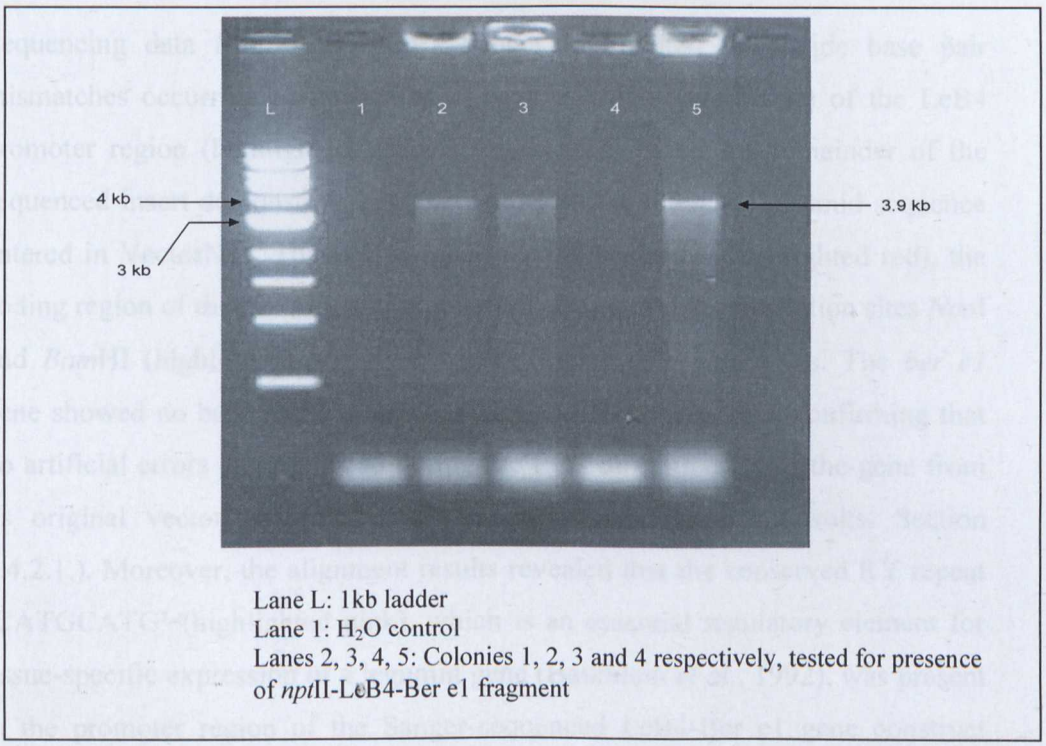


Lanes L: 1kb ladder
Lanes 1-10: Colonies 1-10 respectively, tested for presence of *npt*II-LeB4-Ber e1 fragment

Fig. 4.13. Colony PCR on *E. coli* DH5α colonies transformed with pBI121-LeB4-Ber e1. Detection of the amplified *npt*II-LeB4-Ber fragment was confirmed by the size of the bands which corresponded to the predicted size of 3.9kb.

4.4.4. Transformation of *A. tumefaciens* strain GV3101 with pBI121-LeB4-Ber e1

A. tumefaciens strain GV3101 was transformed with pBI121-LeB4-Ber e1 by electroporation following the method outlined in Section 2.4.6. Four bacterial colonies, which grew on selective medium (MM, Section 4.3.5.1.), were tested for the presence of the 3.9kb-sized *npt*II-LeB4-*Ber e1* fragment *via* colony PCR (MM, Section 4.3.5.2.). The results of the amplification reaction (Fig. 4.14.) showed that out of 4 colonies tested, colonies 1, 2 and 4 were positive for the presence of the desired fragment.



Lane L: 1kb ladder
Lane 1: H_2O control
Lanes 2, 3, 4, 5: Colonies 1, 2, 3 and 4 respectively, tested for presence of *npt*II-LeB4-Ber e1 fragment

Fig. 4.14. Colony PCR on *A. tumefaciens* GV3101 colonies transformed with pBI121-LeB4-Ber e1. Detection of the amplified *npt*II-LeB4-Ber fragment was confirmed by the size of the bands which corresponded to the predicted size of 3.9kb.

4.4.5. Sanger sequencing of LeB4-Ber e1 cassette and multiple sequence alignment using ClustalW

Plasmid extraction was carried out on liquid cultures of *E. coli* DH5α carrying pBI121-LeB4-Ber e1. This plasmid was sequenced using the Sanger sequencing method (Sanger *et al.*, 1977; Section 2.8.1). Using the multiple alignment tool ClustalW (EMBL-EBI), the sequencing data generated was compared against the reference plasmid sequence used in VectorNTI v.11 during construction of the pBI121-LeB4-Ber e1 plasmid map (MM, Section 4.3.6.) and the outcome displayed in Figure 4.15. The reference sequence was denoted as 'LeB4-Bere1 VNTI' while the Sanger sequencing data was denoted as 'LeB4-Bere1 Sanger'.

Sequencing data from LeB4-Bere1 Sanger highlighted nucleotide base pair mismatches occurring in three distinct regions (highlighted blue) of the LeB4 promoter region (highlighted yellow). Aside from these, the remainder of the sequenced insert demonstrated 100% identity to the reference plasmid sequence entered in VectorNTI. The LeB4 signal peptide sequence (highlighted red), the coding region of the *ber e1* gene (highlighted green) and the restriction sites *Nco*I and *Bam*HI (highlighted grey) were identical for both sequences. The *ber e1* gene showed no base pair mismatches between sequences, thus confirming that no artificial errors were induced during the PCR amplification of the gene from its original vector pMJA065 (MM, Sections 4.3.3.1. and Results, Section 4.4.2.1.). Moreover, the alignment results revealed that the conserved RY repeat 'CATGCATG' (highlighted pink), which is an essential regulatory element for tissue-specific expression of a legumin gene (Baumlein *et al.*, 1992), was present in the promoter region of the Sanger-sequenced LeB4-Ber e1 gene construct from pBI121-LeB4-Ber e1.

```
LeB4-Berel VNTI      AGCTTGCATGCCTGCAGGTCGGGGGATCTGCGGTACCCCGATTCAGTTATTTGAGAAAAAG  60
LeB4-Berel Sanger    AGCTTGCATGCCTGCAGGTCGGGGGATCTGCGGTACCCCGATTCAGTTATTTGAGAAAAAG  60
                     ************************************************************

LeB4-Berel VNTI      TAATGCAGACAAAAAGTGGAAAAGACAATCTGACTGTACATAAGAAATTTCCAATTTTTG  120
LeB4-Berel Sanger    TAATGCAGACAAAAAGTGGAAAAGACAATCTGACTGTACATAAGAAATTTCCAATTTTTG  120
                     ************************************************************

LeB4-Berel VNTI      AAATTTTTTTTATAATTATCAGAAATTTTAAAATTTCCGATAAAAACATACATGTATAGAT  180
LeB4-Berel Sanger    AAATTTTTTTTATAATTATCAGAAATTTTAAAATTTCCGATAAAAACATACATGTATAGAT  180
                     ************************************************************

LeB4-Berel VNTI      CGAAAATTTCAAATTTCTAGTACTTTCAAATTTCTTGCAGTAAAAGTTGTAATTTTTTAA  240
LeB4-Berel Sanger    CGAAAATTTCAAATTTCTAGTACTTTCAAATTTCTTGCAGTAAAAGTTGTAATTTTTTAA  240
                     ************************************************************

LeB4-Berel VNTI      AAATTTACGATAATTTACAGTATTTAAAAAAAAATCCAATCTTAAATAAAGGGTATAAGA  300
LeB4-Berel Sanger    AAATTTACGATAATTTACAGTATTTAAAAAAAAATCCAATCTTAAATAAAGGGTATAAGA  300
                     ************************************************************

LeB4-Berel VNTI      ATAAAAGCACTCATGTGGAGTGGCAGGTTTCGTCACACCCTAAGAACATCCCTAAATACA  360
LeB4-Berel Sanger    ATAAAAGCACTCATGTGGAGTGGCAGGTTTCGTCACACCCTAAGAACATCCCTAAATACA  360
                     ************************************************************

LeB4-Berel VNTI      CCACATATGTATAAGTATTAAGTGATTGATGTTAAGTGAAACGAAAATATTTATATGTGA  420
LeB4-Berel Sanger    CCACATATGTATAAGTATTAAGTGATTGATGTTAAGTGAAACGAAAATATTTATATGTGA  420
                     ************************************************************

LeB4-Berel VNTI      AATTTAATATTCAGCTTACTTGATTAAACTCCATAGTGACCCAATAAGTGCTAACTTTTA  480
LeB4-Berel Sanger    AATTTAATATTCAGCTTACTTGATTAAACTCCATAGTGACCCAATAAGTGCTAACTTTTA  480
                     ************************************************************

LeB4-Berel VNTI      CTGTCTTTACCTTTAAATGTTATATTGATTTATTTATGCATTTCTTTTTCCTGCATCTCA  540
LeB4-Berel Sanger    CTGTCTTTACCTTTAAATGTTATATTGATTTATTTATGCATTTCTTTTTCCTGCATCTCA  540
                     ************************************************************

LeB4-Berel VNTI      ATAGTATATAGGGTATCAAATAGTGATTATCCAAACTTAAATAAGTTAGAGGAAACACCA  600
LeB4-Berel Sanger    ATAGTATATAGGGTATCAAATAGTGATTATCCAAACTTAAATAAGTTAGAGGAAACACCA  600
                     ************************************************************

LeB4-Berel VNTI      AGATATGCCATATACTCTCAAATTTGACACTATGATTCAAAGTTGCACTTGCATAAAACT  660
LeB4-Berel Sanger    AGATATGCCATATACTCTCAAATTTGACACTATGATTCAAAGTTGCACTTGCATAAAACT  660
                     ************************************************************

LeB4-Berel VNTI      TATTAATTCAATAGTAAAACCCAACTTGTGCGTGATACAGTTAAAATGACTAAACTACTA  720
LeB4-Berel Sanger    TATTAATTCAATAGTAAAACCAAACTTGTGCGTGATACAGTTAAAATGACTAAACTACTA  720
                     ********************* ************************************

LeB4-Berel VNTI      ATTAAGGTCCCTCCCATTAGTAAATAAGTTATTTTTTTAGAAAAAGAAAATAATAAAAAG  780
LeB4-Berel Sanger    ATTAAGGTCCCTCCCATTAGTAAATAAGTTATTTTTTTAGAAAAAGAAAATAATAAAAAG  780
                     ************************************************************

LeB4-Berel VNTI      AATGACGAGTCTATCTAAATCATATTAACAAGTAATACATATTGATTCATTCGATGGAGG  840
LeB4-Berel Sanger    AATGACGAGTCTATCTAAATCATATTAACAAGTAATACATATTGATTCATTCGATGGAGG  840
                     ************************************************************

LeB4-Berel VNTI      AGGCCAATAATTGTAGCAAACAAGCAGTGCCGAGGTTAATATATGCTCAAGACAGTAAAT  900
LeB4-Berel Sanger    AGGCCAATAATTGTAGTAAACAAGCAGTGCCGAGGTTAATATATGCTCAAGACAGTAAAT  900
                     ***************I********************************************

LeB4-Berel VNTI      AATCTAAATGAATTAAGACAGTGATTTGCAAAGAGTAGATGCAGGAGAGAGAACTAAAGA  960
LeB4-Berel Sanger    AATCTAAATGAATTAAGACAGTGATTTGCAAAGAGTAGATGCAGAGAAGAGAACTAAAGA  960
                     ****************************************   **************

LeB4-Berel VNTI      TTTGCTGCTACACGTATATAAGAATAGCAACAGATATTCATTCTGTCTCTTTGTGGAATA 1020
LeB4-Berel Sanger    TTTGCTGCTACACGTATATAAGAATAGCAACAGATATTCATTCTGTCTCTTTGTGGAATA 1020
                     ************************************************************

LeB4-Berel VNTI      TGGATATCTACTAATCATCATCTATCTGTGAAGAATAAAAGAAGCGGCCACAAGCGCAGC 1080
LeB4-Berel Sanger    TGGATATCTACTAATCATCATCTATCTGTGAAGAATAAAAGAAGCGGCCACAAGCGCAGC 1080
                     ************************************************************

LeB4-Berel VNTI      GTCGCACATATGATGTGTATCAAATTAGGACTCCATAGCCATGCATGCTGAAGAATGTCA 1140
LeB4-Berel Sanger    GTCGCACATATGATGTGTATCAAATTAGGACTCCATAGCCATGCATGCTGAAGAATGTCA 1140
                     ************************************************************
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LeB4-Bere1 VNTI      CACACGTTCTGTCACACGTGTTACTCTCTCACTGTTCTCCTCTTCCTATAAATCACCGCG 1200
LeB4-Bere1 Sanger    CACACGTTCTGTCACACGTGTTACTCTCTCACTGTTCTCCTCTTCCTATAAATCACCGCG 1200
                     ************************************************************

LeB4-Bere1 VNTI      CCACAGCTTCTCCACTTCACCACTTCACCACTTCACTCACAATCCTTCATTAGTTGTTTA 1260
LeB4-Bere1 Sanger    CCACAGCTTCTCCACTTCACCACTTCACCACTTCACTCACAATCCTTCATTAGTTGTTTA 1260
                     ************************************************************

LeB4-Bere1 VNTI      CTATCACAGTCACCATGGCTTCCAAACCTTTTCTATCTTTGCTTTCACTTTCCTTGCTTC 1320
LeB4-Bere1 Sanger    CTATCACAGTCACCATGGCTTCCAAACCTTTTCTATCTTTGCTTTCACTTTCCTTGCTTC 1320
                     ************************************************************

LeB4-Bere1 VNTI      TCTTTACAAGCACATGTTTAGCAGGATCCCAGGAGGAGTGTCGCGAGCAGATGCAGAGAC 1380
LeB4-Bere1 Sanger    TCTTTACAAGCACATGTTTAGCAGGATCCCAGGAGGAGTGTCGCGAGCAGATGCAGAGAC 1380
                     ************************************************************

LeB4-Bere1 VNTI      AGCAGATGCTCAGCCACTGCCGGATGTATATGAGACAGCAGATGGAGGAGAGCACGTACC 1440
LeB4-Bere1 Sanger    AGCAGATGCTCAGCCACTGCCGGATGTATATGAGACAGCAGATGGAGGAGAGCACGTACC 1440
                     ************************************************************

LeB4-Bere1 VNTI      AGACCATGCCCAGGCGGGGAATGGAGCCGCATATGAGCGAGTGCTGCGAGCAGCTGGAGG 1500
LeB4-Bere1 Sanger    AGACCATGCCCAGGCGGGGAATGGAGCCGCATATGAGCGAGTGCTGCGAGCAGCTGGAGG 1500
                     ************************************************************

LeB4-Bere1 VNTI      GGATGGACGAGAGCTGCAGATGCGAAGGCTTAAGGATGATGATGAGGATGATGCAACAGA 1560
LeB4-Bere1 Sanger    GGATGGACGAGAGCTGCAGATGCGAAGGCTTAAGGATGATGATGAGGATGATGCAACAGA 1560
                     ************************************************************

LeB4-Bere1 VNTI      AGGAGATGCAACCCCGAGGGGAGCAGATGCGAAGGATGATGAGGCTGGCCGAGAATATCC 1620
LeB4-Bere1 Sanger    AGGAGATGCAACCCCGAGGGGAGCAGATGCGAAGGATGATGAGGCTGGCCGAGAATATCC 1620
                     ************************************************************

LeB4-Bere1 VNTI      CTTCCCGCTGCAACCTCAGTCCCATGAGATGCCCCATGGGTGGCTCCATTGCCGGGTTCT 1680
LeB4-Bere1 Sanger    CTTCCCGCTGCAACCTCAGTCCCATGAGATGCCCCATGGGTGGCTCCATTGCCGGGTTCT 1680
                     ************************************************************

LeB4-Bere1 VNTI      GAGGCGGACAAGCGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGTTCCA 1740
LeB4-Bere1 Sanger    GAGGCGGACAAGCGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGTTCCA 1740
                     ************************************************************
```

Fig.4.15. Multiple sequence alignment of LeB4-Ber e1 cassette. The gene sequence of the LeB4-Ber e1 expression cassette from pBI121-LeB4-Ber e1 (LeB4-Bere1 VNTI, generated by VectorNTI v.11 software package) was compared with the data generated from Sanger sequencing of the plasmid (LeB4-Bere1 Sanger) extracted from *E. coli* DH5α transformed cells, using ClustalW v.1.83 alignment tool (EMBL-EBI). An almost identical match was obtained between the 2 sequences, with the exception of 3 nucleotide mismatches (highlighted blue) in the LeB4 promoter region.

4.4.6. Comparative sequence analysis of the LeB4 promoter region using BLASTn and ClustalW

The LeB4 promoter region used as the VectorNTI-generated reference sequence of the LeB4-Ber e1 gene construct (Fig. 4.15., highlighted yellow), hereafter referred to as 'LeB4-Pro VNTI', was entered into the BLASTn programme and compared against all available nucleotide sequences in the NCBI database. This procedure was repeated for the Sanger sequencing results obtained for the LeB4 promoter region of this construct (Fig. 4.15., highlighted yellow), hereafter referred to as 'LeB4-Pro Sanger'. LeB4-Pro VNTI demonstrated a perfect match with the *Vicia faba* legumin (LeB4) promoter (GenBank: S60289.1) while LeB4-Pro Sanger showed greater sequence identity to the *V. faba* legumin (LeB4) promoter (GenBank: X03677.1). For both, BLAST results had E values of 0.0, maximum identities of >99% and a query coverage of 100%.

To localise nucleotide mismatches, multiple sequence alignments were employed using LeB4-Pro Sanger, LeB4-Pro VNTI and X03677.1 using ClustalW at default settings. Here, the location of two nucleotide mismatches between LeB4-Pro VNTI and X03677.1 were identical to those identified between LeB4-Pro Sanger and LeB4-Pro VNTI (Section 4.4.5), which were highlighted in red. However, a single nucleotide mismatch was identified between LeB4-Pro Sanger and both LeB4-Pro VNTI and X03677.1, highlighted in blue in Figure 4.16.

```
LeB4-Pro Sanger    ----------------------------------------AGCTTGCATGCCTGCAGGTC 20
X03677.1           ------------------------------------------------------------ 0
LeB4-Pro VNTI      ----------------------------------------AGCTTGCATGCCTGCAGGTC 20

LeB4-Pro Sanger    GGGGATCTGCGGTACCCCGATTCAGTTATTTGAGAAAAAGTAATGCAGACAAAAAGTGGA 80
X03677.1           ATCGGGTAGTTTTTTATCGATTCAGTTATTTGAGAAAAAGTAATGCAGACAAAAAGTGGA 60
LeB4-Pro VNTI      GGGGATCTGCGGTACCCCGATTCAGTTATTTGAGAAAAAGTAATGCAGACAAAAAGTGGA 80
                   ******************************************

LeB4-Pro Sanger    AAAGACAATCTGACTGTACATAAGAAATTTCCAATTTTTGAAATTTTTTTATAATTATCA 140
X03677.1           AAAGACAATCTGACTGTACATAAGAAATTTCCAATTTTTGAAATTTTTTTATAATTATCA 120
LEB4-Berel VNTI    AAAGACAATCTGACTGTACATAAGAAATTTCCAATTTTTGAAATTTTTTTATAATTATCA 140
                   ************************************************************

LeB4-Pro Sanger    GAAATTTTAAAATTTCCGATAAAAACATACATGTATAGATCGAAAATTTCAAATTTCTAG 200
X03677.1           GAAATTTTAAAATTTCCGATAAAAACATACATGTATAGATCGAAAATTTCAAATTTCTAG 180
LeB4-Pro VNTI      GAAATTTTAAAATTTCCGATAAAAACATACATGTATAGATCGAAAATTTCAAATTTCTAG 200
                   ************************************************************

LeB4-Pro Sanger    TACTTTCAAATTTCTTGCAGTAAAAGTTGTAATTTTTTAAAAATTTACGATAATTTACAG 260
X03677.1           TACTTTCAAATTTCTTGCAGTAAAAGTTGTAATTTTTTAAAAATTTACGATAATTTACAG 240
LeB4-Pro VNTI      TACTTTCAAATTTCTTGCAGTAAAAGTTGTAATTTTTTAAAAATTTACGATAATTTACAG 260
                   ************************************************************

LeB4-Pro Sanger    TATTTAAAAAAAAATCCAATCTTAAATAAAGGGTATAAGAATAAAAGCACTCATGTGGAG 320
X03677.1           TATTTAAAAAAAAATCCAATCTTAAATAAAGGGTATAAGAATAAAAGCACTCATGTGGAG 300
LeB4-Pro VNTI      TATTTAAAAAAAAATCCAATCTTAAATAAAGGGTATAAGAATAAAAGCACTCATGTGGAG 320
                   ************************************************************

LeB4-Pro Sanger    TGGCAGGTTTCGTCACACCCTAAGAACATCCCTAAATACACCACATATGTATAAGTATTA 380
X03677.1           TGGCAGGTTTCGTCACACCCTAAGAACATCCCTAAATACACCACATATGTATAAGTATTA 360
LeB4-Pro VNTI      TGGCAGGTTTCGTCACACCCTAAGAACATCCCTAAATACACCACATATGTATAAGTATTA 380
                   ************************************************************

LeB4-Pro Sanger    AGTGATTGATGTTAAGTGAAACGAAAATATTTATATGTGAAATTTAATATTCAGCTTACT 440
X03677.1           AGTGATTGATGTTAAGTGAAACGAAAATATTTATATGTGAA-TTTAATATTCAGCTTACT 419
LeB4-Pro VNTI      AGTGATTGATGTTAAGTGAAACGAAAATATTTATATGTGAAATTTAATATTCAGCTTACT 440
                   ****************************************** ******************

LeB4-Pro Sanger    TGATTAAACTCCATAGTGACCCAATAAGTGCTAACTTTTACTGTCTTTACCTTTAAATGT 500
X03677.1           TGATTAAACTCCATAGTGACCCAATAAGTGCTAACTTTTACTGTCTTTACCTTTAAATGT 479
LeB4-Pro VNTI      TGATTAAACTCCATAGTGACCCAATAAGTGCTAACTTTTACTGTCTTTACCTTTAAATGT 500
                   ************************************************************

LeB4-Pro Sanger    TATATTGATTTATTTATGCATTTCTTTTTCCTGCATCTCAATAGTATATAGGGTATCAAA 560
X03677.1           TATATTGATTTATTTATGCATTTCTTTTTCCTGCATCTCAATAGTATATAGGGTATCAAA 539
LeB4-Pro VNTI      TATATTGATTTATTTATGCATTTCTTTTTCCTGCATCTCAATAGTATATAGGGTATCAAA 560
                   ************************************************************

LeB4-Pro Sanger    TAGTGATTATCCAAACTTAAATAAGTTAGAGGAAACACCAAGATATGCCATATACTCTCA 620
X03677.1           TAGTGATTATCCAAACTTAAATAAGTTAGAGGAAACACCAAGATATGCCATATACTCTCA 599
LeB4-Pro VNTI      TAGTGATTATCCAAACTTAAATAAGTTAGAGGAAACACCAAGATATGCCATATACTCTCA 620
                   ************************************************************

LeB4-Pro Sanger    AATTTGACACTATGATTCAAAGTTGCACTTGCATAAAACTTATTAATTCAATAGTAAAAC 680
X03677.1           AATTTGACACTATGATTCAAAGTTGCACTTGCATAAAACTTATTAATTCAATAGTAAAAC 659
LeB4-Pro VNTI      AATTTGACACTATGATTCAAAGTTGCACTTGCATAAAACTTATTAATTCAATAGTAAAAC 680
                   ************************************************************

LeB4-Pro Sanger    CAAACTTGTGCGTGATACAGTTAAAATGACTAAACTACTAATTAAGGTCCCTCCCATTAG 740
X03677.1           CAAACTTGTGCGTGATACAGTTAAAATGACTAAACTACTAATTAAGGTCCCTCCCATTAG 719
LeB4-Pro VNTI      CCAACTTGTGCGTGATACAGTTAAAATGACTAAACTACTAATTAAGGTCCCTCCCATTAG 740
                   * ************************************************************

LeB4-Pro Sanger    TAAATAAGTTATTTTTTTAGAAAAAGAAAATAATAAAAAGAATGACGAGTCTATCTAAAT 800
X03677.1           TAAATAAGTTATTTTTTTAGAAAAAGAAAATAATAAAAAGAATGACGAGTCTATCTAAAT 779
LeB4-Pro VNTI      TAAATAAGTTATTTTTTTTAGAAAAAGAAAATAATAAAAAGAATGACGAGTCTATCTAAAT 800
                   ************************************************************

LeB4-Pro Sanger    CATATTAACAAGTAATACATATTGATTCATTCGATGGAGGAGGCCAATAATTGTAGTAAA 860
X03677.1           CATATTAACAAGTAATACATATTGATTCATTCGATGGAGGAGGCCAATAATTGTAGCAAA 839
LeB4-Pro VNTI      CATATTAACAAGTAATACATATTGATTCATTCGATGGAGGAGGCCAATAATTGTAGCAAA 860
                   ************************************************** ***
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LeB4-Pro Sanger    CAAGCAGTGCCGAGGTTAATATATGCTCAAGACAGTAAATAATCTAAATGAATTAAGACA 920
X03677.1           CAAGCAGTGCCGAGGTTAATATATGCTCAAGACAGTAAATAATCTAAATGAATTAAGACA 899
LeB4-Pro VNTI      CAAGCAGTGCCGAGGTTAATATATGCTCAAGACAGTAAATAATCTAAATGAATTAAGACA 920
                   ************************************************************

LeB4-Pro Sanger    GTGATTTGCAAAGAGTAGATGCAGAGAAGAGAACTAAAGATTTGCTGCTACACGTATATA 980
X03677.1           GTGATTTGCAAAGAGTAGATGCAGAGAAGAGAACTAAAGATTTGCTGCTACACGTATATA 959
LeB4-Pro VNTI      GTGATTTGCAAAGAGTAGATGCAGGAGAGAGAACTAAAGATTTGCTGCTACACGTATATA 980
                   ************************   *********************************

LeB4-Pro Sanger    AGAATAGCAACAGATATTCATTCTGTCTCTTTGTGGAATATGGATATCTACTAATCATCA 1040
X03677.1           AGAATAGCAACAGATATTCATTCTGTCTCTTTGTGGAATATGGATATCTACTAATCATCA 1019
LeB4-Pro VNTI      AGAATAGCAACAGATATTCATTCTGTCTCTTTGTGGAATATGGATATCTACTAATCATCA 1040
                   ************************************************************

LeB4-Pro Sanger    TCTATCTGTGAAGAATAAAAGAAGCGGCCACAAGCGCAGCGTCGCACATATGATGTGTAT 1100
X03677.1           TCTATCTGTGAAGAATAAAAGAAGCGGCCACAAGCGCAGCGTCGCACATATGATGTGTAT 1079
LeB4-Pro VNTI      TCTATCTGTGAAGAATAAAAGAAGCGGCCACAAGCGCAGCGTCGCACATATGATGTGTAT 1100
                   ************************************************************

LeB4-Pro Sanger    CAAATTAGGACTCCATAGCCATGCATGCTGAAGAATGTCACACACGTTCTGTCACACGTG 1160
X03677.1           CAAATTAGGACTCCATAGCCATGCATGCTGAAGAATGTCACACACGTTCTGTCACACGTG 1139
LeB4-Pro VNTI      CAAATTAGGACTCCATAGCCATGCATGCTGAAGAATGTCACACACGTTCTGTCACACGTG 1160
                   ************************************************************

LeB4-Pro Sanger    TTACTCTCTCACTGTTCTCCTCTTCCTATAAATCACCGCGCCACAGCTTCTCCACTTCAC 1220
X03677.1           TTACTCTCTCACTGTTCTCCTCTTCCTATAAATCACCGCGCCACAGCTTCTCCACTTCAC 1199
LeB4-Pro VNTI      TTACTCTCTCACTGTTCTCCTCTTCCTATAAATCACCGCGCCACAGCTTCTCCACTTCAC 1220
                   ************************************************************

LeB4-Pro Sanger    CACTTCACCACTTCACTCACAATCCTTCATTAGTTGTTTACTATCACAGTCA-------- 1272
X03677.1           CACTTCACCACTTCACTCACAATCCTTCATTAGTTGTTTACTATCACAGTCACAATGTCC 1259
LeB4-Pro VNTI      CACTTCACCACTTCACTCACAATCCTTCATTAGTTGTTTACTATCACAGTCA-------- 1272
                   ************************************************
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Fig. 4.16. Multiple alignment results of the LeB4 promoter region using LeB4-Pro Sanger, LeB4-Pro VNTI and X03677.1 sequences. Comparative sequence analysis identified 3 mismatches (highlighted red and blue).

4.5. DISCUSSION

4.5.1. Choice of recombinant DNA strategies

The strategy employed to design the multigene construct running from the RB to the LB of the pBI121 T-DNA region was mirrored on the construct designed by Molvig *et al.* (1997) whereby the candidate gene encoding for the methionine-rich sunflower seed albumin (SSA) was placed under the control of a vicilin promoter, and the *vic-ssa* cassette introduced between the selectable marker and reporter gene cassettes *35S-bar* and 35S-*uidA* respectively. Molvig and co-workers (1997) reported the successful transformation of lupin with the multigene construct, *via Agrobacterium*-mediated techniques. The similar approach, used to construct the vector pBI121-LeB4-Ber e1, was chosen to improve the chances of successful biolistic- and *Agrobacterium*- mediated transformation of Bambara groundnut.

The *LeB4-Ber e1-35S* expression cassette also carried the gene sequences encoding the c-myc tag and KDEL proteins (Fig. 4.6.). They play an important role during the molecular characterisation of putative transgenics, as they encode proteins which allow for the retention and detection of the expressed protein of interest in transformed plants.

The carboxy-terminal amino acid sequence KDEL (Lys-Asp-Glu-Leu) is a signal which activates the permanent retention of proteins in the endoplasmic reticulum (Pelham, 1989). The KDEL' sequence is recognized by a membrane-bound receptor that continually retrieves the proteins from a later compartment of the secretory pathway and returns them to the endoplasmic reticulum (Pelham, 1989). A study conducted by Wandelt and co-workers (1992) revealed that the accumulation and half-life of the pea seed protein vicilin was higher in tobacco and alfalfa plants transformed with a vicilin gene fused to the KDEL coding sequence, as compared to plants transformed with a gene construct lacking the KDEL sequence.

The c-myc tag can be used in many different assays that require recognition by an antibody. If no antibody exists against the protein of interest, adding a c-myc

tag to this protein allows the protein to be tracked with an antibody against the Myc-sequence. The *c-myc* gene codes for this epitope tag, which is useful to determine the expression of the protein of interest in a cell lysate, for example, using anti-myc antibodies in a Western blot.

4.5.2. Determination of cassette orientation in transformed *E. coli* DH5α

Using PCR analysis to determine insert orientation in transformed bacteria can be a tedious method of testing for positive colonies, as the PCR amplification cycles and gel electrophoresis are time consuming, and also because primers need to be designed and synthesised specifically for this application. Another method is by restriction digest analysis, whereby the plasmid is digested with two restriction enzymes which target two unique restriction sites, one located off-centre within the insert, and the other located in the vector backbone. However, since such unique restriction sites were not present in pBI121-LeB4-Ber e1, the confirmation of correct LeB4-Ber e1-35S insert orientation in transformed bacteria was not possible *via* this method.

4.5.3. Sanger sequencing of the LeB4-Ber e1 gene construct and comparative sequence analyses using BLASTn and ClustalW

The Sanger sequencing method can produce very high quality reads with a generally low error rate of approximately 1 error in every 10,000 – 100,000 nucleotides (Kirscher and Kelso, 2010). In this study, the alignment of Sanger-generated DNA sequences with the reference Vector NTI-generated DNA sequence was performed to highlight any nucleotide variation that might have occurred during the PCR amplification of the *ber e1* gene, which was subsequently used in the construction of the LeB4-Ber e1-35S cassette. The alignment results indicated that the LeB4-Bere1 Sanger sequence contained an identical copy of the *ber e1* gene. Interestingly, however, three nucleotide mismatches were produced which belonged to the LeB4 promoter region (Section 4.4.5., Fig. 4.15.).

Comparative sequence analysis of the LeB4 promoter region using BLAST and ClustalW (MM, Section 4.4.6.) demonstrated 99% sequence identity to two *Vicia*

fabia sequences (GenBank: S60289.1 and X03677.1), deposited in NCBI, which have been characterised as seed specific promoter regions (Baumlein *et al.*, 1992). Disparity between the donated reference sequence data and that obtained following Sanger sequencing could be due to a number of reasons. Amplification of the fragment from the original genomic DNA could have been performed using non-proof reading polymerase, and thus may have introduced artificial base pair errors. Secondly, promoter regions could have been amplified from different accessions of *Vicia fabia* which contained some sequence variation within this region. Finally, sequencing errors could have been introduced into this promoter region through base pair mis-calls which have been shown to be particularly common in regions containing homopolymers and base pair repeats (Pettersson *et al.*, 2009). This could explain the location of two distinct sequence mismatches between LeB4-Pro Sanger and LeB4-Pro VNTI occurring in both "AAA" homopolymer and "GAGA" repeat regions. Despite some sequence variation, the LeB4-Ber e1 construct was confirmed, through Sanger sequencing, to contain the 'CATGCATG' regulatory element (Fig. 4.15.; highlighted pink), which has been shown to regulate seed-specific expression of *ber e1* (Baumlein *et al.*, 1992), and was thus taken forward as part of an *in planta* functional assay in Bambara groundnut.

CHAPTER 5. BIOLISTIC-MEDIATED TRANSFORMATION OF BAMBARA GROUNDNUT

5.1. INTRODUCTION

Biolistic-mediated transformation is a process whereby genetic material is introduced into cells *via* micro-sized particles (Johnston and DeVit, 1996). The biolistic PDS-1000/He instrument (Fig. 5.1.) is capable of accelerating sub-cellular-sized tungsten or gold microparticles coated with DNA over a range of velocities and target distances thus enabling optimistion of each transformation system based on the target cell type. The helium pressure and vacuum circuits in the PDS-1000/He system effectively accelerate the microcarriers into the target cells. When all the materials are in place, the chamber door is closed and a vacuum is applied. The vacuum reduces the frictional resistance on the DNA-coated microcarriers and provides a safety interlock, ensuring that the instrument cannot be activated unless a vacuum is in place. Activating the fire switch allows helium to flow into the gas acceleration tube at a rate regulated by the helium-metering valve and monitored by the helium pressure gauge. The gas is held until the burst pressure of the rupture disc is reached. This generates a helium shock wave into the bombardment chamber which hits the microcarrier launch assembly and propels a plastic macrocarrier holding DNA-coated microcarriers towards the target cells. A stopping screen placed between the macrocarrier assembly and the cells retains the macrocarrier disc, while allowing the coated microprojectiles to pass through and penetrate the target cells.

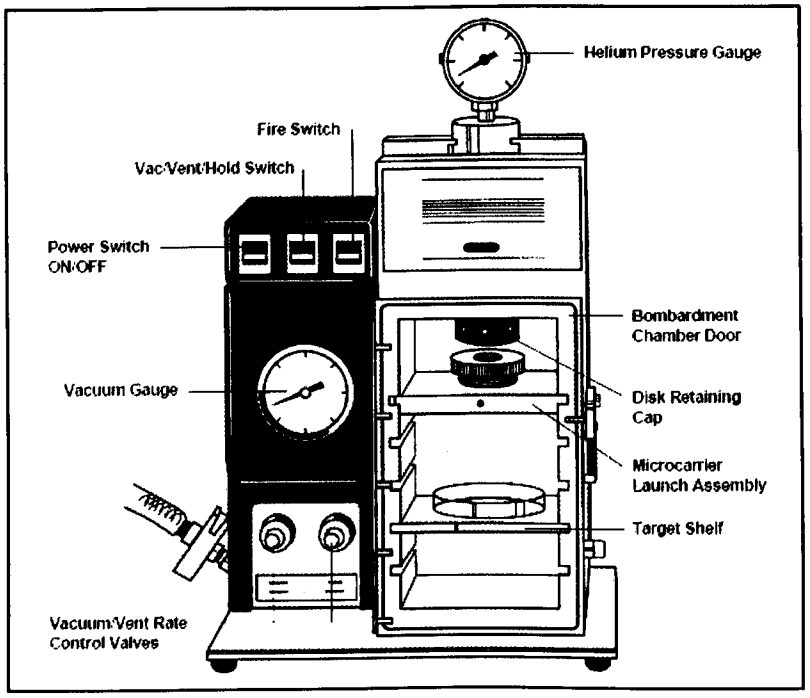

Fig. 5.1. Front view of the PDS-1000/He unit.
Source: Biolistic® PDS-1000/He Particle Delivery System, Bio-Rad instruction manual.

Plant species belonging to the Leguminosae have generally been considered difficult to transform (Hirsch *et al.*, 2010). However, despite the persistent recalcitrance of several legume species towards genetic manipulation, transformation systems have been optimised for legumes such as soybean (Inaba *et al.*, 2007; Rech *et al.*, 2008) and common bean (Rech *et al.*, 2008), using biolistic-mediated gene transfer.

Inaba and co-workers (2007) studied the tissue specificity of the tobacco anthranilate synthase (ASA2) promoter in soybean *via* biolistic-mediated transformation. Embryogenic suspension cultures of the legume were bombarded with pHAG and GUSpAPCH7, which carried the *uidA* gene driven by the ASA2 promoter and the CaMV35S promoter respectively. Selection of transgenics was using hygromycin, and regenerated tissue and plants were assayed for histochemical GUS activity. The results showed that GUS expression driven by the ASA2 promoter was greater in regenerated embryogenic tissues, and in pollen and mature seeds of hygromycin resistant plants as compared to vegetative tissues of the plants. ASA2-driven GUS expression in mature seeds was also found to be comparatively lower than in seeds transformed with the

CaMV35S-*uidA* gene cassette. The tissue specificity of ASA2 expression may be useful in future transformation studies of crops to drive genes of interest in specific tissue types.

The study by Rech *et al.* (2008) focused on developing an efficient and reproducible biolistic-mediated transformation protocol using mature zygotic embryos of soybean, common bean and cotton as the starting explants, and the selectable marker gene *ahas* from *Arabidopsis thaliana.* The *ahas* gene carried a mutation that confers resistance to imidazolinones, a class of herbicides which include imazapyr. Imazapyr usually inhibits the activity of the enzyme acetohydroxyacid synthase, responsible for the catalysis of the three essential amino acids isoleucine, leucine and valine in the apical meristem. Rech and co-workers argued that following particle bombardment of embryonic axes, the putatively transformed apical meristematic cells cannot be selected using agents such as kanamycin and hygromycin because they are not in contact with the selective agent in the culture medium. Under *in vitro* conditions, imazapyr added to the culture media would translocate through the embryonic axis and concentrate in the apical meristem without damaging the explant. Therefore, the addition of imazapyr as the selective agent would allow putatively transformed cells in the apical region, which carry the imazapyr-resistant *ahas* gene, to be selected for effectively. Soybean, common bean and cotton embryos bombarded with pCA321 harbouring the *ahas* gene were cultured onto selective medium containing 500nM, 80nM and 300nM imazapyr, respectively. The anticipated results of this study were an average frequency of transformation of 9, 2.7 and 0.5% for soybean, common bean and cotton respectively.

This chapter discusses the attempts at transforming Bambara groundnut mature zygotic embryos with pBI121-LeB4-Ber e1 *via* biolistic-mediated gene transfer. The plasmid used in this experiment harboured three gene cassettes between the T-DNA left and right borders, namely *35S.gus.nos* and *nos.npt*II*.nos*, which served as reporter and selectable marker genes, respectively, as well as *LeB4.ber e1.35S* which contained the gene of interest for methionine accumulation, driven by the legumin B4 seed-specific promoter.

5.2. AIMS AND OBJECTIVES

The aim of the work carried out in this chapter was to employ the biolistic particle delivery system to transform Bambara groundnut zygotic embryos (from landraces DipC, Uniswa-Red and S19-3) with the recombinant vector pBI121-LeB4-Ber e1 which carried the methionine-rich *ber e1* gene under the control of the seed-specific LeB4 promoter, as well as the selectable marker gene *npt*II and the reporter gene *uidA*. Putatively transformed explants were selected and maintained on a tissue culture régime previously established in Chapter 3. Confirmation of plasmid integration was by histochemical GUS activity in transformed embryos.

The objectives of the experiments carried out were to:

i. Determine the level of endogenous kanamycin resistance in Bambara groundnut embryos *via* the production of kill curves for each landrace.
ii. Prepare the explant material under axenic conditions for biolistic-mediated transformation.
iii. Sterilise the Biolistic® PDS-1000/He Particle Delivery System and ensure that the correct settings were in place.
iv. Coat the microprojectiles with pBI121-LeB4-Ber e1 and prepare macrocarriers for plasmid DNA delivery into target explants.
v. Transform Bambara groundnut zygotic embryos with pBI121-LeB4-Ber e1 using the Biolistic® PDS-1000/He Particle Delivery System.
vi. Select for putatively transformed Bambara groundnut explants on medium supplemented with kanamycin as the selective agent, and maintain those explants in culture based on the established protocol reported in Chapter 3.
vii. Confirm plasmid integration in putatively transformed explants *via* histochemical GUS analysis.

5.3. MATERIALS AND METHODS

5.3.1. Endogenous kanamycin resistance in Bambara groundnut

The sensitivity of Bambara groundnut embryos to kanamycin was investigated to determine their endogenous tolerance to the antibiotic since the transformation vector used in this experiment carried the kanamycin-resistant *npt*II selectable marker gene. A kanamycin kill-curve was set up in order to determine the optimum concentration of the antibiotic necessary to select for putatively transformed regenerants.

Zygotic Bambara groundnut embryos (DipC, Uniswa-Red and S19-3) were excised from whole mature seeds (Appendix I) and cultured for 6 d on basal medium supplemented with $1mgL^{-1}$ NAA and $1mgL^{-1}$ BAP. Embryos were then transferred onto Bambara shoot regeneration (BSR) medium (basal medium with $1.5mgL^{-1}$ BAP) supplemented with 0, 10, 25, 50, 75, 100, 125 and 150 mgL^{-1} kanamycin sulphate. The cultures were placed under a 16 h photoperiod at 23 ± 1°C, and monitored for shoot regeneration over 8 wk. Two independent kill-curve experiments were set up for each Bambara groundnut landrace. Each experiment contained 3 replicates for each antibiotic concentration and each replicate carried 9 whole embryo explants.

After 8 wk in culture, the percentage of embryos which produced adventitious shoots was calculated at each antibiotic concentration for each landrace (Results, Section 5.4.1.). The lowest concentration of kanamycin sulphate which turned all of the tested embryos necrotic and gave no regeneration was determined and subsequently used in the selection medium.

5.3.2. Plant material for biolistic-mediated transformation

Whole zygotic embryos of Bambara groundnut (DipC, Uniswa-Red and S19-3) were used as starting explants in this transformation experiment. Mature seeds were surface-sterilised and prepared for embryo excision following the procedure

outlined in Chapter 2, Sections 2.3.5.1 and 2.3.5.2. The excised embryos were cultured for 6 d prior to bombardment in 9cm Petri dishes containing 20ml basal medium (Appendix I, Section AI.3.) supplemented with $1mgL^{-1}$ NAA and $1mgL^{-1}$ BAP. Embryos were carefully positioned in a circle, equidistant between 6 and 12 mm from the centre of the 9cm Petri dish, with the apical meristematic region facing upwards, as proposed by Rech *et al.* (2008). Two independent bombardment experiments were carried out for each Bambara groundnut landrace. Each experiment contained 3 replicates and each replicate contained 15 whole embryo explants. Control experiments had an identical setup.

5.3.3. Sterilisation of the Bio-Rad PDS-1000/He biolistics device

The laminar air-flow cabinet containing the biolistics apparatus as well as the inside of the biolistics chamber were sterilised by wiping the surfaces with 70% (v/v) ethanol. The other items required for the transformation process included macrocarriers and stopping screens which were autoclaved at 121°C for 20 min, as well as macrocarrier holders, a pair of forceps and a macrocarrier insertion tool which were all sterilised by immersion in 70% (v/v) ethanol for 30 min and allowed to dry in the laminar air-flow cabinet.

5.3.4. Preparation of the gold microcarrier stock suspension

Thirty micrograms of gold powder [1.2µm in diameter, (Alfa Aesar, Heysham, UK)] was weighed in a microfuge tube. One millilitre of absolute (100% v/v) ethanol was added to the gold powder. The mixture was vortexed on a 'medium' setting for 3 min and centrifuged for 1 min at 10,000 rpm. The supernatant was slowly pipetted out in order not to disturb the loose pellet, and the pellet resuspended in 500µl of sterile distilled water. The sterile stock suspension of gold particles was stored at 4°C until required, and replaced monthly.

5.3.5. Preparation of macrocarriers

Each macrocarrier was wiped with a sheet of lens tissue to remove any static charges, and placed in individual macrocarrier holders using a pair of sterile forceps. Care was taken to ensure that the macrocarrier was firmly in place under the lip of the holder by using the macrocarrier insertion tool. The prepared macrocarriers/holders were kept in a 9cm Petri dish in the laminar air-flow cabinet until required.

5.3.6. DNA coating of microprojectiles

This protocol prepared enough DNA coated particles for 6 bombardment shots. The sterile gold particle stock suspension was thoroughly vortexed and a 50µl aliquot was transferred into a sterile microfuge tube. The vortex was set at medium speed, and the tube placed on it and carefully opened. While vortexing, the following was added to the gold suspension in order: 10µl of pBI121-LeB4-Ber e1 DNA (from a 168ng μl^{-1} stock), 50µl of 2.5M $CaCl_2$, and 20µl of 0.1M spermidine (Sigma-Aldrich Co.). The $CaCl_2$ facilitates the precipitation of the DNA onto the gold particles, while the spermidine, which is a polyamide, increases the static charges on plasmid GC-rich regions, thereby enhancing the overall coating efficiency. The mixture was vortexed at medium speed for 3 min.

The gold particles were then pulse-centrifuged for 6 s, and the supernatant was carefully removed to as not to dislodge the loose pellet. Absolute ethanol (250µl) was added to the tube to wash the coated gold particles, and the mixture was resuspended by briefly vortexing at medium speed, followed by pulse-centrifugation for 6 s. The supernatant was discarded, and the gold particles thoroughly resuspended in 65µl of absolute ethanol. The coated microprojectiles were immediately loaded onto the macrocarriers. For each experiment, enough DNA-coated gold suspension was prepared for 12 shots. Any unused suspension was discarded at the end of the experiment.

5.3.7. Loading of macrocarriers

Using a Gilson pipette, 10µl of the freshly coated microprojectile suspension was placed onto the centre of each inverted macrocarrier. The loaded macrocarriers were transferred into an incubator set at 37°C and allowed to dry completely. The loaded macrocarriers were then used immediately for particle delivery.

5.3.8. Biolistic-mediated particle delivery

Each Petri dish containing 15 zygotic embryos of Bambara groundnut was placed in the holder on the 3rd shelf of the biolistics chamber (Fig. 5.1.) Following the manufacturer's instructions (Section 2.5.1.), the embryos from each Petri dish were bombarded once with microprojectiles coated with pBI121-LeB4-Ber e1. A helium delivery pressure of 1,300 psi was set on the helium cylinder regulator, and rupture discs of 1,100 psi were used. Immediately after bombardment, the embryos were transferred into 9cm Petri dishes each containing 20ml of non-selective BSR medium which consisted of basal medium (Appendix I, Section AI.3.) supplemented with 1.5mgL^{-1} BAP, for 48 h.

After 48 h on non-selective medium, the embryos were transferred into screw-capped sterile 175ml Round Powder jars containing the selective medium (5 explants per jar), the latter being basal medium supplemented with 1.5mgL^{-1} BAP and 50mgL^{-1} kanamycin. The explants were subcultured onto fresh selective medium (50ml per jar) every 2 wk and monitored for any signs of shoot regeneration and development (Results, Section 5.4.2.).

5.3.9. Experimental controls

Two types of experimental controls were set up for this experiment. In control I, zygotic Bambara groundnut embryos (30 per landrace) were bombarded with non-coated microprojectiles suspended in absolute ethanol. Following bombardment, the embryos were cultured on non-selective BSR medium for 48

h, before being subcultured periodically on selective medium, as described in Section 5.3.8.

Control II consisted of non-bombarded Bambara groundnut embryos cultured on non-selective BSR medium for 48 h, before being periodically subcultured onto selective medium, as described in Section 5.3.8.

5.3.10. Analysis of transient GUS expression in bombarded embryos

The protocol used for the histochemical detection of GUS activity was based on the method described by Jefferson *et al.* (1987), and was carried out 48 h after the bombardment of the explants with pBI121-LeB4-Ber e1. Ten embryo explants of each Bambara groundnut landrace were selected randomly for histochemical GUS assay. At the time of this random selection, the assayed explants were cultured on non-selective BSR medium (Section 5.3.8.).

The explants from each landrace were placed in sterile glass universal vessels (10 explants per bottle) each containing 10ml of GUS assay solution (Appendix IV. Sections AIV.1. and AIV.2.) made up of 5mg of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; Melford Laboratories Ltd, Ipswich, UK) dissolved in 100µl of dimethyl formamide (DMF), phosphate buffer (0.2M NaH_2PO_4 plus 0.2M Na_2HPO_4, pH 7.0), 0.5M Na_2EDTA, 10mM $K_3Fe(CN)_6$, 10mM $K_4Fe(CN)_6.3H_2O$ and 0.1% (v/v) Triton-X-100 (Sigma-Aldrich Co.). The explants were incubated in the dark at 37°C for 16h, after which they were immersed in a solution of 70% (v/v) ethanol to remove the chlorophyll from the tissues, and observed under the stereomicroscope for traces of indigo staining to confirm the presence of GUS activity. As a control, non-bombarded cultured embryos (5 from each landrace) (Section 5.3.9.) were subjected to the same treatment. Assayed explants were photographed (Results, Section 5.4.3.).

5.4. RESULTS

5.4.1. Kanamycin kill-curve of Bambara groundnut

Embryos from all 3 Bambara groundnut landraces exhibited a decrease in adventitious tissue regeneration when cultured on increasing concentrations of kanamycin (Fig. 5.2.). Uniswa-Red (U-R) embryos showed less resistance to the antibiotic, as all of the embryos cultured on 25mgL^{-1} kanamycin had turned necrotic, while 61.1% of DipC and 27.8% of S19-3 embryos still exhibited adventitious shoot formation at that same antibiotic concentration. In the 3 landraces, all cultured embryos turned necrotic and regeneration was not observed on medium supplemented with 50mgL^{-1} kanamycin. Based on the results of the kill-curve, a concentration of 50mgL^{-1} kanamycin was subsequently used to select for putatively transformed regenerated Bambara groundnut shoots which carried the *npt*II selectable marker gene from pBI121-LeB4-Ber e1.

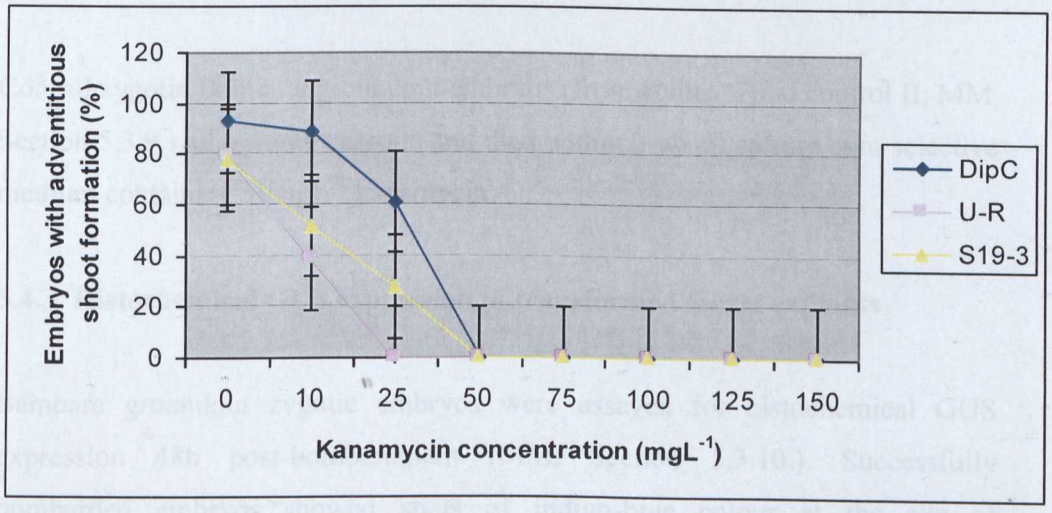

Fig. 5.2. The effect of increasing kanamycin concentrations on the survival *in vitro* and formation of adventitious shoots from embryos of Bambara groundnut (landraces DipC, Uniswa-Red and S19-3). Bars = Standard error.

5.4.2. Fate of Bambara groundnut embryos post-bombardment with pBI121-LeB4-Ber e1

Mature zygotic Bambara groundnut embryos were bombarded with microprojectiles coated with pBI121-LeB4-Ber e1 and cultured onto shoot regeneration medium supplemented with 1.5mgL^{-1} BAP, with or without antibiotics (MM, Section 5.3.8.). For the first 48 h of culture on BSR medium without antibiotic, all the bombarded Bambara groundnut embryos were observed to survive. Following subculture onto selective medium, the survival rate of the bombarded embryo dropped considerably. Out of a total of 80 bombarded embryos for each landrace, the number of surviving embryos was 24 for DipC, 27 for Uniswa-Red and 19 for S19-3, after 4 wk of culture on selective medium. The numbers declined to 6 DipC, 7 Uniswa-Red and 9 S19-3 surviving explants after 8 wk in culture, and by the 12th wk, all of the bombarded embryos had turned necrotic. Embryos of all 3 landraces which survived past the 8th wk showed signs of adventitious bud formation, but the buds also later turned necrotic and did not develop further.

Control zygotic Bambara groundnut embryos (from control I and control II; MM, Section 5.3.9.) all became necrotic and died within 3 wk of culture onto selective medium containing 50mgL^{-1} kanamycin.

5.4.3. Histochemical GUS expression in transformed target explants

Bambara groundnut zygotic embryos were assayed for histochemical GUS expression 48h post-bombardment (MM, Section 5.3.10.). Successfully bombarded embryos showed spots of indigo-blue colour at the site of microprojectile delivery. Out of 10 embryos randomly assayed from each landrace, positive results were obtained in 4 DipC embryos, 6 Uniswa-Red embryos and 2 S19-3 embryos. Figure 5.3. shows DipC embryos which stained positive for histochemical GUS production. The blue spots are scattered over the side of the embryo (Fig. 5.3.) which faced the microprojectile pathway during biolistic-mediated particle delivery (MM, Section 5.3.8.). The positively assayed

embryos from the other landraces Uniswa-Red and S19-3 also exhibited similar patterns of indigo spots.

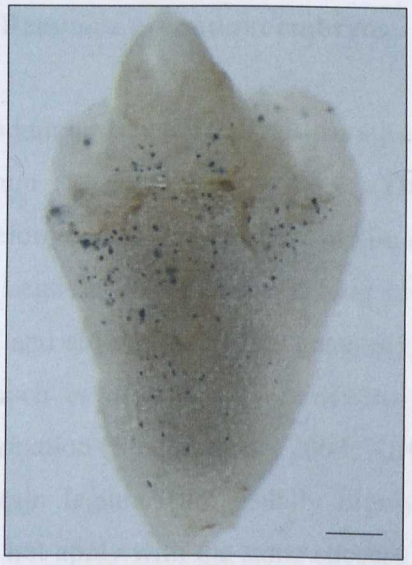

Fig. 5.3. Histochemical GUS staining in a transformed 8 day-old Bambara groundnut DipC embryo, 48h post-bombardment with pBI121-LeB4-Ber e1. Bar = 0.5mm

5.5. DISCUSSION

5.5.1. Fate of bombarded Bambara groundnut embryos

Bombarded Bambara groundnut embryos (DipC, Uniswa-Red and S19-3) did not survive on selective medium (Results, Section 5.4.2.) The biolistic-mediated protocol employed therefore requires further optimisation to increase transformation efficiency. Legume species have a long history of inbreeding, reduced genetic variability, and are reported to be generally recalcitrant towards *in vitro* manipulation (Hirsch *et al.*, 2010; Wisznewska and Pindel, 2009). Reviews on legume transformation (Somers *et al.*, 2003; Kirti, 2008) suggest that protocols developed for grain legumes are usually highly species or variety specific, and therefore may not apply with the same success rate to other species of the family. The *in vitro* regeneration protocol employed in this project was based on a method developed for Bambara groundnut landraces Nkorenza and Yoroba from Ghana (Lacroix *et al.*, 2003), but not for the landraces investigated here. Future work needs to concentrate on optimising an explant-to-plant regeneration system for the DipC, Uniswa-Red and S19-3 landraces with a focus on the use of different basal media types, media supplements, plant growth regulators and growth-room conditions. It may be that while the modified protocol by Lacroix *et al.*, (2003) can be applied with moderate success to non-transformed embryos (Chapter 2), bombarded embryos of the same landraces become too stressed following transformation and cannot survive under the same tissue culture régime.

Other factors may have influenced the regeneration potential of putatively transformed adventitious tissue from bombarded Bambara groundnut embryos. The use of cotyledonary nodes and embryonal axes as starting explants in transformation studies on grain legumes has been reported to result in the successful recovery of transgenic plants (Babaoglu *et al.*, 2000). In the study by Rech and co-workers (2008), the removal of the primary leaves from soybean, common bean and cotton embryos to expose the meristematic region was carried

out under a stereomicroscope prior to bombardment. This step was overlooked in the present experiment, and future work could investigate the effect of removing primary leaves from starting embryo explants on the regeneration potential of putative Bambara groundnut transgenics.

Future studies should also aim to optimise the microprojectile bombardment system in order to maximise the delivery of plasmid DNA into the target explants. Investigations into the optimisation of parameters including the diameter of microparticles, target distance, helium pressure and concentration of plasmid DNA should be carried out (Hunold *et al.*, 1994). The orientation of embryonic explants in Petri dishes prior to bombardment could be altered, with apical meristems positioned more favourably in the path of microprojectiles in order to maximise microprojectile delivery into meristematic tissues. The alteration of the biolistic particle delivery system for the microtargeting of explant tissue is an optimisation step that could also be investigated. This would allow for a more precise delivery of foreign DNA into targeted cells, and could potentially increase the efficiency of the transformation system. Thomas and co-workers (2001) modified the original PSD/1000-He BioRad® particle delivery system by replacing the original macrocarrier with a more rigid one, to minimise the effects of the helium blast. A focusing nozzle was also attached to the biolistic launching module which allowed for a more precise microparticle delivery into the target tissue. The use of the altered biolistic device resulted in a more effective transfection of fragile insect tissues including epithelial wing disks and ovarian follicle cells from *Bombix mori*.

5.5.2. Transient histochemical GUS expression

The positive histochemical GUS staining in Bambara groundnut embryos assayed 48h post-bombardment confirmed that the gene delivery had been successful. Each blue spot represented a single transformation event which confirmed that pBI121-LeB4-Ber e1 had been successfully been delivered into the cells and that the *uidA* expression cassette had been integrated in the nuclear genomes of the transformed cells. The *uidA* gene reporter system is an important

tool to study plant gene expression in transformation systems. The transient expression of the *uidA* reporter gene in bombarded hypocotyls of *Feronia limonia* L. led to the first report of successful transformation in this recalcitrant woody tree (Purohit *et al.*, 2007). Studies on biolistic-mediated transformation of meristematic tissues of important legumes including peanut (Deng *et al.*, 2001) and soybean (Inaba *et al.*, 2007) have relied on the *uidA* reporter gene system to visually screen for transformed tissue and quantitatively assess the efficiency of the transformation protocol. There is a need for further work on the optimisation of the Bambara groundnut regeneration protocol, so that regenerated adventitious shoots can be analysed histochemically by GUS activity to determine if *uidA* gene expression still occurs at the later stages of the regeneration of bombarded tissues. PCR and RT-PCR analyses as well as Southern blotting experiments, could also be carried out on regenerated shoots to confirm the integration and expression of the *nos.nptII.nos* and *LeB4.Ber e1.35S* cassettes.

CHAPTER 6. *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF BAMBARA GROUNDNUT

6.1. INTRODUCTION

In plant transformation studies, foreign gene transfer can be achieved either by mechanical or biological methods. Popular forms of mechanical gene transfer include electroporation, polyethyleneglycol (PEG)-mediated transformation and microprojectile bombardment of plant cells and tissues. A common biological form of plant transformation has originated from the naturally occurring *Agrobacterium tumefaciens*, a soil-borne pathogen with the unique ability to transfer a DNA fragment (T-DNA) of its tumour-inducing (Ti) plasmid into plant hosts. The *Agrobacterium*-mediated transformation system employs disarmed strains of *A. tumefaciens* which have been modified to carry genes of interest on their T-DNA for the purpose of plant transformation. During plant transformation, the genes inserted into the T-DNA are co-transferred and integrated into the host genome, making *Agrobacterium*-mediated gene transfer a popular method of altering traits in plant breeding programmes. Conventional *A. tumefaciens* transformation consists of an initial co-culture step where T-DNA transfer to plant cells occurs, followed by the selection of transformed cells and the regeneration of transgenic plants.

Despite their economic importance, grain legumes have received less scientific attention than cereal crops in terms of improvement by *in vitro* genetic manipulation. This could be due to the fact that grain legumes are less amenable to *in vitro* manipulation, with the limiting step often being the regeneration of transformed cells into transgenic plants. Over the last few decades, however, attempts at the genetic improvement of important legume crops, including the model crop *Medicago truncatula*, have been reported, using the *Agrobacterium*-mediated gene transfer system (Trieu and Harrison, 1996; Chabaud *et al*, 2003; Wright *et al*. 2006; Kirti, 2008).

Agrobacterium-mediated transformation and regeneration protocols have been established for two main *M. truncatula* genotypes, namely Jemalong and R108, and their derivatives, using different explant types and *A. tumefaciens* strains.

Regeneration following inoculation and co-culture was either *via* somatic embryogenesis or organogenesis. Wright and co-workers (2006) used cotyledonary nodes as starting explants in the transformation of *M. truncatula* Jemalong (line A17) and R108-1 with *A. tumefaciens* strain AGL1 carrying the *npt*II selectable marker gene. Regeneration of transformants was *via* organogenesis, using kanamycin as the selective agent. Shoot induction and development was on MS-based medium supplemented with $1mgL^{-1}$ BAP and $0.1mgL^{-1}$ NAA, while root development on regenerated plants was on MS-based medium supplemented with $0.2mgL^{-1}$ IBA. This study reported the regeneration and development of transgenic shoots in 5% of A17 explants and 12% of R108-1 explants. In a study by Iantcheva and co-workers (2005), embryo clusters generated from *in vitro* cultured cotyledons and petioles were used as starting explants and inoculated with *A. tumefaciens* strain LBA4404 carrying pBI121 with the *npt*II selectable marker and *uidA* reporter genes. Regeneration of putative transgenics was *via* secondary embryogenesis on MS-based medium supplemented with $0.5mgL^{-1}$ TDZ and with kanamycin as the selective agent. The development of putatively transformed secondary embryos into plants was achieved on medium supplemented with $0.05mgL^{-1}$ BAP and $50mgL^{-1}$ kanamycin. This study reported that 65% of inoculated embryo clusters produced kanamycin-resistant secondary embryos. Molecular analysis of putatively transformed regenerated plants was by PCR and Southern blotting to confirm transgene integration. Confirmed transformants were acclimatised to glasshouse conditions and produced seeds after 4 – 5 mth. The inheritance of the *npt*II gene in T_1 seedlings followed a Mendelian segregation. Other transformation studies have employed explants such as folioles, petioles and roots, and *A. tumefaciens* strains including AGL1, EHA105 and C58C1, with 25 – 70% of inoculated explants producing transgenic shoots (Chabaud *et al.*, 2003; de Sousa Araujo *et al.*, 2004; Crane *et al.*, 2006).

Soybean is one of the most economically important legume crops, as it is the world's primary source of vegetable protein and oil. Although the efficiency of *Agrobacterium*-mediated transformation methods developed for the genetic improvement of soybean has improved over the last two decades following the

simple protocol published by Horsch and co-workers (1985), tissue culture and transformation procedures remain labour-intensive, with the regeneration frequency of transformants still relatively low compared to previously recalcitrant species such as maize and rice, which are currently routinely and efficiently transformed with *Agrobacterium* (Kirti, 2008). The efficiency of *Agrobacterium*-mediated transformation in soybean is dependent on several factors, including genotype, *A. tumefaciens* strain, explant type and *in vitro* culture and regeneration protocol. The soybean cultivar Peking was one of the first cultivars to respond favourably to *Agrobacterium*-mediated transformation (Hinchee *et al.*, 1988), and subsequent studies demonstrated that most soybean cultivars were amenable to such transformation methods but with varying levels of efficiency (Kirti, 2008). *A. tumefaciens* strains which have been successfully employed in soybean transformation include EHA101, EHA105 and KYRT1 (Meurer *et al.*, 1998; Zhang *et al.*, 1999; Yan *et al.*, 2000). Explants with pre-existing meristematic tissues are favoured for *in vitro* plant regeneration, as reported in studies using cotyledonary nodes (Wright *et al.*, 1986; Olhoft *et al.*, 2006), epicotyls (Dan and Reichert, 1998) and the meristem region of the apical dome (Aragão *et al.*, 2000; Dang and Wei, 2007).

The successful transformation of soybean was reported by Dang and Wei (2007) using meristematic embryonic tips from mature dry seeds as starting explants. Embryonic tips were inoculated and cocultured with *A. tumefaciens* strains EHA105, KYRT1 or LBA4404 carrying the binary vector pCAMBIA3301 which harboured the *uidA* reporter gene and the *bar* selectable marker gene. Transformation efficiency was optimum with strain KYRT1, and an acidic regeneration medium (pH 5.4). Regeneration and development of putatively transformed shoots from embryonic tips was on agar-based MS medium with 0.2mgL^{-1} BAP, 0.2mgL^{-1} IBA and PPT (0.5 – 1.25mgL^{-1}) as the selective agent. The transformation frequency for the tested cultivars ranged from 4.29% to 18.0% and insect resistance in the T_1 progeny was inherited in a Mendelian manner.

Agrobacterium-mediated gene transfer techniques have been developed for the improvement of *Vicia faba* (Faba bean), a commercially important grain legume, to produce transgenic lines with improved resistance to biotic and abiotic stresses and with enhanced nutritional value. The recovery of fertile transgenic plants has been reported *via* 2 regeneration systems, namely *de novo* regeneration from internodal stem segments using TDZ (Böttinger *et al.*, 2001) and direct shoot organogenesis from mature and immature embryo axes (Hanafy *et al.*, 2005). The *de novo* regeneration protocol employed stem segments of *V. faba* inoculated with *A. tumefaciens* strains EHA101 or EHA105, followed by callus induction on MS-based medium with 0.5mgL^{-1} each of TDZ, 2,4-D and NAA, using kanamycin (100mgL^{-1}) or PPT (2mgL^{-1}) as selective agents. Transgenic shoot regeneration was induced *via* organogenesis using media supplemented with 7.5mgL^{-1} TDZ and 0.75mgL^{-1} NAA, and plants were recovered by micrografting, due to poor root formation from the regenerates. This transformation and regeneration system was laborious, time consuming and had low regeneration efficiency, with inoculated explants producing limited quantities of regenerable tissue, and fertile primary transformants produced after 16 – 24 mth. Low regeneration efficiency was also reported by Hanafy and co-workers (2005), where immature and mature zygotic embryo axes were used as starting explants and inoculated with *A. tumefaciens* strains EHA101 and EHA105 harbouring various binary vectors. Regeneration of transformed tissues was *via* direct shoot organogenesis without an intermediate callus phase, using kanamycin or PPT as selective agents. This regeneration system was advantageous over the *de novo* method because it reduced the possibility of somaclonal variation to a minimum. Transformation frequencies ranged from 0.15 – 2.0 %, with 3 – 4 shoots regenerating from each explant. Two out of 6 tested *V. faba* cultivars, namely cv. Mythos and Albatross, produced a total of 7 stable transformed lines after 9 – 10 mth.

There are, to date, no reports of Bambara groundnut transformation using the *Agrobacterium*-mediated gene transfer method and attempts at establishing such a protocol was chosen in this study due to the simplicity of the transformation system (Veluthambi *et al.*, 2003) and also because this transformation method

has been reported to result in stable transgene insertion and integration in a wide range of dicotyledonous plants, including several legume species (Babaoglu *et al.*, 2000; Oger *et al.*, 1996; Popelka *et al.*, 2006; and Trieu and Harrison, 1996).

6.2. AIMS AND OBJECTIVES

This chapter discusses the attempts at *Agrobacterium*-mediated transformation of Bambara groundnut landraces DipC, Uniswa-Red and S19-3. This study aimed at transforming Bambara groundnut zygotic embryos with *A. tumefaciens* strains 1065 and GV3101. Strain 1065 was LBA4404 harbouring the binary vector pVDH65, while strain GV3101 harboured either pBI121 or pBI121-LeB4-Ber e1. All 3 plasmids carried the *npt*II selectable marker and *uidA* reporter genes. pBI121-LeB4-Ber e1 also carried the *ber e1* gene which coded for 18% methionine. It was intended that the tissue culture methods employed for regeneration of putatively transformed Bambara groundnut shoots *in vitro* would include and expand on the work reported in Chapter 3 of this thesis. Molecular analysis of putative transformants was by histochemical GUS assays, PCR and RT-PCR.

Since there were no published reports of Bambara groundnut transformation protocols at the time of reporting, the objective of the experiments carried out was to attempt to set up an efficient and reproducible tissue culture-based *Agrobacterium*-mediated transformation system for Bambara groundnut. The protocols designed were varied and included investigations into:

i. The effect of acetosyringone on transformation efficiency
ii. The effect of L-cysteine on explant viability and regeneration potential.
iii. The effect of antibiotic-free inoculation broth on transformation efficiency.
iv. The incorporation of a non-selective, transitional period following explant co-cultivation with *A. tumefaciens* to improve explant survival and regeneration frequency.

6.3. MATERIALS AND METHODS

All *in vitro* procedures described in this chapter were carried out under aseptic conditions (Section 2.3.1.). *In vitro* procedures involving the manipulation of *Agrobacterium tumefaciens* were carried out in a Class II laminar-flow biological safety cabinet.

6.3.1. Bacterial strains and plasmids

In this study, 2 disarmed *A. tumefaciens* strains harbouring different transformation vectors were used. They were:

(i) Strain 1065 harbouring pVHD65

The 2 gene cassettes in pVDH65, located between the T-DNA left and right borders, were 35S.*uidA*-intron.35S and nos.*npt*II.nos, which served as reporter and selectable marker genes respectively. The *uidA*-intron gene, under the control of the constitutive CaMV 35S promoter (35S Pro) and CaMV 35S terminator (35S Ter) was situated near to the T-DNA right border (RB), while the *npt*II gene, driven by the nopaline synthase promoter (P*nos*) was inserted near to the left border (LB) of the T-DNA, as shown in Fig. 6.1

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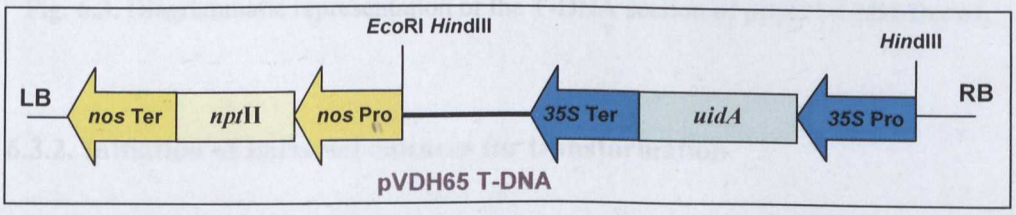

Fig. 6.1. Diagrammatic representation of the T-DNA section of pVHD65.
Source: Adapted from Curtis *et al.*, 1999.

(ii) Strain GV3101 harbouring pBI121

The 2 gene cassettes in pBI121 were nos.*npt*II.nos and 35S.*uidA*.nos, located between the T-DNA right and left borders (Fig. 6.2.). The *npt*II selectable marker gene, driven by the *nos* promoter and *nos* terminator, was situated near the T-DNA right border, while the *uidA* reporter gene was situated near the T-DNA left border, under the control of the CaMV 35S promoter and *nos* terminator.

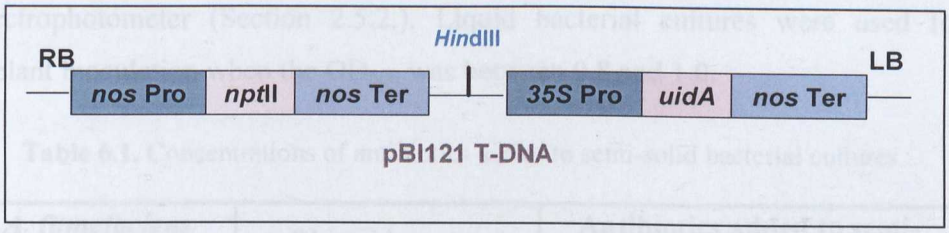

Fig. 6.2. Diagrammatic representation of the T-DNA section of pBI121.

(iii) Strain GV3101 harbouring pBI121-LeB4-Ber e1

The 3 gene cassettes in pBI121-LeB4-Ber e1, located between the T-DNA right and left borders, were nos.*npt*II.nos, LeB4.*Bere1*.35S and 35S.*uidA*.nos (Fig. 6.3.). The *npt*II selectable marker gene, driven by the *nos* promoter and *nos* terminator, was situated near the T-DNA right border, while the *uidA* reporter gene was situated near the T-DNA left border, under the control of the CaMV 35S promoter and *nos* terminator. The LeB4.*Bere1*.35S gene cassette was situated between the *npt*II and *uidA* cassettes, with the *Ber e1* gene of interest coding for methionine accumulation under the control of the LeB4 promoter and CaMV 35S terminator.

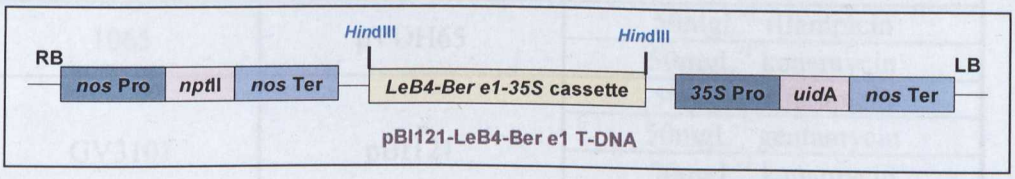

Fig. 6.3. Diagrammatic representation of the T-DNA section of pBI121-LeB4-Ber e1.

6.3.2. Initiation of bacterial cultures for transformation

Bacterial cultures were initiated following the method outlined in Section 2.5.2. Semi-solid and liquid cultures were set up using agar-solidified LB and APM medium respectively, each containing the appropriate antibiotics (Tables 6.1 and 6.2.). The addition of antibiotics was to select for the *Agrobacterium* colonies that had taken up the plasmid vector during bacterial transformation. To monitor the growth of the bacteria in liquid cultures, the optical density (OD) of the culture was measured at a wavelength of 600nm (OD_{600}) using a

spectrophotometer (Section 2.5.2.). Liquid bacterial cultures were used for explant inoculation when the OD_{600} was between 0.8 and 1.0.

Table 6.1. Concentrations of antibiotics added to semi-solid bacterial cultures.

***A. tumefaciens* strain**	**Plasmid vector**	**Antibiotics added to semi-solid cultures**
1065	pVDH65	$100mgL^{-1}$ rifampicin
		$50mgL^{-1}$ kanamycin
GV3101	pBI121	$50mgL^{-1}$ rifampicin
		$50mgL^{-1}$ gentamycin
		$50mgL^{-1}$ kanamycin
		$5mgL^{-1}$ tetracycline
GV3101	pBI121-LeB4-Ber e1	$50mgL^{-1}$ rifampicin
		$50mgL^{-1}$ gentamycin
		$50mgL^{-1}$ kanamycin
		$5mgL^{-1}$ tetracycline

Table 6.2. Concentrations of antibiotics added to liquid bacterial cultures.

***A. tumefaciens* strain**	**Plasmid vector**	**Antibiotics added to liquid cultures**
1065	pVDH65	$50mgL^{-1}$ rifampicin
		$50mgL^{-1}$ kanamycin
GV3101	pBI121	$50mgL^{-1}$ rifampicin
		$50mgL^{-1}$ gentamycin
		$50mgL^{-1}$ kanamycin
		$5mgL^{-1}$ tetracycline
GV3101	pBI121-LeB4-Ber e1	$50mgL^{-1}$ rifampicin
		$50mgL^{-1}$ gentamycin
		$50mgL^{-1}$ kanamycin
		$5mgL^{-1}$ tetracycline

6.3.3. Endogenous kanamycin resistance in Bambara groundnut

Kanamycin sulphate was the selective agent used in the transformation experiments. The concentration of kanamycin added to the selective media used in the *Agrobacterium*-mediated transformation of Bambara groundnut zygotic embryos was $50mgL^{-1}$, as determined in Chapter 5, Sections 5.3.1. and 5.4.1.

6.3.4. *Agrobacterium*-mediated transformation

6.3.4.1. General experimental set-up

Mature zygotic embryos of Bambara groundnut (landraces DipC, Uniswa-Red and S19-3) were used as target explants in this transformation study. Seeds were surface-sterilised and prepared for embryo excision following the procedure outlined in Sections 2.3.5.1 and 2.3.5.2. Wounding was induced in explants by gently stabbing whole embryos 4 – 5 times with a sterile needle and by using a sterile scalpel blade to transversely slice (approx. 2mm in thickness) or bisect embryos. Inoculation was carried out when the *A. tumefaciens* culture had reached an OD_{600} of 0.8-1.0, by immersing explants in the corresponding bacterial suspension for 25min, followed by blotting on sterile 70mm filter paper (Whatman International Ltd., Maidstone, England) and co-cultivation for 3 d on MS-based medium (Appendix I, Section AI.3) supplemented with 1.5mgL^{-1} BAP lacking antibiotics. Following co-cultivation, explants were washed thoroughly thrice in sterile reverse-osmosis water supplemented with cefotaxime or Timentin™, followed by one wash in sterile reverse osmosis water, before being blotted on sterile 70mm filter paper and finally transferred onto their corresponding selection medium for shoot regeneration. Explants were subcultured on fresh selection medium every 2wk until regenerated shoots reached approximately 3 – 4 cm in height. Root formation was then induced by transferring putatively transformed adventitious shoots to MS-based medium (Appendix I, Section AI.3) supplemented with 1mgL^{-1} NAA without antibiotics.

For each tested transformation protocol, three independent experiments were carried out with each Bambara groundnut landrace. Each experiment contained 12 replicates and each replicate contained explants derived from 5 embryos. Control experiments had an identical setup. Two types of control experiments were set up for each protocol, hereafter referred to as control I and control II. In control I, non-transformed wounded explants were cultured on selection medium which consisted of MS-based medium (Appendix I., Section AI.3.) supplemented with 1.5mgL^{-1} BAP, and the corresponding antibiotics. In control II, non-

transformed wounded explants were cultured on non-selective medium, which was MS-based medium (Appendix I., Section AI.3.) supplemented with 1.5mgL^{-1} BAP without antibiotics. The control explants were cultured in 9cm Petri dishes (5 explants per dish) each containing 20ml of the appropriate culture medium. Inoculated and control explants were maintained under standard tissue culture conditions (Section 2.3.2.), subcultured onto fresh medium every 2 wk and observed at regular intervals for signs of regeneration.

6.3.4.2. Transformation with *A. tumefaciens* strain 1065 harbouring pVDH65

Prior to inoculation with *A. tumefaciens* strain 1065, freshly excised, whole zygotic embryos were cultured for 6 d *in vitro* on MS-based medium (Appendix I, Section AI.3.), supplemented with 1mgL^{-1} NAA and 1mgL^{-1} BAP. A 1:10 (v/v) dilution was made by adding 2ml of the bacterial suspension to 18ml of half-strength liquid MS-based medium (Appendix I, Section AI.1.) in 9cm Petri dishes, and used for the inoculation of explants. Inoculation was carried out by immersing wounded whole and transversely sliced embryos in the diluted bacterial broth for 25min. Inoculated explants were co-cultured in 9cm Petri dishes (5 explants per dish) each containing 20ml of MS-based medium (Appendix I., Section AI.3.) supplemented with 1.5mgL^{-1} BAP for 3 d, under standard tissue culture conditions (Section 2.3.2.).

Following co-cultivation, explants were washed and transferred onto selection medium SM1 which consisted of MS-based medium (Appendix I., Section AI.3.) supplemented with 1.5mgL^{-1} BAP, 50mgL^{-1} kanamycin and 100mgL^{-1} cefotaxime, contained in sterile 175ml Powder Round jars (5 explants per jar). The explants were subcultured onto fresh selection medium (50ml per jar) every 2 wk and monitored for signs of regeneration and development (Results, Section 6.4.1.)

6.3.4.3. Transformation with *A. tumefaciens* strain GV3101 harbouring pBI121

For transformation with pBI121, freshly excised, whole zygotic embryos were cultured for 6 d *in vitro* on MS-based medium (Appendix I, Section AI.3.), supplemented with 1mgL^{-1} NAA and 1mgL^{-1} BAP, prior to inoculation with *A. tumefaciens*. Liquid cultures of *A. tumefaciens* GV3101 harbouring pBI121 were set up as detailed in Section 6.3.2.

6.3.4.3.1.Basic transformation experiment (TA1)

Transformation was carried out on whole, transversely sliced and transversely bisected embryos. Wounded explants (Section 6.3.4.1.) were transferred into 16ml of half-strength liquid MS-based medium contained in 9cm Petri dishes (20 explants per dish) and 4.0 ml of an overnight culture of *Agrobacterium* (OD_{600} = 0.8 – 1.0) was added to the mix, giving a final bacterial dilution of 1:5 (v/v). Explants were left immersed in this mixture at room temperature for 25 min before being blotted dry on sterile 70mm diameter filter paper (Whatman), and transferred onto 20ml co-culture medium (MS-based medium supplemented with 1.5mgL^{-1} BAP) for 3 d. Following this co-cultivation period, the explants were washed thrice in sterile reverse osmosis water supplemented with 150mgL^{-1} Timentin™, followed by one wash in sterile reverse osmosis water before being plated, 5 explants per Petri dish, on 20.0 ml of selection medium SM2 (MS-based medium supplemented with 150mgL^{-1} Timentin™, 50 mgL^{-1} kanamycin and 1.5mgL^{-1} BAP). Subsequently subcultures were performed every 2 wk on SM2 medium and the explants monitored for signs of shoot regeneration (Results, Section 6.4.2.1.).

6.3.4.3.2. Effect of acetosyringone on transformation efficiency (Transformation experiment TA2)

To study the effect of acetosyringone on transformation efficiency, transformation experiment TA1 (Section 6.3.4.3.1.) was repeated with or without the addition of acetosyringone (0, 100 and 200µM) to the bacterial liquid culture. Acetosyringone was added to overnight cultures of *A. tumefaciens* strain

GV3101 harbouring pBI121, 15 min prior to inoculation. Whole and transversely bisected Bambara groundnut embryos were used as starting explants in this experiment. Explant inoculation, co-cultivation and culture onto selection medium was performed as for experiment TA1. Explants were subcultured on fresh selection medium SM2 every 2 wk and monitored for signs of regeneration. Transformation efficiency was assessed by histochemical GUS assay on 50% of inoculated explants, 2 d following explant transfer to SM2 (Results, Section 6.4.2.2.).

6.3.4.3.3. Effect of L-cysteine on transformation efficiency (Transformation experiment TA3)

The effect of the anti-necrotic compound L-cysteine on the viability and regeneration potential of Agro-infected Bambara groundnut explants was investigated in this experiment, as an attempt to overcome the high rate of necrosis that developed in cultured explants from previous experiments (Results, Sections 6.4.2.1 and 6.4.2.2.). Whole and transversely bisected Bambara groundnut embryos were used as starting explants in this experiment. Explant inoculation, co-cultivation and culture onto selection medium was performed as for experiment TA1, but with or without the addition of L-cysteine (0, 20 and 40mgL^{-1}) in the selection media SM2. Following culture on SM2 supplemented with L-cysteine, the viability and regeneration frequency of inoculated explants was evaluated over a 12 wk period (Results, Section 6.4.2.3.).

6.3.4.4. Transformation with *A. tumefaciens* strain GV3101 harbouring pBI121-LeB4-Ber e1

For transformation with pBI121-LeB4-Ber e1, freshly excised, whole zygotic embryos were cultured for 6 d *in vitro* on MS-based medium (Appendix I, Section A1.3.), supplemented with 1mgL^{-1} NAA and 1mgL^{-1} BAP, prior to inoculation with *A. tumefaciens*. Liquid cultures of *A. tumefaciens* GV3101 harbouring pBI121-LeB4-Ber e1 were set up as detailed in Section 6.3.2.

6.3.4.4.1. Basic transformation experiment (TB1)

Transformation was carried out using whole and transversely bisected embryos as starting explants. Wounded explants were transformed following the protocol detailed in Section 6.3.4.3.1., but using a 1:5 (v/v) dilution of an overnight culture of *A. tumefaciens* harbouring pBI121-LeB4-Ber e1 with full-strength liquid MS-based medium (Appendix I, Section AI.1.). Following inoculation and co-cultivation, explants were cultured on selection medium SM2 (MS-based medium supplemented with 1.5mgL^{-1} BAP, 50mgL^{-1} kanamycin and 150mgL^{-1} Timentin™). Explants surviving on selection medium SM2 were monitored for signs of adventitious shoot regeneration (Results, Section 6.4.3.1.).

6.3.4.4.2. Effect of antibiotic-free inoculation broth on transformation efficiency (Transformation experiment TB2)

This experiment studied the effect of antibiotic-free bacterial inoculation broth on the regeneration potential of Agro-infected Bambara groundnut explants. Whole and transversely bisected embryos were used as starting explants. An overnight liquid culture of *A. tumefaciens* was set up as detailed in Section 6.3.2. Prior to explant inoculation the bacterial culture was centrifuged at 3000 g for 15min and the supernatant was discarded. The bacterial pellet was resuspended in full-strength liquid MS medium (Appendix I, Section AI.1.) without sucrose to an OD_{600} of 0.8, and used for inoculation by immersing freshly wounded explants in the bacterial solution for 25min. Following inoculation and co-cultivation, explants were cultured on selection media SM2, as described in Section 6.3.4.4.1. Explants surviving on SM3 were monitored for signs of adventitious shoot regeneration (Results, Section 6.4.3.2.).

6.3.4.4.3. Effect of non-selective transition stage on transformation efficiency (Transformation experiment TB3)

The incorporation of a non-selective transition stage following explant inoculation and co-culture was investigated in this experiment. Transformation was carried out using whole and transversely bisected embryos as starting explants. Wounded explants were transformed following the protocol detailed in

Section 6.3.4.4.2. Following inoculation and co-cultivation, explants were washed and cultured on non-selection medium (NSM1), which was MS-based medium supplemented with 1.5mgL^{-1} BAP and 150mgL^{-1} Timentin™, but no kanamycin. After 4wk on this medium, explants were transferred on selection media SM3, which was MS-based medium supplemented with 3mgL^{-1} BAP, 50mgL^{-1} kanamycin and 150mgL^{-1} Timentin™. The increase in BAP concentration was to attempt to boost shoot development and elongation from any adventitious buds that may have regenerated from the explants while on NSM1 medium. Explants surviving on selection medium SM3 were monitored for signs of such development (Results, Section 6.4.3.3.).

6.3.5. Analysis of transient GUS expression in putatively transformed Bambara groundnut explants

The protocol used for the histochemical detection of GUS activity was based on the method described by Jefferson *et al.* (1987), and was carried out on 8 day-old Bambara groundnut explants, 2 d following co-cultivation with *A. tumefaciens*. From each experiment, whole, sliced and bisected explants (30 of each explant type, unless stated otherwise) from each Bambara groundnut landrace were selected randomly for histochemical GUS assay. The assay was carried out as described in Chapter 5, Section 5.3.10. As a control, non-inoculated explants (30 of each explant type from each landrace) from control II (Section 6.3.4.1.) were subjected to the same treatment. Photographs of assayed explants were taken and analysed for the presence of blue colouring in transformed tissue.

6.3.6. Molecular characterisation of putatively transformed Bambara groundnut shoots

6.3.6.1. Genomic DNA extraction

Genomic DNA extraction was performed using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co.) as per the manufacturer's instructions, as detailed in Appendix V, Section AV.I. Genomic DNA was extracted from fully opened leaves of putatively transformed Bambara groundnut shoots, regenerated from *Agrobacterium*-inoculated explants (Section 6.3.4.), surviving on their respective selection medium. DNA was also extracted from fully opened

leaves of untransformed, regenerated Bambara groundnut shoots from control II experiments (Section 6.3.4.1.).

6.3.6.2. PCR analyses

PCR analysis was performed to confirm the presence of transgenes in putatively transformed regenerated Bambara groundnut shoots. PCR reactions were set up as described in Chapter 2, Section 2.7.3. PCR amplification was performed using the 96-well GeneAmp® PCR System 9700 machine (Applied Biosystems). Amplification was carried out on extracted genomic DNA (Section 6.3.6.1.) to detect for the presence of *npt*II, *uidA* and *ber e1* gene fragments in putatively transformed Bambara groundnut shoots. For each set of amplification reactions, positive control reactions were set up using the corresponding isolated plasmids, while control reactions used DNA extracted from non-transformed, wildtype Bambara groundnut leaves. Negative or no-template controls were also set up using sterile water instead of DNA in the reaction mix. The primer sequences used in the amplification of each gene fragment are given in Table 6.3., while their annealing temperatures and expected product sizes are detailed in Table 6.4. The PCR cycling conditions for the amplification of *npt*II, *uidA* and *ber e1* gene fragments are detailed in Figures 6.4, 6.5 and 6.6. respectively.

Table 6.3. Forward and reverse primer sequences for amplification of *npt*II, *uidA* and *ber e1* gene fragments.

Target gene	Primer sequences	
***npt*II**	Forward	5′-AGACAATCGGCTGCTCTGAT-3′
	Reverse	5′-ATACTTTCTCGGCAGGAGCA-3′
uidA	Forward	5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′
	Reverse	5′-ATCGCCGCTTTGGACATACCATCCGTA-3′
ber e1	Forward	5′-AGGAGGAGTGTCGCGAGCAGAT-3′
	Reverse	5′-GCCTCATCATCCTTCGCATC-3′

Table 6.4. Annealing temperatures of primers and expected product size of amplified *npt*II, *uidA* and ber e1 gene fragments.

Target gene	Annealing temperature/°C	Expected product size (bp)
*npt*II	55	261
uidA	57	1053
ber e1	59	255

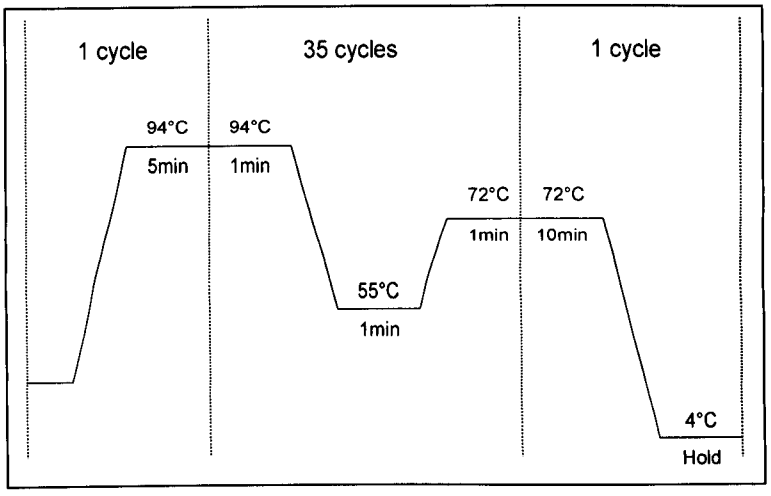

Fig. 6.4. PCR cycling conditions for amplification of *npt*II gene fragment.
N.B: Diagram not drawn to scale.

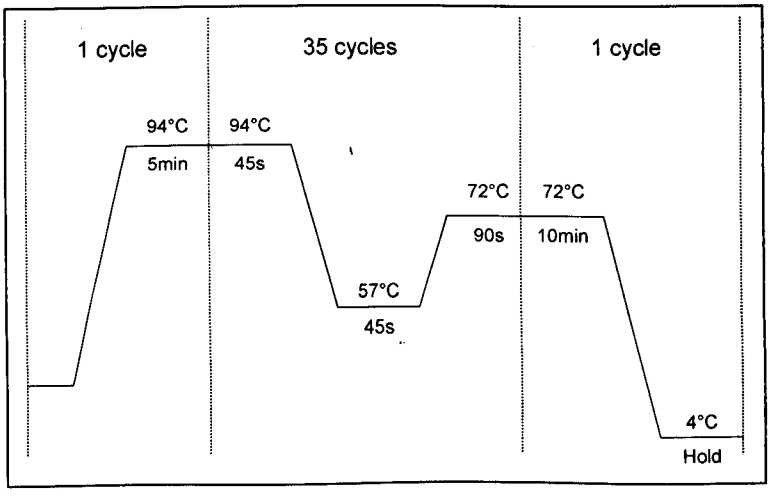

Fig. 6.5. PCR cycling conditions for amplification of *uidA* gene fragment.
N.B: Diagram not drawn to scale.

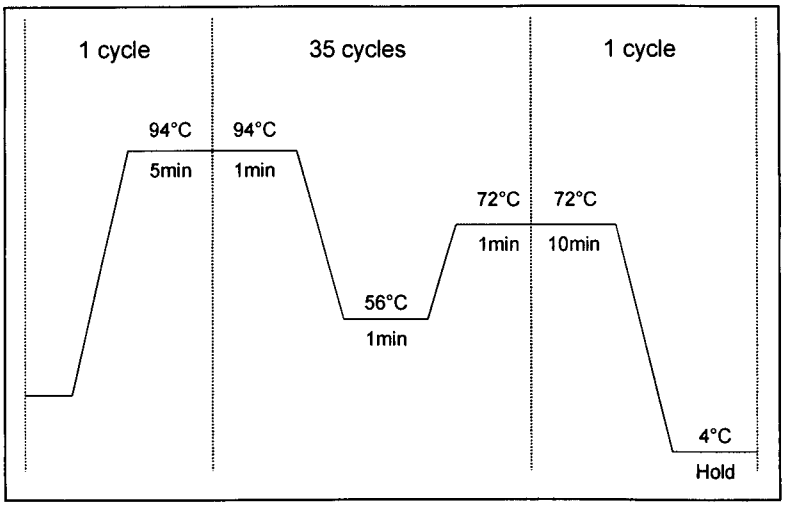

Fig. 6.6. PCR cycling conditions for amplification of *ber e1* gene fragment.
N.B: Diagram not drawn to scale.

6.3.6.3. Total RNA extraction

Total RNA extraction was performed using the RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions, as detailed in Appendix V, Section AV.2. RNA was extracted from fully opened leaves of putatively transformed Bambara groundnut shoots, regenerated from *Agrobacterium*-inoculated explants (Section 6.3.4.), surviving on their respective selection medium. RNA was also extracted from fully opened leaves of untransformed, regenerated Bambara groundnut shoots from control II experiments (Section 6.3.4.1.).

6.3.6.4. Reverse Transcription-PCR analyses

Reverse Transcription-PCR (RT-PCR) analysis was performed on RNA extracted from Bambara groundnut leaves (Section 6.3.6.3) to determine expression of the *npt*II and *uidA* transgenes in putatively transformed Bambara groundnut shoots. This experiment used the One-Step RT-PCR kit (Qiagen), which allowed the reverse transcription and PCR reactions to be carried out sequentially in the same reaction tube. The reaction mixture was set up as described in Appendix VI, Section AVI. 3. Appropriate volumes of the Master Mix were dispensed in the reaction tubes, before adding 350ng of template RNA (from putatively transformed or wildtype shoots). No-template control reactions were set up by substituting the template RNA with 1µl RNase-free water. The

reactions were run in the 96-well GeneAmp® PCR System 9700 machine (Applied Biosystems) using the cycling programme detailed in Figure 6.7. Annealing temperatures were modified accordingly for each tested gene fragment, as detailed in Table 6.4.

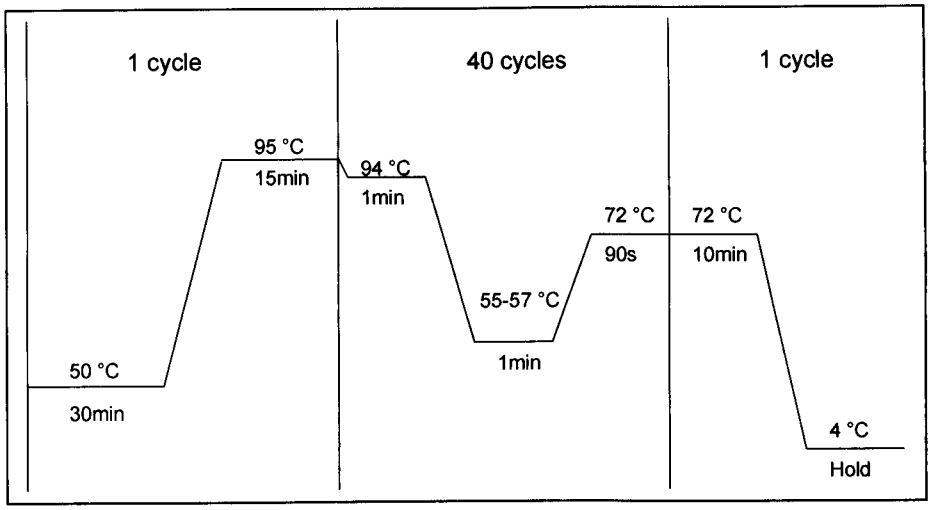

Fig. 6.7. RT-PCR cycling conditions for amplification of *npt*II and *uidA* gene fragments
N.B: Diagram not drawn to scale.

6.3.6.5. Gel electrophoresis of amplified DNA

Amplified PCR and RT-PCR products were electrophoresed on 1% (w/v) agarose gel containing 10mg mL^{-1} ethidium bromide (Section 2.6.2.) Fifteen microlitres of each amplified PCR product was loaded into a well, and 7µl of corresponding DNA ladder loaded in a well adjacent to the first and last loaded PCR product.

A 100bp DNA ladder (Promega) was used when running amplified *npt*II and *ber e1* PCR products, while a 1kb DNA ladder (New England Biolabs) was used when running amplified *uidA* PCR products. Each gel was prepared in 1X TAE buffer (Appendix VII., Section AVII.2.), loaded, and run for 1.5h at 90V and 250mA. Once the run completed, the gel was viewed under UV light in a SynGene transilluminator (Scientific Laboratory Supplies Ltd., Nottingham, UK). Pictures of the gels were captured using the Genesnap software programme from SynGene (Cambridge, UK).

6.4. RESULTS

The results obtained from each experiment revealed no significant differences between the investigated Bambara groundnut landraces DipC, Uniswa-Red and S19-3. Therefore, the results reported below did not attempt to make qualitative or quantitative distinctions between observations made between landraces during analysis of results.

6.4.1. *Agrobacterium*-mediated transformation with pVDH65

Whole and transversely sliced Bambara groundnut embryos were inoculated with *A. tumefaciens* strain 1065 harbouring pVDH65 and following a co-cultivation period, were transferred on to selection medium SM1 (MM, Section 6.3.4.2.). Histochemical GUS assays were carried out on inoculated explants, 2wk after their transfer on to selection medium. Positive GUS staining was obtained only in DipC embryos (4 out of 30 assayed embryos), as shown by the regions of indigo colouring in Fig 6.8., confirming the integration of the pVDH65 T-DNA, and expression of the *uidA* gene at the sites of staining. Assayed Uniswa-Red and S19-3 whole embryos tested negative for GUS staining, as were assayed sliced embryo explants from all 3 landraces.

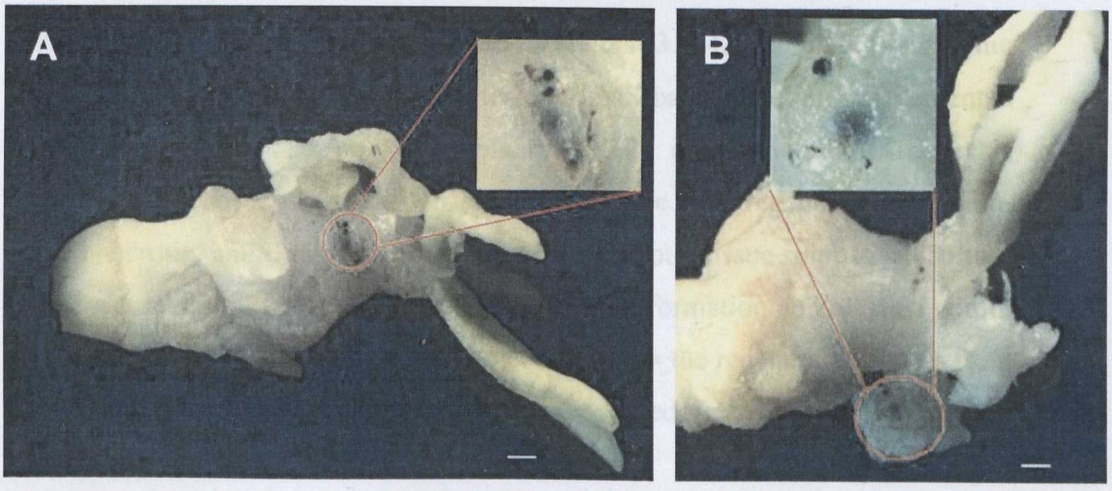

Fig. 6.8. Expression of *uidA* in Bambara groundnut embryos inoculated with *A. tumefaciens* strain 1065. **A:** Histochemical blue staining at the site of wounding of a DipC embryo assayed for GUS expression 2 wk after co-cultivation (bar = 1mm). **B:** Indigo colouring observed in what appeared to be regenerated adventitious tissue of the same DipC embryo as in (A). **Bar** = 0.5 mm.

Following co-cultivation with *A. tumefaciens* strain 1065, Bambara groundnut explants were transferred to selection medium SM1 containing $50mgL^{-1}$ kanamycin and $100mgL^{-1}$ cefotaxime. For all 3 landraces, transversely sliced inoculated embryo explants turned necrotic within 1wk on SM1 and no regeneration was observed. Non-inoculated sliced explants from controls I and II also exhibited the same necrotic tendencies, with control I explants dying within 1wk of culture on SM1, and control II explants dying within 20 d on non-selective medium.

Adventitious shoot regeneration was observed from inoculated whole Bambara groundnut embryos surviving on SM1. Out of 180 inoculated embryos per landrace, 24 DipC, 27 Uniswa-Red and 19 S19-3 explants produced adventitious shoots. The average number of shoots regenerated per embryo was 2 for DipC, 2.1 for Uniswa-Red and 1.6 for S19-3. The shoots were excised from the parent embryo once they grew to approx. 1 - 2 cm in height (approx. 8 wk following co-cultivation with *A. tumefaciens*), and transferred to new SM1 medium for further elongation (Fig. 6.9., A). Control, uninoculated embryos cultured on SM1 medium did not survive and became necrotic within 3wk (Fig. 6.9., B). Non-inoculated embryos from control II survived on non-selective medium and produced adventitious shoots as reported in Chapter 3, Section 3.4.2.2.1.

Putatively transformed Bambara groundnut shoots (3 – 4cm in height) surviving on selection medium SM1 were transferred to MS-based medium supplemented with $1mgL^{-1}$ NAA and no antibiotics to initiate root formation. Upon this transfer, the base of the shoots in contact with the root regeneration medium turned necrotic, and despite the removal of the necrotic tissue prior to subculture onto fresh medium, the shoots did not initiate root formation and all died within 4wk of culture on this medium. This was observed in the regenerated shoots from the 3 landraces. Non-transformed shoots recovered from control II explants rooted upon transfer to root regeneration medium.

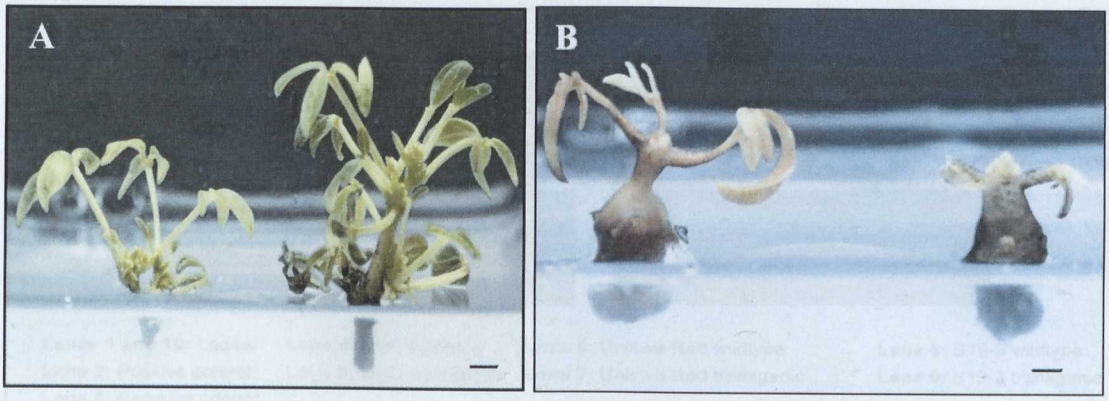

Fig. 6.9. Fate of inoculated and uninoculated Bambara groundnut explants on selection medium. **A:** *In vitro* regenerated shoots of Bambara groundnut (DipC) excised from the parent embryo and surviving on selection medium supplemented with 50mgL^{-1} kanamycin and 100mgL^{-1} cefotaxime, 8 wk following co-cultivation with *A. tumefaciens*; **B:** Uninoculated (untransformed) Bambara groundnut (DipC) embryos showing necrosis within 3 wk of culture on same selection medium as in A. **Bars** = 0.3cm.

Molecular characterisation of putatively transformed regenerated Bambara groundnut shoots was carried out by PCR and RT-PCR analyses. Genomic DNA was extracted from fully opened leaves and was amplified using primers specific to the *npt*II and *uidA* transgenes (Table 6.4.) and PCR cycling conditions detailed in Figures 6.4. and 6.5. respectively. Gel electrophoresis of PCR products showed the amplification of bands corresponding to the expected 261bp *npt*II gene fragment (Fig. 6.10., a) and to the expected 1053bp *uidA* gene fragment (Fig. 6.10., b) in DNA extracted from putatively transformed shoots. The size of the amplified bands also corresponded to their respective amplified positive controls. This suggested that T-DNA transfer with the *npt*II and *uidA* transgenes in the tested Bambara groundnut shoots had occurred. As expected, amplification of control DNA from wildtype Bambara groundnut leaves did not produce any bands. For confirmation of transgene integration in the host cell genome, molecular characterisation analyses such as iPCR and Southern blotting should be performed in future studies.

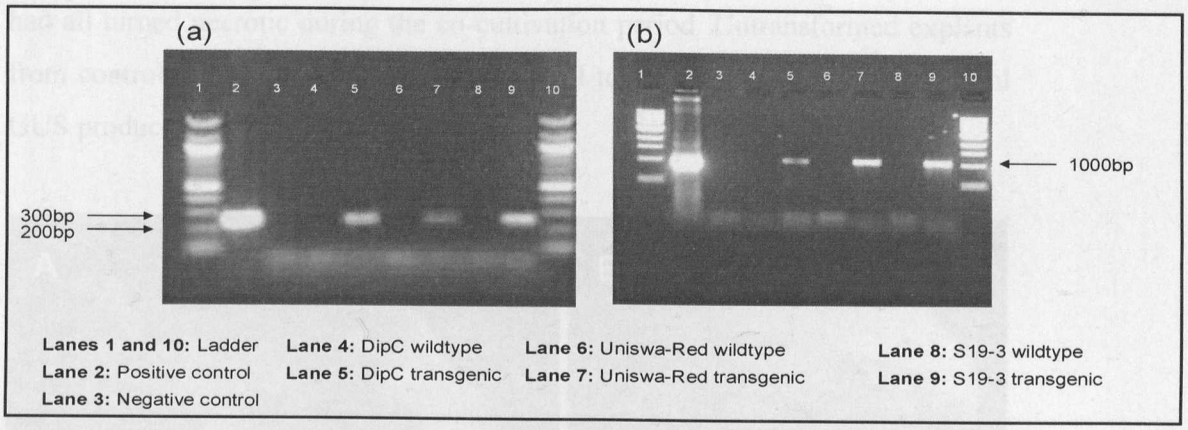

Fig. 6.10. Detection of amplified *npt*II (a) and *uidA* (b) gene fragments by PCR in Bambara groundnut shoots transformed with pVDH65.

To investigate the expression of the *npt*II and *uidA* transgenes in transformed Bambara groundnut shoots, RNA was extracted from fully opened leaves of shoots whose DNA tested positive for the *npt*II and *uidA* gene fragments (Fig. 6.10.), and subjected to RT-PCR analysis, using the Qiagen One-Step RT-PCR kit (MM, Sections 6.3.6.3. and 6.3.6.4.) and the corresponding primer pairs (Table 6.4.). Gel electrophoresis of the RT-PCR products did not produce any bands, thus suggesting that cDNA synthesis and amplification did not occur, possibly due to non-expression of the *npt*II and *uidA* genes in the tested shoots, which would in turn not result in their respective mRNA production.

6.4.2. *Agrobacterium*-mediated transformation with pBI121

6.4.2.1. Basic transformation experiment (TA1)

Zygotic Bambara groundnut embryos (whole, transversely sliced and transversely bisected) were inoculated with *A. tumefaciens* strain GV3101 harbouring pBI121 and transferred to selection medium SM2 following a co-cultivation stage (MM, Section 6.3.4.3.1.). Randomly selected inoculated explants were subjected to histochemical GUS assay to test for transient *uidA* expression 2 d after their transfer to SM2. In all 3 landraces, whole and transversely bisected embryos tested positive for GUS staining by producing extensive blue colouration at the site of transfection (Fig. 6.11.), confirming T-DNA transfer and *uidA* expression in the inoculated explants. Transversely sliced, 2mm thick embryo explants were not subjected to GUS staining, as they

had all turned necrotic during the co-cultivation period. Untransformed explants from controls I and II which were assayed all tested negative for histochemical GUS production.

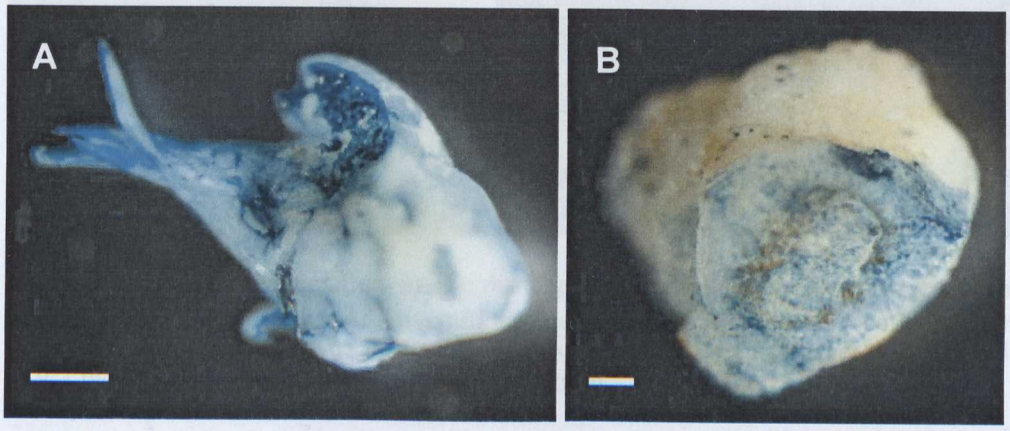

Fig. 6.11. Expression of *uidA* in 8 day-old Bambara groundnut explants, 2 d following co-cultivation with *A. tumefaciens* strain GV3101 carrying pBI121. **A:** Ubiquitous histochemical GUS staining (blue colouration) observed in a whole DipC embryo; **B:** Radicle explant from transversely bisected DipC embryo with indigo staining on the wounded surface. Similar blue staining was observed on the cut surface of the plumule sections of bisected embryos. **Bars** = 200 μm.

Inoculated whole and bisected embryos surviving on selection medium SM2 were monitored for signs of regeneration. Whole and bisected embryo explants visibly expanded and turned green as a result of chlorophyll production. Within 5 wk of culture on SM2 medium, bud initials were observed emerging at the embryonic-cotyledonary node of whole embryos, and a thin layer of green callogenic tissue was observed on the cut surface of radicle explants (Fig. 6.12.). However, adventitious shoot regeneration failed to occur in either explant type, and within 8 wk of culture on SM2, all of the surviving explants had turned necrotic. Plumule explants visibly expanded but regeneration did not occur.

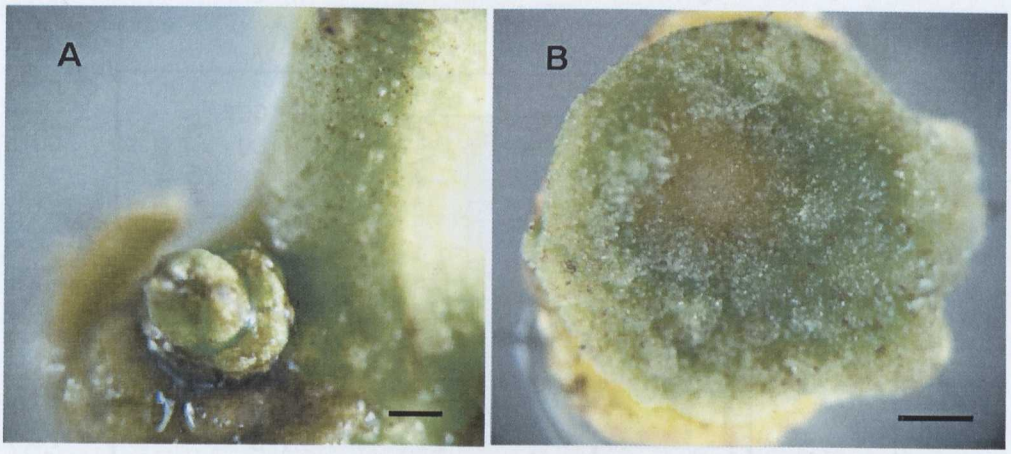

Fig. 6.12. *In vitro* regeneration in Bambara groundnut putatively transformed with pBI121 after 5 wk in culture. **A:** Adventitious bud formation at the embryo-cotyledonary node in whole DipC embryo. **B:** Callogenesis on cut surface of transversely bisected S19-3 radicle explant. **Bars:** A = 0.3mm; B = 0.2mm.

6.4.2.2. Effect of acetosyringone on transformation efficiency (Transformation experiment TA2)

The primary aim of this experiment was to study the effect of acetosyringone on transformation frequency following the co-cultivation stage. Whole and transversely bisected Bambara groundnut embryos (landraces DipC, Uniswa-Red and S19-3) were inoculated with *A. tumefaciens* strain GV3101 harbouring pBI121. The inoculation broth was supplemented with acetosyringone at 0, 200 and 400 µM (MM, Section 6.3.4.3.2.). Transformation frequency was assessed by performing histochemical GUS assays on inoculated explants 2 d after their transfer to selection medium SM2. In this experiment, a larger proportion of explants (90 randomly selected from each explant type) were tested for GUS activity. The proportion of whole embryo explants that stained positive for GUS across the 3 landraces suggested that there were no significant differences in transformation efficiency between explants inoculated in acetosyringone-deficient and explants inoculated in acetosyringone-supplemented broths, as shown in Figure 6.13. Similar results were obtained for bisected embryo explants from all 3 landraces, with the proportion of GUS-positive explants ranging from 90 – 92 % at 0µM acetosyringone, 91 – 92.5% at 200µM acetosyringone and 90 – 92% at 400µM acetosyringone. Uninoculated control explants showed no blue staining when subjected to the GUS assay.

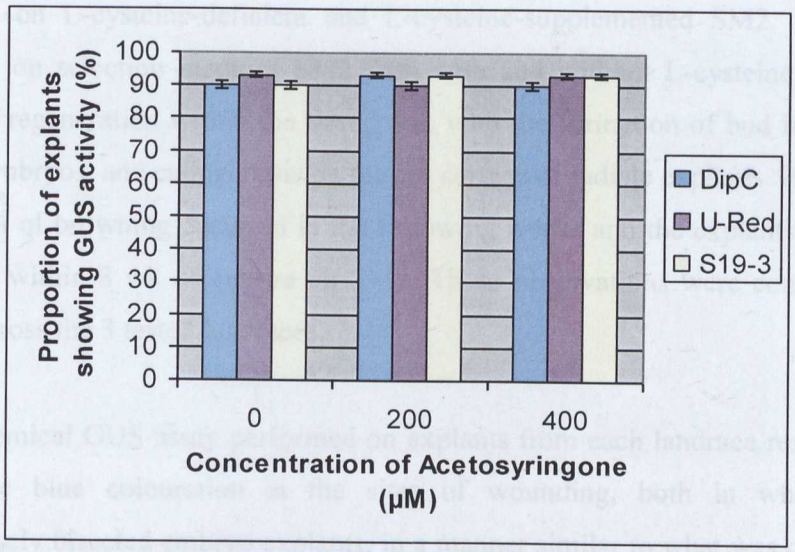

Fig. 6.13. Effect of acetosyringone on transformation frequency of Bambara groundnut whole embryo explants, assessed 2 d following co-cultivation with *A. tumefaciens* GV3101 carrying pBI121. The number of assayed explants per landrace for each acetosyringone concentration was 90.

The remaining inoculated explants surviving on selection medium SM2 were monitored for signs of regeneration. Observations made were similar to those reported for experiment TA1 (Section 6.4.2.1.), with whole embryos and radicle explants exhibiting initial signs of regeneration (*i.e.* formation of adventitious buds in whole embryos, and callogenesis in the radicle portion of bisected embryos), but no further growth or development of these adventitious tissues. Plumule explants visibly expanded and turned green, but did not exhibit signs of regeneration. As observed in experiment TA1, all explants failed to survive on selective medium SM2 past 8 wk in culture.

6.4.2.3. Effect of L-cysteine on transformation efficiency (Transformation experiment TA3)

The effect of the anti-necrotic compound L-cysteine on the viability and regeneration potential of Agro-infected Bambara groundnut explants was investigated in this experiment. Following inoculation and co-cultivation with *A. tumefaciens* strain GV3101 harbouring pBI121, whole and transversely bisected embryo explants were transferred to selective medium SM2 containing 0, 20 or 40mgL^{-1} L-cysteine and monitored for signs of necrosis and/or regeneration over

a 12 wk period. No significant differences were observed in the fate of explants cultured on L-cysteine-deficient and L-cysteine-supplemented SM2. Explants cultured on selection medium SM2 both with and without L-cysteine showed signs of regeneration within the first 5 wk, with the formation of bud initials in whole embryos, and callogenesis on the cut surface of radicle explants. However, the onset of browning occurred in the following weeks and the explants became necrotic within 8 wk of culture on SM2. These observations were consistently made across the 3 tested landraces.

Histochemical GUS assay performed on explants from each landrace resulted in extensive blue colouration at the sites of wounding, both in whole and transversely bisected embryo explants, in a manner similar to what was observed in assayed explants from transformation experiment T1 (Section 6.4.2.1., Fig. 6.11.).

6.4.3. *Agrobacterium*-mediated transformation with pBI121-LeB4-Ber e1

6.4.3.1. Basic transformation experiment (TB1)

Whole and transversely bisected zygotic embryos of Bambara groundnut were inoculated with *A. tumefaciens* strain GV3101 harbouring pBI121-LeB4-Ber e1 and transferred to selection medium SM2 following a co-cultivation stage (MM, Section 6.3.4.4.1.). Randomly selected inoculated explants were subjected to histochemical GUS assay to test for transient *uidA* expression 2 d after their transfer to SM2. The production of indigo colouring at the sites of wounding confirmed T-DNA integration and *uidA* expression in the assayed explants (Fig. 6.14.). This occurrence was observed consistently across the 3 tested landraces DipC, Uniswa-Red and S19-3. Untransformed explants from controls I and II which were assayed all tested negative for histochemical GUS production.

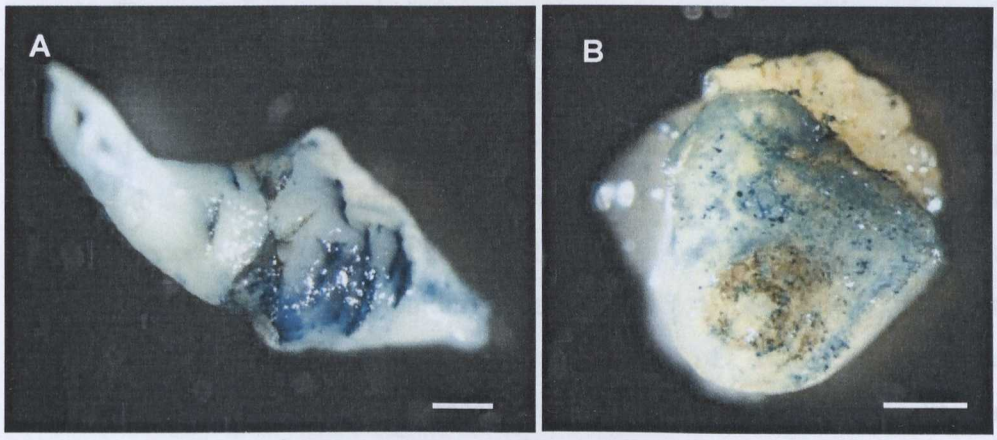

Fig. 6.14. Expression of *uidA* in 8 day-old Bambara groundnut explants, 2 d following co-cultivation with *A. tumefaciens* strain GV3101 carrying pBI121-LeB4-Ber e1. **A:** Histochemical GUS staining (blue) observed at the sites of wounding in a whole DipC embryo, assayed 3 d post-cocultivation; **B:** Radicle explant from transversely bisected Uniswa-Red embryo with indigo staining on the wounded surface. Similar blue staining was observed on the cut surface of the plumule sections of bisected embryos. **Bars:** A = 0.5mm; B = 0.2mm.

Explants surviving on selection medium SM2 were monitored for signs of regeneration. In the 1st week of culture on SM2, whole and bisected embryo explants visibly expanded and turned green as a result of chlorophyll production. Regeneration was not observed in plumule explants. In the following 4 wk, bud initials were observed emerging at the embryonic-cotyledonary node of whole embryos, and a thin layer of green callogenic tissue was observed on the cut surface of radicle explants, similar to results obtained with explants inoculated with pBI121 (Section 6.4.2.1., Fig. 6.12.). However, adventitious shoot regeneration failed to occur in either explant type, and within 8 wk of culture on SM2, all of the surviving explants had turned necrotic.

6.4.3.2. Effect of antibiotic-free inoculation medium on transformation efficiency (Transformation experiment TB2)

In this experiment, *Agrobacterium*-mediated transformation of Bambara groundnut was attempted using an antibiotic-free inoculation broth. An overnight liquid culture of *A. tumefaciens* strain GV3101 harbouring pBI121-LeB4-Ber e1 was centrifuged and the supernatant (antibiotic-supplemented APM) decanted

before resuspending the bacterial pellet in full-strength, sucrose-free liquid MS medium to an OD_{600} of 0.8 (MM, Section 6.3.4.4.2.). Whole and transversely bisected zygotic embryos inoculated in this bacterial broth were assayed for histochemical GUS staining 2 d after their transfer on selection medium, and monitored for signs of regeneration. Varying intensities of GUS staining were observed in assayed whole embryos, ranging from restricted localisation at the embryo-cotyledonary node or root tip (Fig. 6.15., A and C respectively) to a more widespread staining at wounding sites (Fig. 6.15., B), and finally, to almost a ubiquitous staining of the whole embryos (Fig. 6.15., D. This occurrence was consistent across all 3 tested landraces, with staining reported between 85 and 87% of assayed explants. Bisected embryo explants exhibited GUS staining on their cut surface, with 89.3 – 91.5% of assayed explants testing positive for *uidA* expression. This confirmed that integration of the pBI121-LeB4-Ber e1 T-DNA and expression of the *uidA* transgene was successful in positively-tested explants.

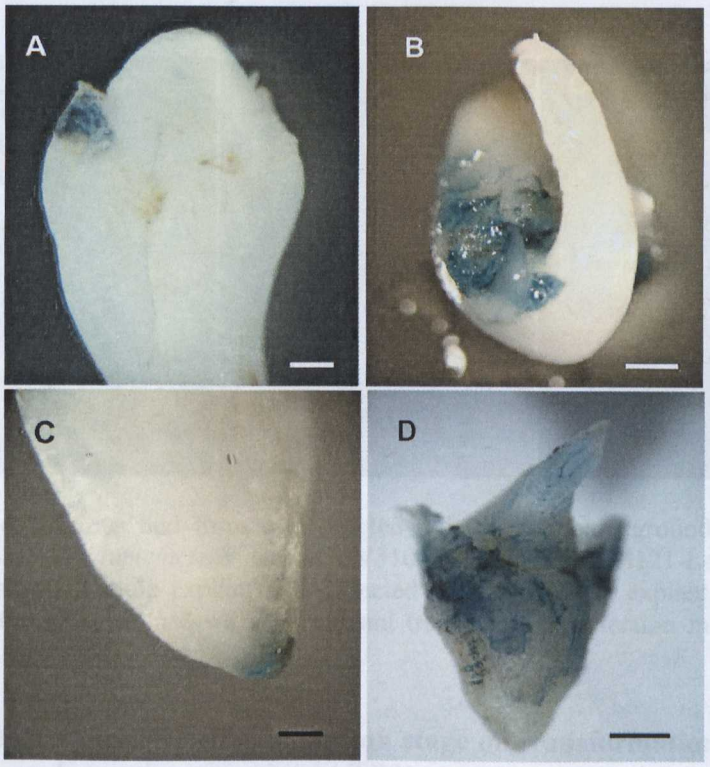

Fig. 6.15. Expression of *uidA* in 8 day-old Bambara groundnut whole embryos, 2 d following co-cultivation with pBI121-LeB4-Ber e1 using an antibiotic-free *A. tumefaciens* broth. Blue colouration was either localised (A – C) or ubiquitously distributed throughout the whole explant (D). Bars: A, C = 0.5mm; B = 0.7mm; D = 1mm

The regeneration frequency of inoculated whole and bisected embryos surviving on selection medium SM2 was assessed in this experiment. Whole and bisected embryo explants visibly expanded and turned green as a result of chlorophyll production. Within 5 wk of culture on SM2, bud initials were observed emerging at the embryonic-cotyledonary node of the explants. Within the following 3 wk, these bud initials expanded in size, as shown in Figure 6.16., and remained in this state for the next 4 wk, with no further development, and eventually turned necrotic, despite regular subcultures on to fresh SM2. This transformation protocol had kept inoculated explants alive for an average of 12 wk, which is 4 wk longer than the previously attempted protocols (TA1-3 and TB1) had achieved. Regenerated adventitious buds had also developed further than what had been achieved in the previous transformation experiments, suggesting that the use of an antibiotic-free inoculation broth for the transfection of Bambara groundnut embryos could be a key factor towards establishing an improved protocol for the efficient transformation of this crop.

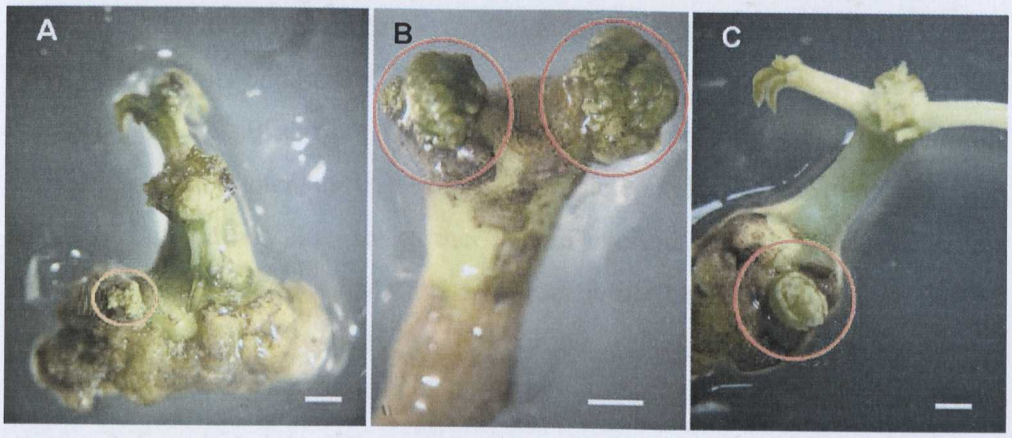

Fig. 6.16. Adventitious bud formation (circled red) on Bambara groundnut explants inoculated with *A. tumefaciens* strain GV3101 harbouring pBI121-LeB4-Ber. **A:** Bisected embryo (plumule explant), **B:** Bisected embryo (radicle explant), **C:** Whole embryo. Photographs taken 8 wk after explant transfer on to selection medium SM2. **Bars:** A, C = 0.8mm; B = 1mm.

6.4.3.3. Effect of non-selective transition stage on transformation efficiency (Transformation experiment TB3)

This experiment investigated the regeneration efficiency of Agro-infected Bambara groundnut explants by incorporating a transitional non-selective culture

period immediately after explant inoculation and co-cultivation, followed by an increase in BAP concentration in the selection medium. Whole and transversely bisected embryos were inoculated with *A. tumefaciens* strain GV3101 carrying pBI121-LeB4-Ber e1, and following co-cultivation on medium with no antibiotics, were washed and transferred onto MS-based medium without the selective agent kanamycin (medium NSM1) for 4 wk (MM, Section 6.3.4.4.3.). Following this transitional period, explants were transferred onto SM3, which contained a higher BAP concentration of $3mgL^{-1}$, and were monitored for signs of adventitious regeneration.

Observations made occurred consistently across the 3 tested landraces DipC, Uniswa-Red and S19-3. During the 4-week culture on NSM1, inoculated whole and transversely bisected embryos visibly expanded (as shown in Fig. 6.17. E, in the case of a plumule explant) and exhibited signs of adventitious bud formation at the embryonic-cotyledonary node region, as shown in Fig. 6.17. (A, C and F). Following explant transfer on to SM3, these regions of adventitious tissue grew larger in size within 3wk on this selection medium. During the next 11 weeks on SM3, adventitious buds from whole embryo and radicle explants developed into shoots (Fig. 6.16., B and D), while the state of adventitious buds from plumule explants remained unchanged. Shoots from radicle explants grew to approx. 1cm in height but did not elongate further. When excised from their parent explant and transferred on to new SM3 medium, radicle-derived shoots became necrotic within 1 wk and did not survive. In contrast, adventitious shoots regenerated from whole embryo explants developed fully opened leaves and elongated to approx. 3-4 cm in height. When excised from their parent explant and transferred on to root regeneration medium (MS-based medium supplemented with $1mgL^{-1}$ NAA and no antibiotics; MM, Section 6.3.4.1.), the shoots turned necrotic within 4 wk, an occurrence also observed during the initiation of root regeneration in Bambara groundnut shoots putatively transformed with pVDH65 (Section 6.4.1.).

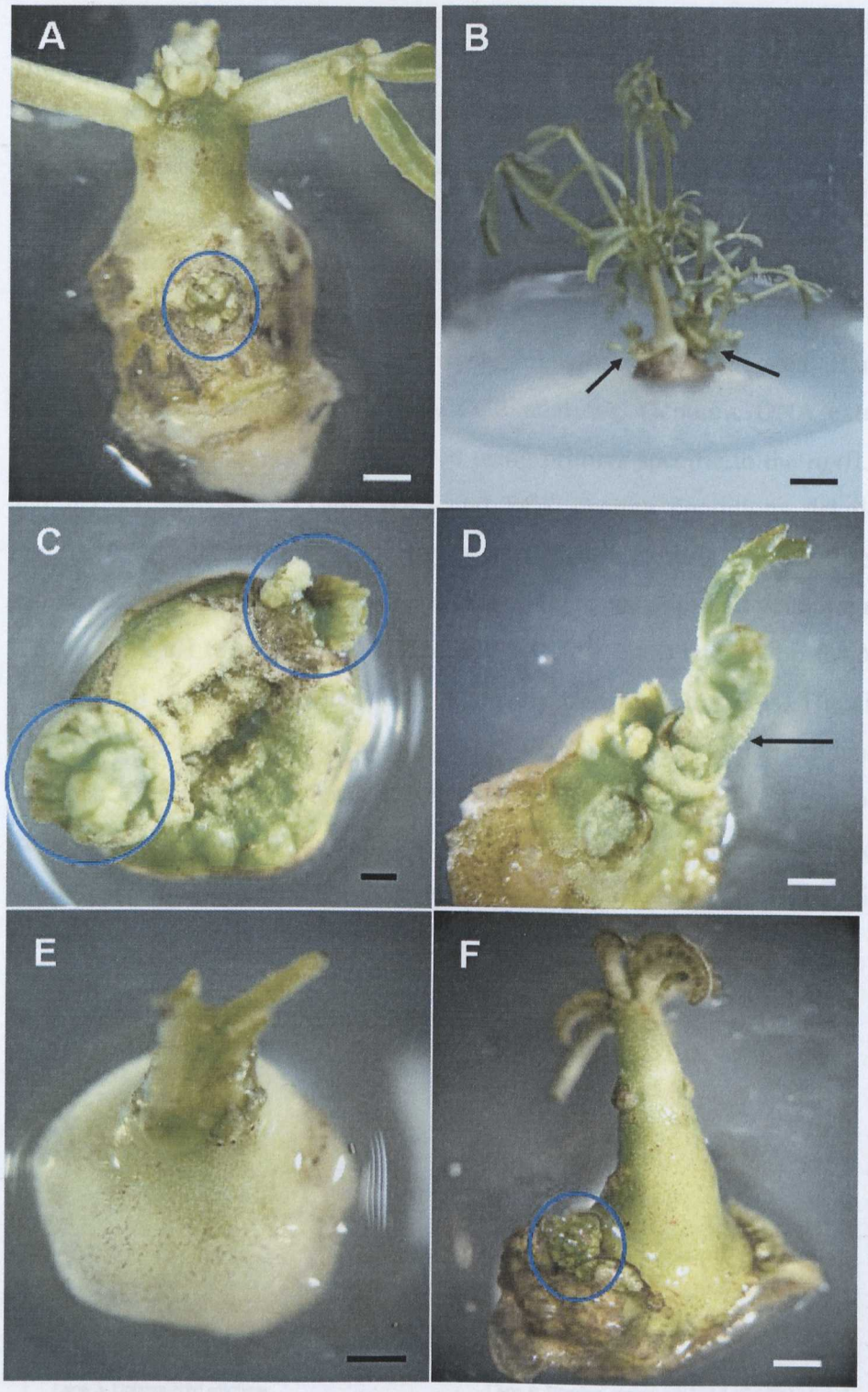

Fig. 6.17. Adventitious bud formation (circled blue; after 4 wk on NSM1) and shoot elongation (arrows; after 11 wk on SM3) from Bambara groundnut explants putatively transformed with pBI121-LeB4-Ber e1. Sets of photographs show the chronological development of shoots from adventitious bud in whole embryos (A and B) and radicle explants (C and D). For plumule explants, adventitious buds (F) regenerated from an expanded explant (E) but failed to elongate further. **Bars:** A = 1mm; B, D = 2mm; C = 0.2 mm; E = 0.25 mm

Histochemical GUS assay was performed on putatively transformed explants, 2 d after their transfer to non-selective medium containing $150mgL^{-1}$ timentin (medium NSM1). Assayed whole embryos produced varying intensities of GUS staining, similar to what was reported in Section 6.4.3.2. (Fig. 6.15.), while bisected embryo explants produced blue colouration at the cut surfaces. Control, non-transformed explants all tested negative for GUS production.

Molecular characterisation of putatively transformed regenerated Bambara groundnut shoots was carried out by PCR analyses. Genomic DNA extracted from fully opened leaves was amplified using primers specific to the *npt*II, *uidA* and *ber e1* transgenes (Table 6.4.) and PCR cycling conditions detailed in Figures 6.4., 6.5. and 6.6. respectively. Gel electrophoresis of PCR products showed the amplification of bands corresponding to the expected gene fragments [261bp for *npt*II (Fig. 6.17.), 1053bp for *uidA* (Fig. 6.18) and 255bp for *ber e1* (Fig. 6.19.)] in DNA extracted from putatively transformed shoots. The size of the amplified bands also corresponded to their respective amplified positive controls. This suggested that T-DNA transfer with the *npt*II, *uidA* and *ber e1* transgenes in the tested Bambara groundnut shoots had occurred. As expected, amplification of control DNA from wildtype Bambara groundnut leaves did not produce any bands.

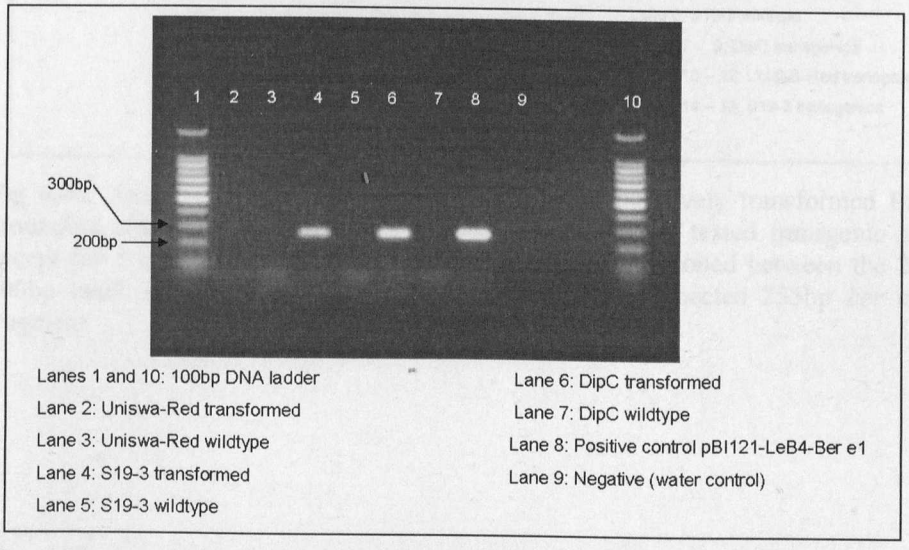

Fig. 6.18. Amplification of *npt*II gene fragment in putatively transformed Bambara groundnut shoots. DNA from the putatively transformed Uniswa-Red shoot tested here showed amplification of the *npt*II gene fragment (Lane 3), while that of S19-3 and DipC did.

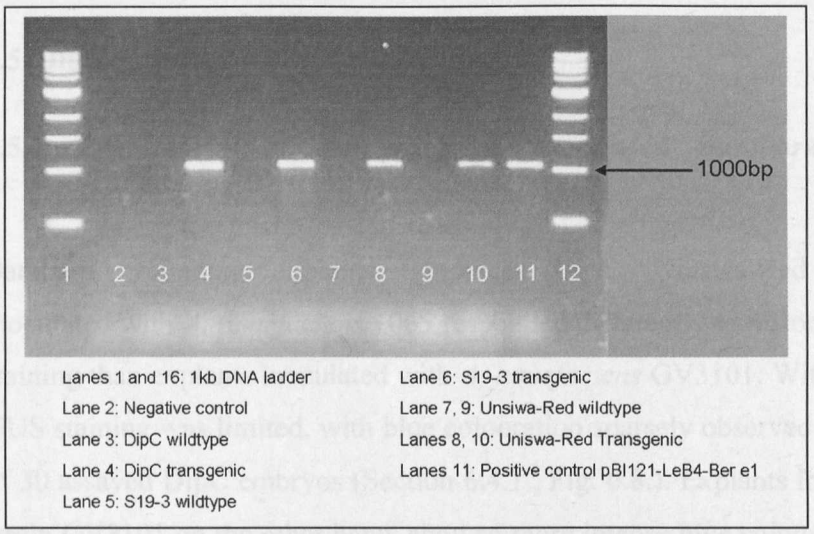

Fig. 6.19. Amplification of *uidA* gene fragment in putatively transformed Bambara groundnut shoots. Amplified bands were generated for all tested transgenic samples and were positioned slightly above the 1kb mark of the DNA ladder, corresponding to the expected 1053bp *uidA* gene fragment.

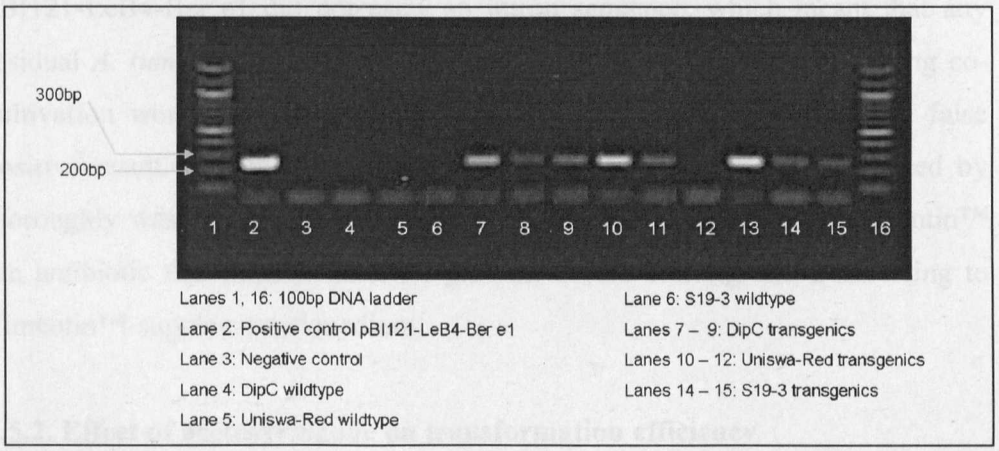

Fig 6.20. Amplification of *ber e1* gene fragment in putatively transformed Bambara groundnut shoots. Amplified bands were generated for all tested transgenic samples except for 1 Uniswa-Red sample (Lane 12) and were positioned between the 200 and 300bp mark of the DNA ladder, corresponding to the expected 255bp *ber e1* gene fragment.

6.5. DISCUSSION

6.5.1. Transient *uidA* expression in inoculated Bambara groundnut explants

Bambara groundnut explants (landraces DipC, Uniswa-Red and S19-3) inoculated with *A. tumefaciens* 1065 responded differently to histochemical GUS staining than explants inoculated with *A. tumefaciens* GV3101. With strain 1065, GUS staining was limited, with blue colouration sparsely observed on only 4 out of 30 assayed DipC embryos (Section 6.4.1., Fig. 6.8.). Explants inoculated with strain GV3101 on the other hand, showed more intense blue colouration on most of the assayed explants, with the staining ubiquitously distributed in some of the whole embryo explants inoculated with pBI121 (Section 6.4.2.1., Fig. 6.11, A) or with pBI121-LeB4-Ber e1 (Section 6.4.3.2., Fig. 6.15., D). Compared to strain 1065 which carried a *uidA*-intron gene on pVDH65, the *uidA* gene in pBI121 and pBI121-LeB4-Ber e1 did not carry an intron sequence, which meant that any residual *A. tumefaciens* GV3101 present on the explants assayed following co-cultivation would also be reacting with the GUS mixture to produce a false positive result. However, the possibility of this occurrence was eliminated by thoroughly washing the explants in sterile water supplemented with Timentin™ (an antibiotic that inhibits bacterial growth) before blotting and transferring to Timentin™-supplemented medium.

6.5.2. Effect of acetosyringone on transformation efficiency

The addition of acetosyringone to the bacterial broth prior to explant inoculation did not produce any significant increase in the transformation efficiency of Bambara groundnut (Results, Section 6.4.2.2.). Since explant wounding was induced prior to inoculation by gently stabbing whole embryos with a fine sterile needle or by transversely bisecting embryos with a sterile scalpel blade, it was possible that this action allowed for the explants to produce bacterial virulence-activating compounds *in situ*, thus eliminating the action of acetosyringone during inoculation. Future work will need to investigate the effect of

acetosyringone on transformation efficiency of Bambara groundnut by using non-wounded explants for inoculation with *A. tumefaciens*.

6.5.3. Effect of L-cysteine on explant viability and regeneration frequency

One of the main reasons for the low transformation frequency of grain legumes is their inability to regenerate under tissue culture conditions. This recalcitrance might due to the accumulation of high levels of phenolic compounds in cells adjacent to wounding sites which results in the oxidation and subsequent death of explants (Raveendar and Ignacimuthu, 2010). However, in transformation experiment TA3, the addition of the anti-necrotic compound L-cysteine to the selection medium SM2 did not result in an increase in the survival rate of inoculated Bambara groundnut explants, nor did it have any significant effect on the regeneration frequency (Results, Section 6.4.2.3.). This could be because the concentrations of L-cysteine used in this experiment (20 and 40mgL^{-1}) were too low. Olhoft and Somers (2001) reported an increase in transformation frequency from 37% to 91% when using 400mgL^{-1} cysteine in the co-coltivation medium during the transformation of soybean. The effects of higher concentrations of L-cysteine in the co-cultivation and selection media on transformation efficiency of Bambara groundnut could be investigated in future studies.

6.5.4. Effect of antibiotic-free *Agrobacterium* inoculation broth on transformation and regeneration efficiency

An improvement in the protocol for *Agrobacterium*-mediated transformation of Bambara groundnut was the switch from an antibiotic-containing to an antibiotic-free bacterial broth during explant inoculation with *A. tumefaciens* strain GV3101 carrying pBI121-LeB4-Ber e1 (Results, Section 6.4.3.2.). The increase in the frequency of adventitious bud regeneration, as well as the fact that explants cultured on selection medium SM2 were kept alive for 4 wk longer than explants inoculated with bacterial broth containing rifampicin, gentamycin, tetracycline and kanamycin, suggested that an antibiotic-free bacterial broth (bacterial pellet resuspended in full-strength liquid MS medium) was less toxic to the wounded explants than its counterpart during inoculation and co-cultivation. Although the

regenerated adventitious buds did not develop further into shoots, the 4 wk increase in the lifespan of inoculated explants was an important achievement towards establishing an optimised and efficient transformation protocol for the genetic improvement of Bambara groundnut.

6.5.5. Importance of a transitional non-selective stage in the transformation system

The incorporation of a 4 wk-long non-selective culture period following co-cultivation resulted in an improved regeneration frequency in Bambara groundnut whole embryos and radicle explants inoculated with pBI121-LeB4-Ber e1 (Results, 6.4.3.3.). Following this 4-wk transitional culture period, these explants were kept alive on selection medium SM3 (supplemented with $3mgL^{-1}$ BAP) for an additional 11wk, during which time regenerated adventitious buds developed into shoots. This occurrence was also partly due to the increased BAP concentration (from $1.5mgL^{-1}$ to $3mgL^{-1}$) in SM3. Shoots regenerated from whole embryos developed fully opened leaves, while shoots regenerated from radicle explants did not. The non-selective transitional culture period was incorporated in this protocol with the aim of prolonging the longevity of inoculated explants following co-cultivation, while the increased BAP concentration in SM3 was introduced as an attempt to boost the development of adventitious buds regenerated during culture on NSM1 into shoots. This was the first successful report in this study of shoot formation and elongation from explants inoculated with pBI121-LeB4-Ber e1.

6.5.6. Molecular characterisation of putative transgenic shoots

Putatively transformed regenerated Bambara groundnut shoots with fully developed leaves were recovered only from whole embryos inoculated with pVDH65 (Results, Section 6.4.1.) and pBI121-LeB4-Ber e1 (Results, Section 6.4.3.3.). Results of PCR analyses revealed the presence of amplified DNA bands corresponding to the *npt*II, *uidA* and *ber e1* gene fragments, thus suggesting the presence of transgenes in the shoot genome. However, RT-PCR analyses on shoots transformed with pVDH65 were negative, suggesting that transgene

expression did not occur. In the case of shoots transformed with pBI121-LeB4-Ber e1, the quality and quantity of extracted RNA per shoot was quite low, and was not enough to be analysed by RT-PCR. Future transformation studies should focus on the further optimisation of the transformation and regeneration system set up in Section 6.4.3.3., in order to successfully establish an efficient protocol for the genetic improvement of Bambara groundnut. Also, molecular characterisation tools such as iPCR and Southern blotting should be employed to confirm transgene integration in putatively transformed Bambara groundnut shoots.

CHAPTER 7. GENERAL DISCUSSION

7.1. Plant biotechnology for crop improvement in developing countries

For centuries, plants with desirable traits have been produced using traditional crop improvement strategies including selective breeding and hybridisation through the controlled pollination of plants. In today's world of rapidly changing climates and ever-increasing human population growth, competition for space and demands on food and energy productions are higher than ever. However, these classical plant breeding approaches are failing to adapt to such requirements. Global food production must increase by 70% by 2050 in order to prevent worldwide starvation, representing an annual increase of 38% per annum over current crop improvement initiatives (FAO, 2009; Beddington, 2010; Tester and Langridge, 2010). It is therefore necessary to develop plant breeding strategies and crop improvement techniques that will rapidly respond to the demands for high-yielding and nutritionally-enhanced crops. Plant biotechnology is an extension of traditional breeding strategies and offers a promising alternative to classical crop improvement methods by allowing the manipulation of genetic information in a more precise and controlled manner (Beddington, 2010). It is an important tool that includes the study of genetic variability and diversity within and among different germplasm collections, tissue culture coupled with recombinant DNA and genetic transformation technologies, as well as techniques employed in the molecular characterisation of putative transgenic plants (Stewart and Ow, 2008).

The application of biotechnology to reduce malnutrition in impoverished developing countries presents a more sustainable alternative to strategies such as dietary diversification and nutrient supplementation, which are highly dependent on political and financial stability (Bouis *et al.*, 2003; Timmer, 2003; White and Broadley, 2009). Malnutrition remains to date a major public health concern particularly in developing countries, with various publications and surveys associating it with continuous inadequate nutrient consumption among infants and young children, as well as pregnant and lactating women from impoverished

communities (Matta *et al.* 2009; White and Broadley, 2009; FAO, 2010). It is mainly a consequence of poverty and is the primary global risk factor responsible for illness and death in poorer communities which rely heavily on low cost, high energy staple crops as their principal source of food (Toenniessen, 2002; FAO, 2010). The recent escalating food prices in 2007 resulted in worldwide malnutrition for an additional 75 million people indicating, that at current production levels, mass malnutrition and food insecurity are at perilous levels (Beddington, 2010). Protein is a vital component of the human diet as it is essential for growth processes and tissue repair (Bouis and Welch, 2010). Long-term, insufficient protein intake may lead to protein-calorie malnutrition (PCM), a condition which affects on average 70% and 26% of children in Asia and Africa respectively (Singh *et al.*, 2007). Impoverished communities are unable to consume adequate quantities of protein from premium sources such as egg, milk, poultry, fish and meat, which provide optimal ratios of essential amino acids for human nutrition, due to their proportionally higher costs compared to plant sources (Matta *et al.*, 2009), therefore relying heavily on legume crops as their primary, and cheaper, source of protein.

Legume seeds are one of the richest sources of dietary proteins, with contents ranging from 20 – 40% (dry weight) (Baudoin and Maquet, 1999; Duranti, 2006; Kirti, 2008). However, despite their high protein content, these seeds generally do not carry a balanced ratio of essential amino acids, and are usually deficient in the sulphur-containing amino acid, methionine (Baudoin and Maquet, 1999; Wang *et al.*, 2003; Arulbalachandran and Mullainathan, 2009; Singh and Yadav, 2010). Recent scientific studies have aimed at improving legume seed crops in terms of their nutritional value through the use of plant tissue culture and transformation technologies. However, the vast majority of efforts have been focused mainly on well-established crops cultivated on a large scale, such as soybean (Krishnan, 2005; Kita *et al.*, 2010).

Underutilised legumes are crops which have received limited attention from the scientific community, and constitute an unexploited resource with comparable agronomical and nutritional qualities to major cultivated legume crops. The use

of genetic transformation technologies in the biofortification of underutilised crops for their nutritional enhancement could therefore provide developing countries with cost-effective alternatives to imported nutritionally comparable food, which has been cultivated and produced abroad. This will allow underutilised crops to become important components of a nutritionally well-balanced diet, especially in areas of cultivation where they are better adapted to the biotic and abiotic stresses present in their local environments, with the ability to survive on low-nutrient soils, and with limited water supply, compared to their commercial counterparts such as soybean and chickpea.

The work presented in this thesis has investigated the genetic improvement of Jicama and Bambara groundnut, two underutilised legume crops with nutritional and agronomic qualities that could potentially allow them to become part of sustainable agricultural systems in their respective areas of cultivation. Work on jicama has focused on attempting to establish an explant-to-plant regeneration system for the crop, an achievement not yet reported to date. For Bambara groundnut, work described in this thesis aimed at optimising and adapting *in vitro* regeneration systems, previously characterised for distinct landraces including Nkorenza and Yoroba (Lacroix *et al.* 2003), and to develop novel unreported regeneration pathways for DipC, Uniwa-Red and S19-3 landraces. Conditions which produced maximum regeneration frequencies were thus employed as the basis of genetic transformation assays for Bambara groundnut, using both biolistic and *Agrobacterium*-mediated techniques. All putatively transformed explants and regenerated shoots were subjected to molecular characterisation including PCR and histochemical analyses to confirm transfer and expression of transgenes.

7.2. Tissue culture and regeneration studies

7.2.1. Tissue culture of jicama

There was, at the time of reporting, no publicly available report on the regeneration potential of jicama *in vitro*. An investigation into the

micropropagation of jicama using tissue culture methodologies was carried out, with the aim of establishing an explant-to-plant regeneration system for the crop. From preliminary tissue culture experiments, it was determined that whole mature zygotic embryos gave optimal regenerative response for shoot and root formation. Shoot regeneration was optimum in whole zygotic embryos at $2.5mgL^{-1}$ BAP, while root formation was optimum at $0.5mgL^{-1}$ NAA. Cotyledon, hypocotyl, leaf, stem, root and transversely sliced embryo sections were similarly examined, but these explants did not exhibit any signs of regeneration during *in vitro* culture.

From these initial experiments, explant-to-plant regeneration was attempted by culturing whole zygotic embryos on MS-based medium supplemented with $2.5mgL^{-1}$ BAP to initiate shoot regeneration. Despite the successful formation of adventitious shoots, leaves from these shoots did not fully expand, giving them an under-developed appearance. Upon transfer of the shoots to MS-based medium supplemented with $0.5mgL^{-1}$ NAA (but no BAP), root formation failed to initiate and the shoots eventually became necrotic, potentially due to an endogenous auxin deficiency exacerbated by a dependency on exogenously supplied BAP. Due to time constraints (these initial results necessitated approximately 1.5 years of experimentation), and limitations on availability of starting material, it was decided to concentrate efforts on the development and optimisation of tissue culture and genetic transformation techniques within Bambara groundnut.

Future studies should aim however, to optimise the developmental stages of *in vitro* regenerated shoots by evaluating the impact of important variables which could not be investigated here, such as genotype, carbohydrate sources, salt composition of basal culture medium and culture conditions, all of which are important contributors to an established and reproducible regeneration and micropropagation system (Birch, 1997). The data produced and recorded in this research programme have contributed towards the design of a robust regeneration protocol for jicama, for the development of a fundamental prerequisite as the basis of future tissue culture based investigations including genetic

transformation, *in vitro* mutagenesis, and somaclonal variation induction techniques (Birch, 1997; Mohan Jain, 2001).

7.2.2. Tissue culture of Bambara groundnut

Recent studies on the regeneration of Bambara groundnut *in vitro* have been carried out on several landraces including Nkorenza and Yoroba from Ghana (Lacroix *et al.*, 2003) and Ci1 – Ci4 from Ivory Coast (Koné *et al.*, 2007; Mongomaké *et al.*, 2009). However, there are, to date, no reports of tissue culture studies performed on the landraces investigated in this research programme, namely DipC, Uniswa-Red and S19-3. The tissue culture work carried out to establish an explant-to-plant regeneration system for these 3 landraces included, and expanded upon, previously published methods by Lacroix *et al.* (2003) and Koné *et al.* (2007).

Experiments carried out to identify explants which demonstrated maximum shoot regeneration revealed that leaf and stem material failed to respond to *in vitro* culture under all conditions examined. For root tip and hypocotyl explants, the optimum regenerative responses were recorded from landraces DipC and S19-3, with 72.2 – 77.8% of DipC explants and 68.5 – 75.9% S19-3 explants exhibiting limited non-proliferative callus formation, but no shoot regeneration, on MS-based medium supplemented with $3mgL^{-1}$ BAP. Mongomaké *et al.* (2009) reported that 73.33% of hypocotyl cuttings from landraces Ci1 – Ci6 regenerated on average 3.7 shoots per explant when cultured on MS-based medium supplemented with $2mgL^{-1}$ BAP. Future studies should test this medium composition on landraces DipC, Uniswa-Red and S19-3 to determine whether similar regenerative responses can be obtained.

The regenerative response of cotyledon and zygotic embryo explants were investigated using conditions which were adapted and optimised from previous work published by Koné *et al.* (2007) and Lacroix *et al.* (2003) respectively. Cotyledon explant culture resulted in callus formation on the cut edges of the explants, an observation also reported by Koné *et al.* (2007) for landraces tested

on MS-based medium supplemented with 5mgL^{-1} BAP and 0.5mgL^{-1} NAA. However, the absence of shoot regeneration from cotyledon explants was not consistent with results reported by Koné and co-workers (2007), who observed highest shoot regeneration in landrace Ci4, with approximately 6% of cultured explants on MS-based medium supplemented with 3 – 5 mgL^{-1} BAP alone or in combination with 0.01 – 0.1mgL^{-1} NAA.

Lacroix and co-workers (2003) reported that highest shoot regeneration occurred in landrace Nkorenza, with transversely cut zygotic embryos producing 100% regeneration frequency with 7.0 ± 0.7 shoots obtained per explant when cultured on basal medium supplemented with 1.5mgL^{-1} BAP. Application of these conditions in the present study to landraces DipC, Uniswa-Red and S19-3, resulted in shoot formation solely from the radicle sections of transversely bisected embryos, with 85.2 – 100% regeneration frequency, and a comparatively lower number of shoots per explant (1.8 ± 0.8, 1.5 ± 0.9 and 2.1 ± 0.7 shoots for DipC, Uniswa-Red and S19-3 respectively).

The use of whole zygotic embryos as starting explants produced the highest incidence of shoot regeneration when cultured on basal medium containing 1.5mgL^{-1} BAP, with 96.3 – 100% regeneration frequency, and with 5.2 ± 1.2, 5.5 ± 0.6 and 5.1 ± 0.9 shoots per explant for DipC, Uniswa-Red and S19-3 respectively. Therefore, whole zygotic embryos were selected as the optimal explant type to test for an efficient explant-to-plant regeneration system for Bambara groundnut. Regenerated shoots from DipC, Uniswa-Red and S19-3 were rooted *in vitro* on basal medium supplemented with 1mgL^{-1} NAA, an occurrence consistent with results obtained by Lacroix *et al.* (2003) for landrace Nkorenza. However, the survival rate of *in vitro* regenerated plantlets was very low upon their transfer *ex vitro*, with only 3 DipC and 4 S19-3 plants surviving to maturity. Furthermore, these plants were comparatively smaller in size than seed-derived Bambara groundnut plants, and did not set seed after flowering.

Results from this study highlighted two main limitations in the proposed explant-to-plant regeneration protocol for landraces DipC, Uniswa-Red and S19-3. The first was the lower incidence of shoot regeneration compared to values obtained in previously published studies by Lacroix *et al.* (2003) and Koné *et al.* (2007). This occurrence could be genotype-dependent, suggesting that existing genetic variations between landraces could affect their individual regenerative response *in vitro* culture. Interactions between the explant's genotype and the culture environment may sometimes have genotype-specific effects, *e.g.* the use of certain PGRs in the culture medium may favour regeneration in one, but not the other landrace. Reports on the genetic diversity in Bambara groundnut have suggested that high levels of polymorphism exist both within and between Bambara groundnut landraces (Amadou *et al.*, 2001; Massawe *et al.*, 2002, 2003). The study by Massawe and co-workers (2002) on 16 Bambara groundnut landraces, including DipC1995, revealed that the genetic diversity generated through AFLP analyses was so extensive that no two landraces produced identical electropherograms. Cluster analysis revealed that, while DipC1995 and Malawi5 were the most genetically related landraces, their cluster was also the most genetically diverse from the clusters generated for the remaining 14 landraces (Massawe *et al.*, 2002).

The final limitation to the establishment of an efficient explant-to-plant regeneration protocol involved the observed low survival rate of regenerated Bambara groundnut plantlets following acclimatisation *ex vitro*. This incidence may have been influenced by abiotic stresses accumulated during the development of plantlets *in vitro*, induced by factors including nutrient composition and hormone levels of the culture medium, as well as environmental aspects, such as the use of air-tight cultivation vessels which may have lead to increased air humidity and decreased irradiation levels. These culture conditions could potentially result in the abnormal physiological and morphological development of plantlets *in vitro*, with, for example, the formation of poor vascular root-stem connections (Pospíšilovà *et al.*, 1999), a reduction in photosynthetic activity (Kane, 1999), as well as reduced thickness of waxy cuticles and impaired stomatal function in leaves (Smith and Spomer, 1995).

Following the transfer of Bambara groundnut regenerated plantlets to *ex vitro* conditions, the sudden exposure to higher irradiance and lower air humidity, as well as the absence of readily available nutrients in the culture environment caused a high proportion of plantlets to wilt due to excessive water loss. Additionally, since the process of adapting to correct such abnormalities can prove lengthy, the plantlets may not have developed sufficiently to restore photosynthetic competence to ensure their survival without an exogenous supply of readily available phytonutrients and carbon sources.

Future studies should focus on determining the degree of genetic diversity between and within landraces DipC, Uniswa-Red and S19-3, and further optimise the tissue culture system to maximise shoot regeneration and plantlet development *in vitro*. Experiments investigating the effect of different nutrient compositions of culture media, gelling agents, combination and concentration of different PGRs, as well as carbon sources should be performed in order to optimise the regenerative responses of individual landraces. The inclusion of an *in vitro* 'hardening' step, such as utilising culture vessels with permeable lids to decrease air humidity, to facilitate acclimatisation of plants to *ex vitro* conditions prior to their transfer to the glasshouse, may help reduce the percentage of plant mortality. Data generated during this study should permit progress towards establishing a more robust and reproducible tissue culture system for Bambara groundnut.

7.3. DNA recombination and molecular cloning strategies

One of the aims of this research programme was to design and construct a plasmid vector carrying a gene encoding for a methionine-rich protein which could be employed in subsequent transformation studies to enhance the methionine levels in Bambara groundnut seeds. The candidate gene of interest was *ber e1*, a gene from Brazil Nut, which codes for 18% methionine (Lacorte *et al.*, 1997). The confirmed allergenicity of this gene (Nordlee *et al.*, 1996; Alcocer *et al.*, 2002) restricted its use in this research programme as a model

investigation in order to demonstrate a 'proof of concept' to advance future Bambara groundnut transformation studies.

Traditional DNA recombination and molecular cloning strategies, including gene amplification by PCR, restriction digestion and ligation methods were employed to place the *ber e1* coding region under the control of the seed-specific LeB4 promoter from *Vicia faba* and the constitutive CaMV 35S terminator, and to introduce the LeB4-Ber e1-35S cassette into the transformation vector pBI121. Following Sanger sequencing of the LeB4-Ber e1-35S cassette, analysis of the data generated revealed 100% homology for the *ber e1* gene to the donated reference sequence data. Comparative sequence analysis of the LeB4 promoter region, however, showed 3 disparities between the Sanger generated data and the donated reference sequence data, which could be attributed either to the introduction of artificial base pair errors during the amplification of the fragment from the original *V. faba* genomic DNA through the use of non-proof reading polymerase, or to the introduction of base pair mis-calls in the promoter region during Sanger sequencing. Nevertheless, the LeB4 promoter region was confirmed to carry the 'CATGCATG' regulatory element necessary for seed-specific expression. The recombinant vector was named pBI121-LeB4-Ber e1 and maintained as glycerol stocks in *E. coli* DH5α and *A. tumefaciens* GV3101 strains at -80°C, to be employed in successive tissue culture-based transformation studies for Bambara groundnut. The construction of the transformation vector pBI121-LeB4-Ber e1 has provided a template that could be exploited in future studies by replacing the *ber e1* gene in pBI121-LeB4-Ber e1 with non-allergenic genes of interest to attempt nutritional enhancement or other seed-specific functional assays in Bambara groundnut and other economically important crops.

7.4. Bambara groundnut transformation studies

The final aim of this research programme was to attempt genetic transformation of Bambara groundnut *via* two DNA delivery methods, namely biolistic-mediated particle delivery and *Agrobacterium*-mediated transformation. At the

time of writing, there were no reports of genetic transformation studies for Bambara groundnut. With a tissue culture regeneration approach optimised, transformation experiments were initiated using whole or transversely bisected zygotic embryos as starting explants. The transformation vectors employed were pVDH65, pBI121 and pBI121-LeB4-Ber e1, with all 3 harbouring the *npt*II selectable marker and *uidA* reporter genes. Vector pBI121-LeB4-Ber e1 additionally harboured the methionine rich *ber e1* gene, and was used in this study to attempt transformation of Bambara groundnut for enhanced methionine production.

7.4.1. Biolistic-mediated transformation

An initial trial, investigating the genetic transformation of Bambara groundnut *via* biolistic-mediated gene transfer was attempted by bombarding whole zygotic embryos once with microcarriers coated with pBI121-LeB4-Ber e1 DNA. Following a 48-hr recovery period on non-selection medium, bombarded embryos subjected to histochemical GUS assay produced indigo coloured spots at the sites of microparticle delivery, confirming transfer of the plasmid T-DNA and *uidA* gene expression. However, upon transfer to selection medium, all the bombarded explants turned necrotic, eliminating any possibility of shoot regeneration. A possible explanation for this occurrence could be that, since embryos were only bombarded once, the area of non-transformed tissue in each explant would have been relatively larger than sites of foreign DNA delivery and therefore could not survive on medium containing kanamycin. This limitation could be rectified in future experiments by subjecting the explant tissue to multiple bombardments. This, coupled with the extension of the recovery period of bombarded embryos on non-selection medium to more than 48 h, may provide explants with a higher chance to maintain their viability and regenerate putatively transformed tissues upon their subsequent transfer to selection medium.

7.4.2. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation of Bambara groundnut was attempted using *A. tumefaciens* strain 1065 harbouring pVDH65, and *A. tumefaciens* strain GV3101 harbouring either pBI121 or pBI121-LeB4-Ber e1, in distinct transformation experiments. In an effort to develop a highly efficient and reliable protocol, several factors were investigated to optimise the transformation system for maximum generation of transgenic plants. Experiments were designed to investigate the effects of acetosyringone (a *vir* gene-inducing phenolic compound) and L-cysteine (an anti-necrotic compound), as well as the use of antibiotic-free inoculation broth, on transformation and regeneration frequency. In addition, the inclusion of a non-selective transitional stage following the co-cultivation of inoculated explants was investigated, as an attempt to improve their survival rate and regeneration frequency, post-infection with *Agrobacterium*.

The addition of acetosyringone to the bacterial broth prior to explant inoculation was found to have no significant effect on the transformation efficiency of Bambara groundnut, since the % of explants inoculated with acetosyringone-supplemented bacterial broth which tested positive for histochemical GUS production (91 – 92.5%) was comparatively similar to that of explants inoculated with acetorysingone-free broth (90 – 92%). Supplementation of the selection medium with the anti-necrotic compound L-cysteine had no significant effect in delaying the onset of necrosis in Agro-infected Bambara groundnut explants. Whole and transversely bisected embryo explants exhibited a similar behaviour on selection medium with or without L-cysteine, with the formation of bud initials or callogenic tissue within the first 5 wk of culture, followed by the onset of necrosis before any further regeneration could occur.

The concentrations of acetosyringone (200 and 400μM) and L-cysteine (20 and 40mgL^{-1}) employed may have been too low to significantly enhance the efficiency of the transformation protocols tested. Future studies should aim to

test the effects of increased concentrations of these compounds on transformation efficiency. Also, the inclusion of L-cysteine in the co-cultivation medium as well as in the selection medium in future studies might enhance the transformation efficiency and the rate of explant viability, thus increasing the regeneration frequency of putative transgenic shoots. Furthermore, the effect of other anti-necrotic compounds such as dithiothreitol (DTT) could be tested, since it was shown to enhance the transformation efficiency in pea (Švábová and Griga, 2008).

The use of antibiotic-free bacterial broth for inoculation improved the viability of Agro-infected Bambara groundnut explants cultured on selection medium, by extending their survival by an additional 4 wk, compared to explants inoculated with antibiotic-containing broth. This was accompanied by an increase in adventitious bud formation, although the regenerated tissue failed to develop into shoots, due to the onset of necrosis. This transformation protocol was therefore modified to include a non-selective transitional culture period following explant co-cultivation with *Agrobacterium*.

The addition of a non-selective transitional stage following the co-cultivation of inoculated Bambara groundnut explants improved their viability and prolonged their survival by 11wk, during which time limited shoot regeneration occurred. However, putatively transformed shoots failed to survive on root regeneration medium. Future studies should aim to further optimise this tissue culture-based transformation protocol, as it has proved to be the most efficient of all the protocols tested in this study. Experiments should be geared towards the elimination of tissue necrosis, since this was the main factor responsible for the loss of viability in inoculated explants and regenerated shoots.

All but two transformation protocols described in this research programme demonstrated limited success producing putatively transformed shoots. Firstly, Bambara groundnut whole embryos inoculated with *A. tumefaciens* strain 1065 harbouring pVDH65 regenerated adventitious shoots, which tested positive for the *npt*II and *uidA* gene fragments when subjected to PCR analyses. Secondly,

whole and transversely bisected Bambara groundnut embryos inoculated with an antibiotic-free broth of *A. tumefaciens* strain GV3101 harbouring pBI121-LeB4-Ber e1 produced putatively transformed shoots when cultured on non-selective medium following co-cultivation, before being transferred to kanamycin-supplemented selection medium (Transformation experiment TB3, Section 6.4.3.3.). PCR analyses revealed that these shoots tested positive for the presence of the *npt*II, *uidA* and *ber e1* gene fragments. These molecular analyses indicated the presence of the respective T-DNAs into the regenerated tissue. However, identification of integrative transformation represents an initial step towards the establishment of stable transgenic lines, and further optimisation of the selection and regeneration protocol will be required to allow shoots to develop and produce sufficient leaf tissue for more thorough molecular analyses such as iPCR, RT-PCR, as well as Southern and northern blotting, all of which require a large amount of DNA or RNA, to be performed to a reliable standard.

7.5. Factors affecting tissue culture-based transformation systems in legumes

The essential requirements in a gene transfer system for the production of transgenic plants include the immediate availability of target tissue carrying competent cells, a method of DNA delivery into these regenerable cells, and an optimised protocol that permits the routine selection and regeneration of transgenic plants (Birch, 1997). Legume species are commonly categorised as recalcitrant to tissue culture and transformation methodologies, since a disproportionate number of species in this family struggle to maintain their viability and regeneration capabilities when subjected to *in vitro* culture conditions or DNA transformation. Despite being a major constraint in transgenic plant production, recalcitrance is, however, not an inherent trait (Birch, 1997), and is instead a limitation that can be overcome by identifying the optimum culture and transformation conditions to trigger the production of proliferating and regenerable cells in target explants, into which foreign DNA can be introduced at high frequency without interfering with the explants' viability and regenerative potential to develop putatively transformed shoots.

Legume recalcitrance to *in vitro* manipulation can be influenced by several factors, including environmental stresses such as high relative humidity and accumulation of gases in the culture vessel due to poor ventilation, as well as excess nitrogen supply or unusual PGR concentrations and combinations in the culture medium (Gaspar *et al.*, 2000; Cassells and Curry, 2001). These stresses can potentially cause hyperhydricity in regenerated shoots, giving them a turgid and watery, sometimes translucent, appearance. Hyperhydric shoot clusters usually exhibit malformed organs such as wrinkled and/or curled, brittle leaves, or thick shoots with shorter internodes. The prolonged subculture of hyperdendric clusters may result in the sudden and simultaneous necrosis of all meristematic apices, causing individual shoots from such clusters to turn brown and eventually die (Gaspar *et al.*, 2000).

Physical factors such as explant wounding during tissue or organ excision or prior to inoculation with *Agrobacterium* can also affect the regeneration ability of the explant and promote its recalcitrance to *in vitro* manipulation (Gaspar *et al.*, 2000; Ozygit *et al.*, 2007). Phenolic compounds are a group of secondary metabolites with important roles in plant defense and in plant growth regulation and development. During wounding, contents of the cytoplasm and vacuoles of damaged cells become mixed and phenolic compounds can become oxidised by air. These oxidised phenols, also known as quinones, are highly toxic and may cause a darkening of the culture medium as well as inhibit enzyme activity in the explants, eventually causing lethal browning and death (Ozygit *et al.*, 2007). Remediation strategies that could help lower the risk of explant necrosis through exposure to quinones include the use of liquid medium to reduce phenolic oxidation. Frequent subculturing, as well as the addition of antioxidants such as citric acid, ascorbic acid and activated carbon in the culture medium can help to minimise phenolic oxidation and improve explant viability and regeneration in culture (Ozygit *et al.*, 2007).

7.6. Directions for future research

Global hunger and malnutrition including protein deficiency affects over 1 billion people globally with current predictions estimating that this figure will increase significantly in the near future due to an unprecedented growth in the human population (FAO, 2010; Henley *et al.*, 2010). To prevent such a catastrophe, crop production must increase by over 38 % more than current rates of increase delivered by conventional crop improvement and breeding strategies (Tester and Langridge, 2010). One possible approach to achieve such ambitious objectives would be through the application of plant biotechnology, including the establishment of genetically enhanced cultivars through transgenic techniques. Already, such approaches have introduced both economically and environmentally desirable qualities into commercially important crop species. Pest resistance introduced into cotton, maize and soybean through the integration of *cry* genes from *Bacillus thuringiensis* has reduced the cost of producing these crops due to a reduction in pesticide applications required, which has consequently reduced the damaging impact of agriculture on both ecology and environment. Due to reduced incidences of pest damage, crop productivity has subsequently increased in these species. Similarly, vitamin, mineral and protein concentrations have been improved in crops through transgenic approaches and could represent a viable and sustainable approach to tackling world malnutrition through biofortification (Palmgren *et al.*, 2008; White and Broadley 2005; 2009).

Adopting such strategies to underutilised crops represents a viable avenue for crop improvement, especially in developing countries, as these crops are often better suited to local extreme conditions. Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landraces are adapted through local breeding to specific harsh drought prone regions *e.g.* sub-Saharan Africa, where common commercial crops such as maize, soybean and wheat are poorly adapted. Similarly jicama [*Pachyrhizus erosus* (L.) Urban] is adapted to South-East Asian regions where many commercial crops are prone to yield loss due to pest damage as well as being heavily dependent on environmentally damaging pesticides, herbicides and fertiliser inputs (Karuniawan, 2004).

The main objective of this thesis therefore was to provide fundamental data regarding the adoption of tissue culture-based transformation strategies for the genetic improvement of both Bambara groundnut and jicama. For both crops, results obtained have provided essential information regarding the optimisation of tissue culture and plant regeneration as a fundamental prerequisite to future transformation-based assays. Additionally, data obtained from attempted transformation studies in Bambara groundnut has offered preliminary insight into the amenability and limitations of the crop towards genetic manipulation.

Expanding on the data produced from this research programme, future work on jicama should focus on optimising the stages of shoot development *in vitro* by assessing the effect of factors including nutrient composition and type of gelling agent used in the culture medium, the incorporation of anti-necrotic agents in the culture medium, as well as environmental conditions such as light intensity and temperature, on explant viability and regeneration frequency. These future studies will contribute towards refining and establishing a reproducible *in vitro* regeneration system for jicama, thus providing a necessary prerequisite for future tissue culture-based studies directed towards the improvement of this underutilised legume.

For Bambara groundnut, future studies on landraces DipC, Uniswa-Red and S19-3 should aim at maximising regeneration frequency from explants cultured *in vitro*, and on limiting the loss of regenerated plantlets to a minimum upon their transfer to *ex vitro* conditions. To this end, tissue culture experiments targeted at individual landraces should investigate the regenerative response of explants to modifications in the culture medium by varying nutrient composition, PGR combinations and concentrations as well as the type of gelling agent used. The effect of environmental and physical factors on cultured explants and regenerated shoots, including light intensity, temperature and type of culture vessel used, should also be considered when setting up future experiments. With regards to Bambara groundnut transformation studies, future experiments could employ transformation vector pBI121-LeB4-Ber e1 as a template to explore non-

allergenic alternatives to the *ber e1* gene to enhance seed methionine content in the crop. Also, since data obtained from histochemical GUS assays have revealed that foreign gene transfer and expression into targeted explants was successfully achieved, the main obstacle in the production of transgenic Bambara groundnut plants remained regeneration and development of adventitious shoots from inoculated explants upon their transfer to selection medium, suggesting that, while future studies should aim at maximising transformation frequency (*e.g.* by performing multiple biolistic bombardments or using higher acetosyringone concentrations in the inoculation broth), efforts should also concentrate on optimising the selection process in order to maximise the survival of inoculated explants and ensure their viability for regeneration of transformed tissue. The optimisation of the regeneration system will favour the production of well-developed shoots, with more available tissue for DNA and RNA extraction, thus enabling a more reliable molecular characterisation of putative transgenics *via* informative Southern, Northern and RT-PCR analyses.

7.7. Considerations for future research

The potential within the jicama and Bambara groundnut genomes is important to maintain and improve through GM approaches, as these have the ability to greatly enhance crop quality and yield. However, despite this positive outlook on the future of crop improvement, some important aspects must be considered when developing future strategies.

It must be remembered that despite being a sustainable and robust system, crop improvement for increased methionine or other essential nutrients *via* biotechnology-assisted strategies has some limitations. Firstly, it is a long term approach, requiring knowledge and careful balancing of a blend of technologies including comparative genomics, transgenic data and traditional breeding strategies. Outcomes cannot be guaranteed, despite requiring a substantial initial investment. Additionally, genetic variation of nutrient accumulation in suitable lines and the stability of the trait must be established across a range of disparate environments with variations in both soil and climatic conditions. Biofortified

crops should be widely accepted by consumers in both developed and developing countries and it must be ensured that appearance, taste, texture and cooking quality should not be compromised (Bouis *et al.*, 2003). Assuming that minute alterations in nutrient content should not significantly alter food quality, important aspects such as taste and colour could nevertheless be affected by these compounds (White and Broadley, 2009). To be accepted, the consumption of such products must be associated with health benefits to humans. If transgenes are to be employed, then the associated risks to human health as well as to wild populations and the environment with must be considered. Moreover, a balance must be struck between these perceived risks, with the economic and sustainable provision of essential nutrients to a growing global population.

7.8. Conclusion

Experiments described in this thesis have, initially, focused on developing an explant-to-plant regeneration system for the underutilised crops, jicama and Bambara groundnut (landraces DipC, Uniswa-Red and S19-3), as a basis for innovative genetic manipulation techniques. In the case of jicama, results observed from tissue culture experiments indicated that, of all six types of plant tissues examined (cotyledon, hypocotyl, leaf, stem, zygotic embryo and root), whole zygotic embryos demonstrated maximum potential for *in vitro* regeneration, while media and hormone supplementation were optimised for maximum regeneration of both shoots and roots. Although establishing an efficient *in vitro* explant-to-plant regeneration system remains a limiting factor due to poor shoot development and premature explant necrosis, it is anticipated that future improvements will be facilitated by the results generated in this thesis. Moreover, results described here have established important parameters and highlighted areas requiring immediate attention and optimisation to advance the development of a robust regeneration protocol for this important underutilised food crop.

For Bambara groundnut, efforts were targeted at adapting an explant-to-plant regeneration system described in Lacroix *et al.* (2003) and Koné *et al* (2007), for

three landraces, DipC, Uniswa-Red and S19-3, for which no published tissue culture data exists to date. Results in this study have thus, provided evidence of the first recorded successful *in vitro* regeneration, *ex vitro* acclimatisation and maturation to flowering of Bambara groundnut landraces DipC, Uniswa-Red and S19-3.

Furthermore, experimental assays performed in the present study have successfully developed a previously unpublished, novel tissue culture-based approach to the genetic transformation of Bambara groundnut. Moreover, PCR and histochemical GUS assays have identified and confirmed the presence of transgenes in regenerated tissues.

Together, these results have developed novel understanding of tissue culture-based regeneration of both jicama and Bambara groundnut plantlets, while innovative transgenic approaches have established previously unpublished protocols for the genetic transformation of Bambara. With food insecurity currently affecting ~900 million people and population growth increasing unsustainably, malnutrition has steadily developed as one of the primary issues threathening mankind. Enhancing the amino acid balance in underutilised crops is one sustainable solution which has been proposed, and it is therefore envisaged that data and protocols produced in this thesis could be employed in future studies to genetically enhance and exploit the enormous agronomic potential of jicama and Bambara groundnut.

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APPENDICES

Appendix I. Composition of media for plant tissue culture

A I.1. MS medium (Murashige and Skoog, 1962)

The components of the MS powder used to prepare the medium are shown below in mgL^{-1}.

Micro elements

$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.6H_2O$	8.60

Macro elements

$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00

Vitamins

Glycine	2.00
myo-Inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10

The above salt mixture was available commercially from Duchefa Biochemie, Haarlem, The Netherlands. The addition of 4.4mg of salt mixture and 30g of sucrose to 1L of reverse-osmosis water produced 1L of full-strength MS medium. Half-strength liquid MS medium was prepared by adjusting the weight of the powder accordingly. The pH of the medium was adjusted to 5.8 using 1M KOH before autoclaving at 121°C under 104 kPa for 20 min.

AI.2. MS0 medium

MS0 medium was MS-based medium (Appendix I, Section A I.1) lacking growth regulators, made semi-solid with 0.8% (w/v) agar (Sigma-Aldrich Co., Steinheim, Germany). MS0 medium was supplied ready to use by the Central Services technical staff at the Plant and Crop Sciences Division.

AI.3. Basal medium

1L of basal medium consisted of 1L of MS-based medium (Murashige and Skoog, 1962), semi-solidified with 0.8% (w/v) agar, and supplemented with Nitsch and Nitsch (1969) vitamin mixture.

Components of Nitsch and Nitsch (NN) vitamins (1969) in mgL^{-1}:

Biotin	0.05
Folic acid	0.50
Glycine	2.00
Myo-inositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	0.50
Thiamine HCl	0.50

10.85g of NN vitamin mixture makes 100ml of a 1000X vitamin stock solution. One millilitre of vitamin solution was added to 1L of MS-based medium.

Appendix II. Composition of media for bacterial culture

AII.1. Liquid Luria Broth (LB)

Medium components are in made up to 1L in reverse-osmosis water.

Tryptone	10.0g
Yeast extract	5.0g
NaCl	10.0g

The pH was adjusted to 7.2 before autoclaving.

AII.2. Semi-solid LB

Medium components are in made up to 1L in reverse-osmosis water.

Tryptone	10.0g
Yeast extract	5.0g
NaCl	10.0g
Agar	18.0g

The pH was adjusted to 7.2 before adding agar and autoclaving at 121°C under 104 kPa for 20 min.

AII.3. APM medium

Medium components are made up to 1L in reverse-osmosis water

Yeast extract	5.0g
Casamino acids	0.5g
Mannitol	18.0g
$(NH_4)_2SO_4$	2.0g
NaCl	5.0g
$MgCl_2$	0.427g

The pH was adjusted to 6.6 before autoclaving at 121°C under 104 kPa for 20 min.

Appendix III. General kit-based protocols used in molecular cloning

AIII.1. Plasmid DNA extraction and purification using the Qiaprep Spin Miniprep Kit

N.B.: This protocol was designed for purification of up to 20µg of plasmid DNA from 1 – 5 ml overnight cultures of *E. coli* in L.B medium.

1. The liquid cultures were centrifuged at 2000 rpm for 10 min, and the supernatant decanted.

2. RNase A and LyseBlue reagent were added to Buffer P1. Pelleted bacterial cells were resuspended in 250µl Buffer P1 and transferred to a 1.5ml microcentrifuge tube. The bacteria was resuspended completely by vigorous vortexing until no cell clumps are visible.

3. Buffer P2 (250µl) was added and the contents of the tube thoroughly mixed by inverting the tube 4-6 times. Vortexing was not recommended as this would result in shearing of genomic DNA.

4. Buffer N3 (350µl) was added and the contents mixed immediately and thoroughly by inverting the tube 4-6 times. If LyseBlue reagent had been used, the suspension would have been mixed until all traces of blue had gone.

5. The samples were centrifuged for 10 min at 13,000 rpm in a table-top microcentrifuge.

6. The supernatants from step 4 were applied to the Qiaprep spin column by pipetting.

7. Centrifugation was at 13,000 rpm for 1 min. The flow-through was discarded.

8. The Qiaprep spin column was washed by adding 0.5ml Buffer PB and centrifuging for 1 min at 13,000 rpm. The flow-through was discarded.

9. The Qiaprep spin column was washed by adding 0.75ml of Buffer PE and centrifuging for 1 min at 13,000 rpm.

10. The flow-through was discarded and the column centrifuged for an additional 1 min to remove residual wash buffer.

11. The Qiaprep column was placed in a clean 1.5ml Eppendorf tube. To elute the DNA, 30µl of sterile H_2O was added to the centre of each column. The column was left to stand for 2 min and centrifuged at 13,000 rpm for 1 min. The concentration of the eluted plasmid DNA was measured on the nanodrop and DNA was stored at -20°C.

AIII.2. Gel extraction and purification of plasmid DNA using the Qiaquick Gel Extraction Kit

i. Ethanol (100% v/v) was added to Buffer PE before use.
ii. All centrifugation steps were carried out at 13,000 rpm in a conventional table-top microcentrifuge at room temperature.

1. The DNA fragment was excised from the agarose gel with a clean sharp scalpel.

2. The gel slice was weighed in a colourless tube and 3 volumes of Buffer QG was added to 1 volume of gel (*e.g.* add 300µl of Buffer QG to 100mg of gel).

3. The mixture was incubated at 50°C for 10 min (or until the gel slice had completely dissolved). To help dissolve the gel, the tube contents were mixed by vortexing the tube every 2 - 3 min during the incubation.

4. After the gel slice had dissolved, the colour of the mixture was confirmed to be still yellow (similar to Buffer QG without dissolved agarose).

5. One gel volume of isopropanol was added to the sample and mixed. For example, if the agarose gel slice weighed 100µg, add 100µl of isopropanol.

6. A Qiaquick spin column was placed in a provided 2ml collection tube.

7. To bind DNA, the sample was applied to the Qiaquick column and centrifuged for 1 min.

8. The flow-through was discarded and the Qiaquick column was placed back in the collection tube.

9. A volume of 0.5ml of Buffer QG was added to the Qiaquick column and centrifuged for 1 min to remove any remaining trace of agarose.

10. To wash, 0.75ml of Buffer PE was added to Qiaquick column and centrifuged for 1 min.

11. The flow-through was discarded and the QIAquick column centrifuged for an additional 1 min to remove traces of residual ethanol.

12. The Qiaquick column was placed into a clean 1.5ml Eppendorf tube.

13. To elute DNA, 30µl of sterile H_2O was added to the centre of the Qiaquick membrane, let stand for 2 min, and centrifuged for 1 min.

14. The concentration of the eluted DNA was measured on the nanodrop and stored at -20°C.

Appendix IV. Preparation of solutions for histochemical GUS assay

AIV.1. Preparation of stock solutions

(1) 0.5M EDTA (pH 8.0)

Add 186.1g of EDTA salt to 800ml of H_2O.
Stir vigorously on a magnetic stirrer.
Adjust the pH to 8.0 with NaOH (Note: the disodium salt of EDTA will not completely go into solution until the pH is adjusted).
Dispense solution into aliquots and sterilise by autoclaving.

(2) 0.1% (v/v) Triton-X-100

Triton-X-100 solution (50µl) was added to 49.95ml of sterile H_2O.
The solution was filter-sterilised and stored in a sterile centrifuge tube at 20°C.

(3) 10mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$)

$K_4Fe(CN)_6.3H_2O$ (21mg) was dissolved in 5ml of sterile deionised H_2O.
The solution was stored in a 30ml sterile plastic universal wrapped in aluminium foil at 4°C for up to 14 days.

(4) 10mM potassium ferricyanide ($K_3Fe(CN)_6$)

$K_3Fe(CN)_6$ (16mg) was dissolved in 5ml of sterile deionised H_2O.
The solution was stored in a 30ml sterile plastic universal wrapped in aluminium foil at 14°C for up to 14 days.

(5) Phosphate buffer

The phosphate buffer was made up of 0.2M NaH_2PO_4 (solution 1) and 0.2M Na_2HPO_4 (solution 2).

Preparation of solution 1:
NaH_2PO_4 (2.758g) was dissolved in 100ml of H_2O. The solution was sterilised by autoclaving at 121°C under 104 kPa for 20 min.

Preparation of solution 2:
Na_2HPO_4 (3.558g) was dissolved in 100ml of H_2O. The solution was sterilised by autoclaving at 121°C under 104 kPa for 20 min.

To prepare 10ml of phosphate buffer, 3.9ml of solution 1 was mixed with 6.1ml of solution 2. Each GUS reaction mixture used 5ml of phosphate buffer.

AIV.2. Preparation of GUS reaction mixture

In a sterile 30ml glass bottle, the following was added:

5mg of X-Gluc dissolved in 100µl of dimethyl formamide

3.79 ml of sterile deionised H_2O

200µl of 0.5M EDTA

10µl of 0.1% (v/v) Triton-X-100

500µl of 10mM $K_4Fe(CN)_6.3H_2O$

500µl of 10mM $K_3Fe(CN)_6$

5ml of phosphate buffer

This made 10ml of reaction mixture and was used for the incubation of 1 explant.

Appendix V. General kit-based protocols used in nucleic acid isolation

AV.1. DNA extraction using the GenElute™ Plant Genomic DNA Miniprep Kit

Prior to carrying out the extraction procedure,

i. The water bath was pre-heated to 65°C
ii. Elution solution was pre-heated to 65°C
iii. Eppendorf tubes were labeled accordingly
iv. Plant tissue was transferred to an Eppendorf tube and the tube was immediately dropped tube in liquid nitrogen. Leave for 2 min.
v. The tissue was ground to a fine powder using a sterile pestle, and the tube dropped back in liquid nitrogen.

Extraction process

1. **Lysis**
   Eppendorf tubes were removed from the liquid nitrogen and placed on ice.
   To each tube, 350µl of Lyse A solution and 50µl of Lyse B solution were added. The tubes were inverted and vortexed. A white precipitate formed. The mixture was incubated at 65°C for 10 min, with occasional inversion.

2. **Precipitation**
   To the mixture, 130µl of Precipitation solution was added. Each tube was mixed by inversion and placed on ice for 5 min.

   The tubes were centrifuged for 5 minutes at maximum speed (12,000 – 16,000 x *g*) to pellet debris.

3. **Filtration of debris**
   The supernatant (530µl) was transferred into a blue tube. Centrifugation was at maximum speed for 1 min. The filtration column was discarded and the collection tube retained.

4. **Binding**
   To the flow-through liquid from step 3, 700µl of Binding solution was added, and the solution mixed by inversion.

   Binding columns were prepared by inserted a red tube in a new collection tube, and adding 500µl of Column preparation solution to each column. Centrifugation was at 12,000 x *g* for 1 min. The flow-through liquid was discarded.

5. **Loading lysate**
   The mixture from step 3 (600µl) was transferrd to the column prepared in step 4. Centrifugation was at maximum speed for 1 min. The flow-through liquid was discarded and the column retained and returned to the collection tube.

The remaining lysate from step 3 was applied onto the column. Centrifugation was at maximum speed for 1 min. The flow-through liquid and collection tube were discarded but the column was retained.

6. **Washing**

1st wash: The binding column was placed into a fresh 2ml collection tube. Diluted Wash solution (500µl) was added to the column and centrifuged at maximum speed for 1 min. The flow-through liquid was discarded. The column and collection tube were retained.

2nd wash: Diluted Wash solution (500µl) was added to the column and centrifuged at maximum speed for 3 min. The flow-through liquid and collection tube were discarded. The column was retained.

7. **Elution**

The binding column was transferred to a fresh 2ml collection tube. Elution solution (100µl) was added to the column. Centrifugation was at max. speed for 1 min. The elution process was repeated and the eluate was stored in the same collection tube at -20°C.

AV.2. Total RNA extraction using the RNeasy Plant Mini Kit

Prior to carrying out the extraction procedure,

i. Microcentrifuge tubes were labelled accordingly
ii. The water bath was pre-heated to 56°C
iii. β-Mercaptoethanol (β-Me) was added to Buffer RLT (10µl β-Me per 1ml Buffer RLT)
iv. Four volumes of ethanol (95 – 100%) were added to Buffer RPE
v. Plant tissue was transferred to a 1.5ml microcentrifuge tube and the tube was immediately dropped tube in liquid nitrogen. Leave for 2 min.
vi. The tissue was ground to a fine powder using a sterile pestle, and the tube dropped back in liquid nitrogen.

Extraction process

1. Eppendorf tubes were removed from the liquid nitrogen and placed on ice. To each tube, 450µl of Buffer RLT was added. The mixture was vortexed vigorously and incubated at 56°C for 1-3min.

2. The lysate was transferred to a Qiashredder spin column (lilac) placed in a 2ml collection tube, and centrifuged at 13,000 rpm for 2min. The supernatant of the flow-through was carefully transferred into a new 1.5ml microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. This supernatant was now a clear lysate and was used in subsequent steps.

3. A 0.5 volume of absolute ethanol was added to the lysate, and immediately mixed by inversion.

4. The mixture from step 3 was transferred into an RNeasy spin column (pink) placed in a 2ml collection tube and centrifuged at max. speed for 15s. The flow-through was discarded.

5. Buffer RW1 (700µl) was added to the RNeasy spin column and centrifuged at max. speed for 15s to wash the spin column membrane. The flow-through was discarded.

6. Diluted Buffer RPE (500µl) was added to the RNeasy spin column and centrifuged at maximum speed for 15s to wash the spin column membrane. The flow-through was discarded.

7. The RNeasy spin column was placed in a new 2ml collection tube and centrifuged at max. speed for 1min to eliminate any trace of residual Buffer RPE.

8. The RNeasy spin column was placed in a new 1.5ml microcentrifuge tube. RNase-free water (30-50µl) was added directly to the spin column membrane and the tube centrigufed at max. speed for 1min to elute the RNA. Long term storage of eluted RNA was at -80°C.

Appendix VI. Composition of Master Mixes

AVI.1. Master Mix used in conventional PCR analyses

Component	Volume used (µl) in 1 reaction tube
RedTaq™ polymerase	7
Sterile H_2O	4
Forward primer (10pmol/µl)	1
Reverse primer (10pmol/µl)	1
DNA or H_2O	2

AVI.2. Master Mix used in Colony PCR analyses

Component	Volume used (µl) in 1 reaction tube
RedTaq™ polymerase	7
Forward primer (10pmol/µl)	1
Reverse primer (10pmol/µl)	1
Sterile water	6

AVI.3. Reaction components for RT-PCR analysis

Component	Volume/Reaction	Final concentration
Master Mix		
Rnase-free water	Variable	
5x One-step RT-PCR buffer	10 µl	1x
dNTP mix (10mM of each dNTP)	2 µl	400 µM each dNTP
Forward primer		0.6 µM
Reverse primer		0.6 µM
One-Step RT-PCR enzyme mix	2 µl	
Rnase inhibitor		5 - 10 units/reaction
Template RNA (350ng)	Variable	1pg - 2 µg/reaction
Total reaction volume	50 µl	

Appendix VII. General methods for gel electrophoresis

AVII.1. Preparation of 50X TAE buffer (pH 8.18 – 8.29)

To make up 1L of buffer solution, the following components were mixed:

242g of Tris-base

57.1 of glacial acetic acid

100ml of 0.5M EDTA (pH8)

Deionised H_2O to make the final volume to 1.0 L.

The buffer solution was sterilised by autoclaving at 121°C under 104 kPa for 20 min.

AVII.2. Gel preparation

1. The water bath was set at 55°C.
2. A solution of 1X TAE buffer (working solution) was prepared from a 50X TAE buffer (stock solution) by adding 20ml of stock solution to 1L of H_2O.
3. Agarose (1g) was weighed and melted in 100ml of 1X TAE buffer.
4. The molten agarose was cooled to approx. 55°C in the water bath.
5. The gel tray was sealed at both ends with adhesive tape, and combs were inserted in the appropriate slots.
6. The cooled agarose was transferred into a conical flask and 5µl of ethidium bromide (EtBr) was added.
7. The cooled agarose was poured onto the gel tray, and bubbles were removed using a pipette tip. The gel was allowed to set.
8. The tape from both ends of the tray was removed and the combs were gently lifted, so as not to tear any well in the gel. The tray was placed into the electrophoresis tank.
9. Sufficient 1X TAE buffer was added into the tank to cover the gel.