Genetic enhancement of pearl millet

by

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Date:

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The aim of this study was to establish a reliable protocol for the production of transgenic pearl millet as this will open new avenues for augmenting the gene pool of this crop. This was achieved by identifying a highly regenerable genotype and optimisation of a tissue culture system, and biolistic protocol for stable integration of selected transgenes. Both a negative, herbicide resistance selectable marker gene, *bar*, and a positive selectable marker gene, *man*A, were individually introduced in order to identify and establish a reliable transformation protocol. The optimised transformation protocol was then used to introduce an antifungal gene in the genome of pearl millet to enhance resistance to the biotrophic fungus *Sclerospora graminicola*. *S. graminicola*, an obligate oomycetous fungal phytopathogen, is the causal agent of downy mildew in pearl millet plants and a major constraint in the production of pearl millet. A single component of antifungal resistance was introduced into the genome of pearl millet, as preliminary work towards determining its role in the total plant defence system. The approach chosen was to introduce a hydrolytic enzyme, β-1,3-glucanase, from *Trichoderma atroviride* (formerly *T. harzianum*), a soil-borne filamentous fungus, capable of parasitizing several plant pathogenic fungi. It was anticipated that introducing this glucanase gene from *T. atroviride* which degrades glucan in the fungal cell walls, would significantly contribute to the improvement of resistance against downy mildew. Constructs were prepared containing the gene (*gluc*78) encoding a 78 kDa beta-1,3-glucanase. The constructs were prepared containing the *gluc*78 gene driven either by a strong constitutive promoter (ubiquitin promoter, exon and intron) or a wound inducible promoter, the potato proteinase inhibitor IIK gene promoter. The wound inducible promoter includes either an AMV leader sequence or the rice *Act*1 intron to obtain higher expression levels in the monocotyledonous plant. The transformation efficiency using the particle inflow gun and the herbicide resistance gene, *bar,* was improved from 0.02% on a MS based medium, to 0.19 or 0.72% with *man*A as selectable marker gene on MS or L3 based medium, respectively. However, individual experiments, introducing *man*A as selectable marker gene, resulted in frequencies of 1.2 and 3%. This translated to one transformation event per plate, which contains on average 31-35 pre-cultured immature zygotic embryos. This is the first report of the successful introduction and expression of a β-1,3-glucanase encoding gene from a biocontrol fungus not only under constitutive expression but also under wound inducible expression in a plant. Optimisation of genetic engineering of pearl millet, a cereal crop recalcitrant to transformation, and the introduction of an antifungal transgene, was accomplished in this study. Initial results hint that expression of this transgene enhances resistance to *S. graminicola*.

Genetiese verbetering van pêrel manna

Die doel van die studie was om 'n betroubare genetiese transformeringsprotokol vir pêrel manna te ontwikkel. Hiervoor moes eerstens 'n regenereerbare genotipe geidentifiseer word. Twedens moes 'n betroubare weefselkultuur en biolistiese transformeringssisteem ontwikkel word. Beide die onkruiddoder bestandheidsgeen, *bar*, en 'n positiewe selektiewe geen, *man*A, is vir die doel van die projek onafhanklik in die genoom van pêrel manna in gekloneer. Die optimale sisteem is vervolgens aangewend om 'n geen wat potensieël verbeterde bestandheid teen die biotrofiese swam *Sclerospora graminicola* wat donsige meeldou by plante veroorsaak, in pêrel manna in te kloneer. 'n Enkele komponent van bestandheid is in die genetiese material van pêrel manna in gekloneer as inleidende werk om die rol van hierdie geen in die totale verdedigingsisteem te bepaal. Die benadering wat gekies was, behels die klonering van 'n hidrolitiese ensiem β-1,3-glukanase, van *Trichoderma atroviride* (voorheen *T. harzianum*), 'n grondgedraagde swam, wat op 'n aantal ander plantpatogene fungus kan parasiteer. Die verwagting is dat klonering van hierdie β-1,3-glukanase geen van *T. atroviride* wat die glukaan verteer in die selwande van swamme, 'n groot verbetering tot die bestandheid teen donsige meeldou sal meebring. Konstrukte is voorberei wat die *gluc*78 geen bevat wat kodeer vir die 78 kDa beta-1,3-glukanase protein. Die konstrukte wat voorberei is bevat die *gluc*78 geen geinduseer deur of 'n sterk konstituwe promoter (ubiquitin promoter, exon en intron) of deur 'n wond geinduseerde promoter, die aartappel proteïnase inhibeerder IIK geen promoter. Hierdie promoter word gevolg deur of 'n AMV leier volgorde of die rys *Act*1 intron om verhoogde uitdruk vlakke in monokotiele plante te verseker. As die partikel invloei geweer in kombinasie met die onkruiddoderbestandheidsgeen gebruik word, was die doeltreffendheid van transformasie 0.02% op 'n MS gebasseerde groeimedium. 'n Transformasie doeltreffendheid van onderskeidelik 0.19 en 0.72% is verkry wanneer die *man*A as selektiewe geen gebruik is op MS of L3 gebasseerde medium. Twee individual eksperimente, waar die *man*A geen as selektiewe geen gebruik is, het gelei tot 'n transformasie doeltreffendheid van 1.2 of 3%. Dit gee 'n gemiddelde van een transformasie per plaat wat 31 tot 35 voorafgekweekte onvolwasse embrios bevat. Hierdie is die eerste verslag van die suksesvolle klonering en uitdrukking van 'n β-1,3-glukanase koderende geen van 'n swam wat as 'n biologiese beheeragent gebruik word. Hierdie is nie alleenlik onder konstitutiewe uitdrukking nie, maar ook onder wond geinduseerde uitdruk in 'n plant. In hierdie studie is die optimisering van genetiese verbetering van pêrel manna, 'n graan gewas wat gehard is teen transformasie, deur die klonering van 'n bestandheidsgeen in die genoom van hierdie gewas gedoen. Aanvanklike resultate dui daarop dat die uitdruk van hierdie geen lei tot verbeterde bestandheid teen *S. graminicola*.

Worthy are You, our Lord and our God,
to receive glory and honor and power;
for You created all things,
and because of Your will they existed,
and were created"

Revelation 4:11

PREFACE

This dissertation is presented as a compilation of 7 chapters, with each chapter being introduced separately.

Chapter 1: Introduction

Chapter 2: Literature review

Chapter 3: Improved regeneration efficiency of a pearl millet (*Pennisetum glaucum* [L.] R.Br.) breeding line

Chapter 4: A protocol to produce transgenic fertile pearl millet (*Pennisetum glaucum* [L.] R.Br.) plants using the particle inflow gun

Chapter 5: Pearl millet (*Pennisetum glaucum* [L.] R.Br.) transformation system using the positive selectable marker gene phosphomannose isomerase

Chapter 6: Genetic enhancement of pearl millet (*Pennisetum glaucum* [L.] R.Br.) for downy mildew resistance

Chapter 7: Concluding remarks and future prospects

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CHAPTER 5

PEARL MILLET (*PENNISETUM GLAUCUM* [L.] R.BR.) TRANSFORMATION SYSTEM USING THE POSITIVE SELECTABLE MARKER GENE PHOSPHOMANNOSE ISOMERASE

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CHAPTER 6

GENETIC ENHANCEMENT OF PEARL MILLET (*PENNISETUM GLAUCUM* [L.] R.Br.) FOR DOWNY MILDEW RESISTANCE

Chapter 1

INTRODUCTION

Genetic engineering of pearl millet – a jewel of Africa

"We are talking about a crop that is virtually unimprovable – a crop that grows where not even weeds can survive. A crop that has been improved by farmers and through natural selection for thousands of years. A crop that produces nourishment from the poorest soils in the driest regions in the hottest climates. A crop that grows straight out of sand dunes. A crop that survives sand storms and flash floods"

"http://www.africancrops.net"

The semi-arid tropics are characterised by unpredictable weather, limited and erratic rainfall and nutrient-poor soils and suffer from a host agricultural constraints (Sharma and Ortiz, 2000). Pearl millet (*Pennisetum glaucum* [L.] R. Br.) is very important for food security in some of the world's hottest, driest cultivated areas and has been a staple food in Africa and India for millennia (http://www.casz.bangor.ac.uk). Pearl millet is hardy, drought-tolerant and more suitable for semi-arid environments than maize or wheat. Nevertheless, downy mildew (*Sclerospora graminicola*), an obligate biotrophic oomycetous fungal phytopathogen, is a widespread and destructive disease of pearl millet in India and in African countries (Singh and Talukdar, 1998) and losses up to 30% amounting to 260 million US dollars are reported (Singh *et al.*, 1993; Shetty and Kumar, 2000). The most important applications of biotechnology for plant protection amongst the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) mandate crops, especially in Africa, include downy mildew in pearl millet (Sharma and Ortiz, 2000).

The available gene pool of pearl millet with traditional breeding methods is restricted by the sexual incompatibility of many interspecific and intergeneric crosses. Genetic manipulation and *in vitro* culture provide a means for the gene pool to be broadened by allowing transfer of specific genes controlling well-defined traits from one organism to another. Furthermore, as chemical reagents for pathogen resistance control are removed from the market due to their toxicity, genetically engineered crops resistant to various fungal pathogens represent an environmental friendly alternative.

In spite of the vast amount of knowledge, the complexity of plant defence response mechanisms against fungal invasion (McDowell and Woffenden, 2003; Somssich and Hahlbrock, 1998), the rapid development of new virulent forms of phytopathogens (Johnson,

2000; Kamoun *et al.*, 1999) and the failure to produce the desired gene product at the expected level in the transgenic plant hindered the release of fungal resistant crops reaching the market to date.

AIM

The aim of this study was to establish a reliable protocol for the production of transgenic pearl millet as this will open new avenues for augmenting the gene pool of this crop regarded as a jewel of Africa. Secondary, it was anticipated that a beta-1,3-glucanase gene from a biocontrol fungus, *Trichoderma atroviride* (formerly *T.harzianum*) would confer enhanced resistance against the oomycetous fungal phytopathogen *Sclerospora graminicola*, the causal agent of downy mildew in pearl millet.

The development of a reliable transformation protocol for pearl millet would form the basis for future genetic enhancement of this crop by complimenting classical breeding programmes for the benefit of India and sub-Saharan Africa. As a first step towards a routine transformation system, it was essential to establish a reliable and highly efficient regeneration system for selected pearl millet breeding lines. It was anticipated that improved regeneration capacity for selected African pearl millet breeding line(s) would underpin the development of a reliable transformation system. I*n vitro* culture of cereals shows strong genotype dependence and production of the appropriate culture is generally limited to selected genotypes. Therefore, eight pearl millet genotypes were assessed for their tissue culture amenability. Genotype purity and cultivation in Africa, or potential use in African breeding programmes, were considered in the initial selection of the genotypes. Both immature zygotic embryos and leaf base segments were screened to identify the most suitable explant source to initiate embryogenic tissue from which fertile plants could be regenerated. One of the highly regenerable genotypes, 842B, was assessed further on various tissue culture media. The MS (Murashige and Skoog, 1962) and L3 (Jähne *et al.*, 1991) based media were chosen in this study, for the following reasons. The MS based medium as described by Pinard and Chandrapalaiah (1991) was successfully used before in our laboratory to induce regenerable calli from pearl millet immature zygotic embryos. The L3 based medium was previously shown to be suitable for callus induction and regeneration of recalcitrant cultures such as microspores of cereals (Mordhorst and Lörz, 1993) and was successfully used to establish a highly efficient regeneration system for oat plants (Gless *et al.*, 1998). This study reports on the efficient induction of a highly embryogenic culture, derived from cultured immature zygotic embryos, as a direct effect of the addition of L-proline and subsequent production of numerous somatic embryos that regenerated to form fertile pearl millet plants. Previous papers reporting on pearl millet tissue culture regenerability rarely quantified the regeneration ability of their systems, which defeats comparison. In this study, the regenerability of pearl

millet was expressed as the number of regenerants per explant and the highest number obtained were 80 regenerants per immature zygotic embryo explant.

A transformation protocol was established using the herbicide resistance selectable marker gene, *bar*, and the particle inflow gun (PIG). However, the transformation efficiency obtained was very low (0.02%). Subsequently, the mannose selection system (POSITECH, Syngenta), using the *manA* as selectable marker gene, was used as an alternative. The system employs the phosphomannose isomerase (PMI) expressing gene (*man*A) as selectable marker gene and mannose, converted to mannose-6-phosphate by endogenous hexokinase, as selective agent. The mannose positive selection system favours regeneration and growth of the transgenic cells while the non-transgenic cells are starved but not killed. Therefore, untransformed tissue is separated from transgenic tissue by carbohydrate starvation of the untransformed cells. In this study, the positive mannose selectable marker gene technology 1) limited the number of escapes obtained with the *bar* as selectable marker gene using the PIG, 2) improved the transformation efficiency and 3) avoided usage of antibiotic or herbicide resistant genes as selectable marker genes in pearl millet transformation.

A single antifungal gene was introduced into the genome of pearl millet in order to elucidate the potential resistance conferred by a single antifungal component to the oomycete phytopathogen *Sclerospora graminicola*, causal agent of downy mildew in pearl millet. Stable introduction and expression of the gene, *gluc78*, encoding the 78 kDa β-1,3-glucanase enzyme from *T. atroviride* is potentially the *Bt* solution for oomycetous fungal pathogens for the following reasons: 1) *T. atroviride* is the best studied biocontrol fungus, which developed specifically to attack other fungi but not plants and is therefore a potential source of powerful antifungal genes, 2) fungal cell wall degrading enzymes from *T. atroviride* is 100 times more active than the corresponding plant enzymes, 3) a previous study showed that a 78 kDa β-1,3-glucanase enzyme from *T. atroviride* exhibited potent antifungal activity to the oomycetous fungal pathogen *Phytophthora* (Fogliano *et al.*, 2002), 4) a soybean β-1,3-endoglucanase in transgenic tobacco increased resistance to *Phytophthora infestans* and 5) a β-1,3-endoglucanase is constitutively expressed in pearl millet cultivars resistant to *S. graminicola.* It is therefore anticipated that introducing this single glucanase gene from the biocontrol fungus *T. atroviride* which degrades glucan in the fungal cell walls, would significantly contribute to the improvement of resistance against downy mildew.

Since the fungal cell wall degrading enzymes of *Trichoderma* is non-toxic to plants even at high concentrations, the *gluc78* gene was placed not only down stream of the wound inducible promoter, the potato proteinase inhibitor IIK gene promoter, but also down stream

of the maize ubiquitin constitutive promoter. Introduction of the *gluc78* transgene under the control of the strong constitutive promoter will provide immediate protection against pathogen ingress, and thereby give the plant an advantage over the pathogen during the early stages of infection, but it may also deplete valuable energy resources of the plant for continual expression of the protein. In contrast, the wound inducible potato proteinase inhibitor II gene (*pin2*) promoter is thought to decrease the investment of cellular metabolic energy and building blocks in transgenic plants.

In this study, the gene from *Trichoderma* encoding a β-1,3-glucanase was chosen as there is no chitin in the cell walls of oomycete phytopathogens (Ait-Lahsen *et al.*, 2001). It is not excluded that the simultaneous introduction of the genes encoding chitinase and β-glucanase would synergistically improve the fungal resistance as the combinations can lead to the onset of systemic acquired resistance (SAR).

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Chapter 2

LITERATURE REVIEW

Pearl millet - a jewel of Africa

The focus of this literature review is to highlight recombinant DNA technology as a potential tool to enhance the gene pool of pearl millet, a crop regarded as a jewel of Africa. It is not my intention in this literature review to summarise development of major cereal crop transformation technology for barley, wheat, maize and rice as these crops are extensively reviewed (Komari *et al.*, 1998; Lemaux *et al.*, 1999; Rakszegi *et al.*, 2001; Repellin *et al.*, 2001; Wisniewski *et al.*, 2002). Rather the focus will be on transformation technology developed for previously neglected, high priority cereal crops for Africa such as sorghum and pearl millet. It is also not my intention to review plant pathogen defence mechanisms, but to focus on mechanisms with the potential to be of immediate relevance to pearl millet resistance against oomycete pathogens and in particular *Sclerospora graminicola*.

2.1 The origin, production and nutritional value of pearl millet

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) is descended from wild grasses native to the central Saharan plateau region of Niger, from where it spread to east Africa and India, and currently ranks as the fourth most important cereal (after rice, wheat and sorghum) (http://www.africancrops.net). Developing countries, mainly in Asia and Africa, account for about 94 percent of global output in millet production. However, pearl millet is also extensively used as a summer annual grazing crop in the southern United States and tropical and subtropical regions of the world (Goldman *et al.*, 2003). In 1998, millet was sixth in production amongst all cereals worldwide with 29 million metric tons harvested on 25 million ha, an area bigger than that used for wheat production in the USA (FAOSTAT http://apps.fao.org/ 1998). Five countries in West Africa (Nigeria, Niger, Mali, Burkina Faso and Senegal) produce 85% of the continent's total pearl millet crop. Almost all millet is produced by small-scale farmers for household consumption and localized trade.

In West Africa, pearl millet is consumed primarily as a thick porridge, or *toh*, but it is also milled into flour to prepare breads and cakes (http://www.africancrops.net). Pearl millet is the most-preferred cereal grain grown in Sahelian countries, Senegal, Mali, Niger and Burkina Faso, and is consumed in preference to sorghum. In northern Nigeria, pearl millet is used in making a popular fried cake known as "masa". Roasted young ears are a popular food for children.

Feeding trials conducted in India have shown that millet is nutritionally superior for human growth to maize and rice (http://www.africancrops.net). It has slightly higher protein content

(average of 16%) than maize and roughly twice the fat content (5-7%) of most maize varieties, and is particularly high in calcium and iron. It has lower levels of fibre and most vitamins, although its vitamin A content is relatively high.

2.2 Disease susceptibility of pearl millet

S. graminicola an obligate, oomycetous fungus causal agent of downy mildew in pearl millet is a major constraint in the production of pearl millet. *S. graminicola* was first reported in India by Butler (1907). However, it became an important disease in 1971 when Tift 23A, a male sterile line from Georgia, USA, became susceptible (Chahal *et al.*, 1994). In 1973, the disease devastated the most popular hybrid, HB-3 that was grown on more than one million ha in India and caused an estimated loss of 10-45% (Safeeulla, 1977).

Pearl millet is the natural host plant of *S. graminicola,* but it has been reported on different graminaceous hosts such as maize, sorghum and sugarcane (http://cygnus.tamu.edu/PLPA/Projects/1/sclerospora_graminicola.html). The members of the class oomycetes produce, with few exceptions, bi-flagellated zoospores with one tinsel flagellum directed forward and one whiplash flagellum directed backward. Characteristic features, which distinguish these fungi from other fungi, include a cell wall, which contains cellulose rather than chitin and a gametic (rather than zygotic) lifecycle (http://cygnus.tamu.edu/PLPA/Projects/1/oomycetes.html).

The life cycle of *S. graminicola* comprises both sexual and asexual phases. The sexual stage produces oospores that become soil- or seed borne, thus providing the primary source of inocula each season. Seedlings growing in soil infested with oospores, become systemically infected and show chlorosis. Sporangia require water to germinate and maximum germination occurs at 22-25˚C (Chahal *et al.*, 1994). Typical symptoms of infection with *S. graminicola* are stunted growth, chlorosis, downy growth of asexual spores on the adaxial surface of the leaves and malformed ear heads (Chahal *et al.*, 1994).

Management of pearl millet downy mildew by chemicals is neither economical nor eco-friendly and hence use of more tolerant cultivars is being practised routinely. Furthermore, induction of systemic acquired resistance (SAR) by exposing the plants to suboptimal levels of *S. graminicola* inoculum (Kumar *et al.*, 1993 and 1998) and biocontrol by combinations of antagonistic fungi and bacterial species (*Trichoderma*, *Chaetomium*, *Bacillus subtilis* and *Pseudomonas fluorescence*) (Shetty and Kumar, 2000) are currently practised to protect pearl millet from invading *S. graminicola*.

A clear understanding of the biochemical basis of pearl millet resistance to downy mildew is essential to obtain a complete picture of pearl millet-downy mildew interactions. Earlier studies identified the involvement of lytic activity, β-1,3-glucanase, arachidonic acid, ribonucleases, manganese, superoxide dismutase and peroxidases in pearl millet-downy mildew interaction as increased activity in highly resistant (constitutive or inducible resistant cultivars) seedlings and a decrease in enzyme activity in highly susceptible seedlings (Babitha *et al.*, 2002; Kini *et al.*, 2000 a, b; Shetty *et al.*, 2001; Shivakumar *et al.*, 2000, 2003; Umesha *et al.*, 2000). Singh and Talukdar (1998) reported on a pearl millet parental line with complete resistance to downy mildew, but only the years to come will confirm the long-term resistance. Even though many cultivars are resistant to the disease, the resistance is not durable and often there is breakdown of resistance in the cultivars. The exact reasons for the breakdown of resistance are not clearly known, as there is a lack of complete understanding of the biochemical basis of resistance of pearl millet to downy mildew.

2.3 Cereal transformation technology

Recombinant DNA technology has significantly augmented the conventional crop improvement and offers great promise to assist plant breeders to meet the increased food demand predicted for the 21st century (Sharma *et al.*, 2002). Monocotyledonous plants, especially the grasses, play a crucial role in the agricultural economy of all nations, and their biotechnological manipulation offers great potential for both developed and developing countries (Iyer *et al.*, 2000). Genes of agronomic importance such as those that confer resistance to insects, soil salinity, aluminium toxicity and environmental stress and as well as nutritional quality and yield improvement have been identified, isolated, inserted and expressed effectively in various dicotyledonous crops (Dunwell, 2000; Hilder and Boulter, 1999; Sharma *et al.*, 2000 and 2002). Efficient transformation systems, both biolistic- and *Agrobacterium*-mediated transformation, for important cereal crops such as maize (Armstrong and Songstad, 1993; Ishida *et al.*, 1996), wheat (Weeks *et al.*, 1993; Cheng *et al.*, 1997), rice (Christou *et al.*, 1991; Chan *et al.*, 1992; Hiei *et al.*, 1994), and barley (Wan and Lemaux, 1994; Tingay *et al.*, 1997), respectively, have more recently been established. Unfortunately, most cereal agricultural crops that have been genetically improved with genes of relevance are temperate crops with high commercial value. The first *Bt* maize trial was already published in 1993 (Koziel *et al.*, 1993) and subsequently various GM maize products have penetrated the commercial market (Brandt, 2003; Dunwell, 2000). It is interesting to note that most of the groundwork for cereal transformation was done in sorghum (*Sorghum bicolor* L. Moench) and pearl millet. The first cereal embryogenic *in vitro* culture systems were established for sorghum (Gamborg *et al.*, 1977) and pearl millet (Vasil and Vasil, 1981). Furthermore, the first report on optimisation of transient expression of the reporter gene *uid*A (GUS) in scutellum cells of cultured immature zygotic embryos, following microprojectile

bombardment, was published for pearl millet (Taylor and Vasil, 1991). Nevertheless, no commercial transgenic sorghum or pearl millet product has reached the market. It is clear that the pearl millet transformation technology lags behind that of the major cereal crops probably because of the low market value, which did not attract company investments for research support. Private sector research is unlikely to take on research focussed on the problems of small farmers in developing countries, given the uncertainty of future profits in these areas (Sharma *et al.*, 2002). It is rarely the case that large foundations or institutes such as the Rockefeller foundation and the Swiss Federal Institute of Technology support the development of a project such as the "golden rice" project for humanitarian purposes. Another example would be that during 2001, the CGIAR investment in millet research was approximately US $5.9 million (http://www.cgiar.org). This represents about 2.0 percent of the total CGIAR commodity investment.

It is critically important to focus on crops relevant to the small farm holders and poor consumers in the developing countries of the humid and semi-arid tropics as 73 million people are added annually, of which 97% live in developing countries (Sharma *et al.*, 2002). Furthermore, grain sorghum and pearl millet, indigenous to Africa, are favoured above maize in many parts of Africa and, are becoming increasingly important staple foods in Africa in the face of growing food scarcity and several prolonged droughts. Increasing economical importance of sorghum and pearl millet necessitates the development of transformation technology to genetically enhance these crops especially against fungal diseases.

2.4 Disease resistance – Transgenic plants an alternative?

One of the major challenges facing modern agriculture is to achieve a satisfactory, but environmentally friendly, control of plant diseases. The extensive use of chemicals remains the main strategy of disease control, but a variety of alternative approaches have been considered which include the use of biocontrol agents and organisms and pathogen resistant crop cultivars. Classical breeding has been very useful in producing some resistant varieties (for review see Johnson, 2000), but has no access to resistance available in sexually incompatible species. Although no single resistance genotype and no single genetic basis for durable resistance exist (Johnson, 2000), a combination of genetic engineering and classical breeding complimenting one another, are anticipated to be the future for a holistic horizontal crop improvement strategy.

Disease resistance in plants is associated with the activation of a wide variety of defence responses that serve to prevent pathogen infection. Plants can activate a very effective arsenal of inducible defence responses, comprised of genetically programmed suicide of infected cells (the hypersensitive response, HR), tissue reinforcement (lignin, callose), the

production of fungal cell wall degrading enzymes and antimicrobial metabolites (phytoalexins) at the site of infection. These local responses can trigger a long lasting systemic response (systemic acquired resistance, SAR) that primes the plant for resistance against a broad spectrum of pathogens. This multicomponent complimentary response requires a substantial commitment of cellular resources, including extensive genetic reprogramming and metabolic re-allocation (Somssich and Hahlbrock, 1998).

Various approaches can be followed to confer enhanced resistance to pathogens in crops but long term durable broadspectrum resistance must be the ultimate goal. Plant disease resistance genes (*R* genes), such as the RPP8 (Cooley *et al.*, 2000) and the RPP13 (Bittner-Eddy *et al.*, 2000) genes, offer several attractive features for disease control (McDowell and Woffenden, 2003; Rommens and Kishore, 2000) even against oomycete pathogens. Unfortunately, only a limited number of pathogens are recognised by the *R* genes, it does not provide broad-spectrum resistance and are often overcome by novel developing pathogens. Overproduction of salicylic acid (SA) by bacterial transgenes (Verberne *et al.*, 2000) or the expression of *NPR*1 from *Arabidopsis thaliana* (Cao *et al.*, 1998) were also shown to have enhanced pathogen resistance in plants, but trigger only those defence responses that are SA or *NPR*-1 dependent (Shah *et al.*, 2001). Another approach would be constitutive or fungal induced expression of genes encoding naturally occurring antimicrobial peptides, plant defensins (Gao *et al.*, 2000; Lai *et al.*, 2002; for review see Thomma *et al.*, 2002) in pearl millet. Although evidence of broad host-range antifungal activity was reported for pea defensins, greenhouse and especially field trials need to be conducted to fully test the potential usefulness of these genes in broad-spectrum durable resistance. Plant defence mechanisms are however not functioning in isolation with its environment. Lorito and co-workers have shown synergistic interaction between a number of cell wall degrading enzymes and cell membrane affecting compounds (from various microbial organisms) that alter cell membrane structure or permeability of phytopathogenic fungi (*Botrytis cinerea* and *Fusarium oxysporum*) and bacteria (mainly Gram-positive) (Lorito *et al.*, 1996; Schirmböck *et al.*, 1994).

One of the prominent mechanisms of disease resistance in plants, and one that we will focus on in this study, entails inducible defence responses in the form of induction of defence-related enzymes that become activated upon pathogen infection (for review see Datta et al., 1999). Tightly correlated with the appearance of HR and SAR is the accumulation of salicylic acid (SA) and the expression of a subset of these pathogenesis-related (*PR*) genes, some of which encode proteins with antimicrobial activities.

2.4.1 Fungal cell wall degrading enzymes as defence mechanism

The production of enzymes capable of degrading the cell walls of invading phytopathogenic fungi is an important component of the defence response of plants. Glucans and chitin are the major constituents of the cell walls of various phytopathogenic fungi (Datta *et al*, 1999). However, β-1,3-glucans are the major components of the cells walls of oomycetes, such as *S. graminicola*, a group of fungi that do not contain chitin (Yoshikawa *et al.*, 1993). β-Glucanases were proposed to be involved in plant defense as early as 1970 (Abeles *et al.*, 1970). Chitinase and β-glucanase are thought to kill the fungus by thinning the cell wall at the hyphal tip by degrading chitin and β-glucan, which subsequently causes swelling and ultimate bursting and death of the hyphal tip (Simmons, 1994) or by promoting the release of fungal cell-wall derived oligosaccharides that can act as elicitors of defense reactions (Leubner-Metzger and Meins, 1999). In addition, β-1,3-glucanase appears to be a key enzyme involved in the generation of phytoalexin elicitor-releasing signals leading to active disease resistance (Yoshikawa *et al.*, 1993).

There are many reports of the induction of β-1,3-glucanase expression during fungal pathogen attack and the positive correlation to the level of pathogen resistance (see Simmons, 1994 for a summary of data older than 1994). It is however important to notice that they are mainly acidic and secreted (extracellular). The basic pI 1,3-β-glucanases and monocot 1,3;1,4-glucanases (Nishizawa *et al.*, 2003) , located in the vacuole (intracellular), are involved in many other important aspects of plant physiology and development, such as endosperm degradation during kernel germination, vegetative cell wall elongation, growth, flowering, cellular and tissue development and differentiation (Leubner-Metzger and Meins, 1999; Noronha *et al.*, 2000; Simmons, 1994; Tsabary *et al.*, 2003).

2.4.2 Evidence of β-glucanases in plants elicited by fungal pathogens: A defence response?

The presence of acidic and basic β-1,3-glucanases associated with fungal infection and defence is well documented for plants. Gheong *et al.* (2000) isolated and characterised a soybean gene (SGN1) encoding a basic beta-1,3-glucanase that is a plant class III isoform of β-1,3-glucanase. Expression of the SGN1 was strongly induced by a variety of defense-related signals, such as treatment with H_2O_2, wounding, or treatment with fungal elicitor prepared *Phytophthora* spp as well as inoculation with *Pseudomonas syringae*. However, the expression level of SGN1 was hardly induced with jasmonate, ethephon and salicylate. Yi and Hwang (1997) purified a basic beta-1,3-endoglucanse (34 kDa) from soybean hypocotyls infected by an incompatible race of *Phytophthora sojae*. The beta-1,3-endoglucanase inhibited spore germination and hyphal growth of the chitin-negative fungus *Phytophthora* sojae, but did not show any antifungal activity against the chitin-containing fungi *Alternaria*

mali, *Colletotrichum gloeosporioides* and *Magnaporthe grisea*. Ji *et al.* (2000) found a 33 kDa isoform of β-1,3-glucanases in maturing maize kernels associated with *Aspergillus flavus* fungal infection. A basic β-1,3-glucanase cDNA clone was isolated from the cDNA library constructed from hypersensitive response lesions of pepper leaves infected with avirulent strain of *Xanthomonas campestris* (Jung and Hwang, 2000).

It is, however, invalid to conclude that increased β-glucanase expression necessarily causes increased fungal resistance. It is, however, likely that β-1,3-endoglucanases offer protection against pathogens through their capacity to hydrolyse the branched (1→3, 1→6) β-glucans that are commonly found in fungal cell walls (Kini *et al.*, 2000a, b). *In vitro* studies showing that chitinase and β-1,3-glucanase leads to enhanced mycelial cell wall disruption leading to efficient cellular lysis of zoospores and hyphae (Kim and Hwang, 1997; Lorito *et al.*, 1993).

2.4.3 β-glucanase elicited by oomycete fungal infection

The level and onset of β-glucanase expression often is positively correlated to the level of pathogen resistance. The work of Kini and co-workers (Kini *et al.*, 2000 a, b) is particularly of interest as it specifically report on the accumulation of β-1,3-glucanase in resistant cultivars of pearl millet seedlings upon inoculation with the downy mildew causing pathogen *S. graminicola*. Not all the isoforms of β-1,3-glucanases identified in the study of Kini and co-workers were associated with resistance of pearl millet to *S. graminicola* infection, which coincide with previous studies. The basic isoform of pI 9.6 has probably a developmental role as it was present in both resistant and susceptible cultivars of pearl millet before and after inoculation. In contrast, the acidic isoform of β-1,3-glucanase (pI 6.2) was unique to resistant cultivars and strongly induced after inoculation with *S. graminicola*. A 30 kDa isoform, which appears to be related to the isoform pI 6.2, was prominently detected by an 20.5 kDa isoform antiserum, in the resistant cultivars but absent in the control and inoculated seedlings of susceptible cultivars (Kini *et al.*, 2000b). The data clearly indicate that particular β-1,3-glucanases are involved in the resistance of pearl millet against the downy mildew pathogen.

2.4.4 Constitutive expression of β-glucanases from various sources in plants

In the past twenty years, the avalanche of knowledge generated and the tremendous advancement in technologies for identification, isolation and transfer of genes amongst species have enabled the expression of traits for disease resistance in plants with minimal effect on their intrinsic properties. Genes with antifungal properties were and are currently identified and isolated from various sources such as plants, viruses, bacteria, yeast and fungi that have potential use for plants. These genes were, introduced into the genome of the plant and successfully expressed in selected crops. Recent studies have shown that ectopic

expression of chitinase and β-1,3-glucanase in transgenic plants can mediate increased protection against phytopathogenic fungi.

Combinatorial expression of transgenes encoding proteins with antifungal properties has been reported to enhance and provide synergistic *in planta* protection (Anand *et al.*, 2003; Jach *et al.*, 1995; Lan *et al.*, 2000; Nishizawa *et al.*, 2003; Zhu *et al.*, 1994). A combination of class-II chitinase, a class-II beta-1,3-glucanase and a Type-I ribosome-inactivating protein (RIP) were expressed in tobacco plants under the control of the constitutive CaMV 35S-promoter (Jach *et al.*, 1995), resulting in 20-60% reduction in disease index against the soilborn fungal pathogen *Rhizoctonia solani,* when compared to wild type or empty vector transgenic plants. In another study, constitutive co-expression of a basic chitinase (rice RCH10) and acidic β–1,3-glucanase (alfalfa AGLU1), severely suppressed the reduction of frogeye lesion size and the number of leaves infected by *Cercospora nicotianae* in T_2 progeny heterozygous for both transgenes, and even more so for the T_3 double homozygous combination, relative to the plants homozygous for either transgene alone (Zhu *et al.*, 1994). Similarly, fungal challenge of transgenic oilseed rape plants constitutively expressing the beta-1,3-glucanase and chitinase genes, showed decreased susceptibility to *Sclerotinia sclerotiorium* compared to untransformed control plants (Lan *et al.*, 2000). Unfortunately, the paper is published in Chinese and the origin of the beta-1,3-glucanase gene and percentage inhibition is not known. These data suggests that the battery of chitinases and glucanases are not redundant defence systems but represent complimentary, interacting protective mechanisms.

No single plant gene encoding β-1,3-glucanase confer sufficient or full protection against oomycete pathogens (Yoshikawa *et al.*, 1993; Masoud *et al.*, 1996; Borowska *et al.*, 1998), but these studies indicate that constitutive expression of high levels of glucanase is an effective strategy for engineering protection against oomycete pathogens. Transgenic potato plants expressing soybean β-1,3-endoglucanase, with up to eight fold higher activity to control plants, resulted in a reduction in sporangia production of the oomycetous pathogen *Phytophthora infestans* (Borkowska *et al.*, 1998). Masoud and co-workers (1996) found that constitutive overexpression of the alfalfa β-1,3-glucanase protein encoded by the chimaeric *Aglu*1 gene, in transgenic alfalfa, did not lead to co-suppression of the endogenous gene, but resulted in improved disease severity where the transgene was expressed up to ten fold higher than control plants. No reduction in disease severity was obtained with the lower level of expression of the *Aglu*1 gene in transgenic plants harbouring the tandem glucanase/chitinase transgenes. However, this may be a result of low levels of expression of both transgenes and not combinatorial expression itself. Furthermore, Yoshikawa *et al.* (1993) showed that transgenic tobacco plants, expressing a soybean β-1,3-glucanase,

showed almost no disease symptom in comparison to the untransformed control plants when challenged with a diverse group of fungi including the oomycete fungus *Phytophthora parasitica* var nicotianae.

Nishizawa and co-workers (2003) constitutively over-expressed the 1,3;1,4-glucanase encoding *Gns* 1 gene of rice which resulted in a stunted lesion-mimic phenotype which simultaneously express the pathogen related genes PR-1 an PBZ1. This is the first report on a 1,3;1,4-glucanase, which hyrolyzes 1,3;1,4-glucosidic linkages on 1,3;1,4-glucan, an important component of cell walls in the Poaceae plant family, playing a significant role in defence resistance as the enzyme degrades glucan in the plant cell wall and not the virulent blast fungus (*Magnaporthe grisea*) fungal cell walls.

Lorito and Scala (1999) published an extensive review of microbial genes expressed in plants to enhance defence response to pathogens or to activate plant defence responses or protect from pathogen toxins. However, this review publication identified only *Ruminococcus flavefaciens*, *Bacillus amyloliquefaciens* and *Bacillus macerans* bacterial β-1,3-glucanases which are expressed in plants for purposes other than fungal resistance e.g. the engineered thermotolerant (1,3-1,4)-β-glucanase which was introduced to improve malting qualities of transgenic barley (Jensen *et al.*, 1996) and transgenic plants as bioreactors for the production of industrial enzymes (Herbers *et al.*, 1996).

It is clear then that in the past only plant β-glucanases were introduced into the genome of plants to confer resistance to fungal phytopathogens. A novel and attractive approach would therefore be to introduce a gene encoding β-1,3-glucanase from a biocontrol fungi, such as *T. atroviride* (=*T. harzianum* P1), to confer broadspectrum resistance to oomycete pathogens.

2.4.5 β-glucanases and chitinases from *T. atroviride*

T. atroviride is a well-known biological control agent, which is regularly used in combination with *Bacillus* spp. to combat *S. graminicola*, causal agent of downy mildew in pearl millet (Shetty and Kumar, 2000). *T. atroviride*, a soil-borne filamentous fungus, is capable of parasitising several plant pathogenic fungi and therefore a potential source of powerful antifungal genes (for review see Markovich and Kononova, 2003). Secretion of lytic enzymes, mainly glucanases and chitinases, is considered the most crucial step of the mycoparasitic process (Cohen-Kupiec *et al.*, 1999). The lytic enzymes degrade the cell walls of the pathogenic fungi, enabling *Trichoderma* to utilize both their cell walls and cellular contents for nutrition. Purified enzymes from *T. atroviride* which have an antagonistic and a nutritional role, are strong inhibitors of many important plant pathogens and are able to lyse

not only the "soft" structure of the hyphal tip but also the "hard" chitin wall of mature hyphae, conidia, chlamydospores and sclerotia (Lorito *et al.*, 1998). These enzymes are substantially more antifungal than chitinolytic and glucanolytic enzymes purified thus far from any other source when assayed under the same conditions and up to one hundred times more active than the corresponding plant enzymes and effective on a much wider range of pathogens (Lorito *et al.*, 1998). These enzymes are also non-toxic to plants even at high concentrations. Finally, the antifungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes are used in combination with pant pathogenesis-related proteins, commercial fungicides, cell-membrane-affecting toxins, peptide antibiotics (including peptaibols) or biocontrol bacteria (Lorito *et al.*, 1993, 1994 and 1998; Markovich and Kononova, 2003).

Chitinolytic enzymes produced by *T. atroviride*, especially combining endochitinase and the chitobiosidase, was shown to be potent *in vitro* against phytopathogens such as *Botrytis cinerea* and *Fusarium solani* (ED_{50} values of 10 and 30 µg ml^{-1}, respectively), but it did not inhibit the sporangia germination nor mycelial growth of the Oomycete *Pythium ultimum* (Lorito *et al.*, 1993). In addition, adding a glucan 1,3- β-glucosidase (EC 3.2.1.58) and an N-acetyl- β-glucosaminidase (EC 3.2.1.30) to the two above mentioned chitinolytic enzymes gave ED_{50} (50% effective dose) values as low as 1.6 µg ml^{-1} for inhibition of conidial germination and 1.7 µg ml^{-1} for inhibition of germ tube elongation of the surviving spores of *B. cinerea* (Lorito *et al.*, 1994). Although chitinolytic enzymes produced by *T. atroviride* was potent against phytopathogens such as *Botrytis cinerea* and *Fusarium solani*, it did not inhibit the Oomycete *Pythium ultimum* (Lorito *et al.*, 1993). Neither sporangia germination nor mycelial growth was affected, even when the fungus was exposed to 1000 µg ml^{-1}. Another example of the powerful enzymes originating from *Trichoderma* was reported by Rey and co-workers (2001). The authors identified a *T. atroviride* mutant that has two to four times more chitinase, beta-1,3-glucanase and beta-1,6-glucanase activities than the wild type which provide improved protection on grapes against *Botrytis cinerea* and *in vitro* against *Rhizoctonia solani*.

Various β-1,3-glucanases were isolated from the fungi *T. atroviride* such as the acidic β-1,3-glucanases of 29 kDa (Noronha and Ulhoa, 2000), 78 kDa (Lorito *et al.*, 1994) and a 110 kDa novel extracellular beta-1,3- exoglucanase containing a unique C-termincal embodying cysteine motifs (Cohen-Kupiec *et al.*, 1999). It was shown, that the glucan β-1,3-glucosidase (GLUC78) [78 kDa β-1,3-glucanase (EC 3.2.1.58)] purified from *T.harzianum*, exhibits antifungal activity (ED_{50} of 35-75 µg ml^{-1}) against the oomycetous fungal pathogen *Phytophthora* spp., also when applied in combination with microbial toxins and chemical fungicides (Fogliano *et al.*, 2002).

2.4.6 *T. harzianum* genes expressed in plants

A full scale application of fungal biocontrol agents in commercial agriculture has been delayed because of: 1) the inconsistent results obtained by introducing these complex microorganism into the ever changing environment (Lorito *et al.*, 1998) and 2) due to the lack of understanding the factors responsible for disease control, the interaction of *Trichoderma* with the agricultural environment (Zeilinger *et al.*, 1999) and even the different regulatory signals which triggers expression of two major chitinase genes of *Trichoderma* (Mach *et al.*, 1999). It would therefore be feasible to transfer and constitutively express these powerful fungal cell wall degrading enzymes directly in the plant.

Since extensive testing *in vitro*, has shown that there are virtually no chitinous pathogens resistant to *Trichoderma* chitinases (Lorito *et al.*, 1993, 1994 and 1996), it was expected that expression of a single chitinase gene from this organism in transgenic plants, should confer broad-spectrum resistance. During 1998, disease resistance in transgenic plants has been improved, for the first time, by the insertion of a gene from a biocontrol fungus. The high level and broad spectrum of resistance obtained against soil-borne and foliar pathogens with a single constitutively expressed endochitinase gene from *T. atroviride* (*chit*42) overcome the limited efficacy of transgenic expression in plants of chitinase genes from plants and bacteria (Lorito *et al.*, 1998). Selected transgenic tobacco and potato plants expressing the *chit* 42 gene, had no visible effect on plant growth and development, yet tolerance or complete resistance was obtained to the foliar pathogens *Alternaria alternata*, *A. solani*, *B. cinerea* and the soilborne pathogen *Rhizoctonia solani* (Lorito *et al.*, 1998). Reduced plant vigor is however in some cases reported when a chitinase gene from *T. atroviride* was expressed in a plant e.g. increased constitutive expression of *Trichoderma* derived fungal endochitinase in apple increased resistance to *Venturia inaequalis*, but also reduced plant growth (Bolar *et al.*, 2000). Emani and co-workers (2003) also obtained significant resistance to both the soil-borne pathogen, *Rhizoctonia solani* and the foliar pathogen *Alternaria alternata* of T_2 transgenic cotton plants with high endochitinase expression of the transgene from *T. virens*.

2.5 Sorghum and pearl millet transformation technology

An efficient transformation system has to be in place in order to enhance the genetic pool of pearl millet and to apply recombinant DNA technology. Furthermore, high frequency plant regeneration from cultured explant material is a prerequisite for the successful transformation of this crop, as the limiting step in the development of genetic engineering technology for the improvement of selected cereal genotypes is the *in vitro* culture step (Lambé *et al.*, 1999). Development and establishment of the *in vitro* culture systems for pearl millet will be discussed, as it is fundamental that the explant of choice produces consecutive induction of

embryogenic calli, which further develop and mature to form somatic embryos from which fertile plantlets can be regenerated.

During the last decade, slow progress was made in the establishing protocol for the genetic engineering of both sorghum and pearl millet, both under-exploited cereal crops of Africa.

2.5.1 History and progress of transformation technology for sorghum

The production of transgenic sorghum plants via particle bombardment of immature zygotic embryos has been reported for the first time by Casas *et al.*, (1993), and subsequently reported by Tadesse (2000) and Emani *et al.* (2002). In addition, transgenic sorghum plants were produced via *Agrobacterium*-mediated transformation using immature zygotic embryos as explant by Zhao *et al.* (2000). Furthermore, transgenic sorghum plants were obtained by particle bombardment of immature inflorescences (Casas *et al.*, 1997) and shoot tips (Tadesse, 2000) introducing mainly reporter and selectable marker genes. Only recently, a trait which codes for a feedback insensitive dihydropicolinate synthase, the first enzyme of the lysine-specific pathway, was introduced into the genome of sorghum with the goal of producing transgenic sorghum plants with increased lysine content (Tadesse, 2000). Currently, a consortium of scientists funded by the European Commission (Contract number: ICA4-CT-2000-30034) is in the process of genetically enhancing the nutritional quality of grain sorghum. The goal is to produce transgenic sorghum plants with elevated lysine and methionine contents by introducing genes encoding the methionine-rich maize beta-zein and engineered lysine-rich barley chymotrypsin inhibitor CI-2 proteins (O'Kennedy *et al.*, 2003).

2.5.2 History and progress of pearl millet transformation technology

2.5.2.1 Pearl millet tissue culture

I*n vitro* culture of cereals show a strong genotype dependence and production of the appropriate culture is generally limited to selected genotypes (Lambé *et al.*, 1999). Similar to other cereal cultures, pearl millet lose their morphogenic capacity after several sub-cultures (Lambé *et al.*, 1999; Pius *et al*, 1993). Mature and immature zygotic embryos, immature inflorescences and shoot apices are commonly used as explant for obtaining embryogenic tissue cultures from various graminaceous species. Similarly, procedures for pearl millet plant regeneration via somatic embryogenesis have been described for immature zygotic embryos (IZEs) (Goldman *et al.*, 2003; Lambé *et al.*, 1999, Oldach *et al.*, 2001; Vasil and Vasil, 1981), mature embryos (Botti and Vasil, 1983), immature inflorescences (Goldman *et al.*, 2003; Vasil and Vasil, 1981), shoot apices (Devi *et al.*, 2000, Lambé *et al.*, 1999) or apical meristem (Goldman *et al.*, 2003).

Morphological and histological studies of cultured immature zygotic embryos of pearl millet have shown that single subepidermal cells at the periphery of the scutellum in the coleorhizal

end undergo internal segmenting divisions, resulting in discrete groups of richly cytoplasmic cells (Vasil and Vasil, 1981 & 1982). These cells, which were differentiated, and attained embryogenic and transformation competence during the early stages of culture, could be perpetuated by subculture, and gave rise to embryoids and plants. Furthermore, there is sufficient evidence in the literature (Lu and Vasil, 1985, Woodward, 1989) to show that either cereal somatic embryos in tissue culture arise directly from single cells, or indirectly from a proembryonal complex, which itself arises from a single cell. Somatic embryos of pearl millet fit the same pattern (Vasil and Vasil, 1982). The single cell origin of cereal somatic embryos is essential if after targeting these cells for transformation, uniformly transgenic plants are to be regenerated and subsequently transgenic progeny.

Various components of cereal tissue culture medium were previously optimised in an attempt to improve overall tissue culture responsiveness of selected explants. In this study, only the potential advantage of L-proline and silver nitrate will be discussed.

The addition of L-proline is commonly used in maize tissue culture media (Armstrong and Green, 1985; Pareddy and Petolino, 1990) and recently for pearl millet cultures (Chapter 3). L-proline was essential for routine initiation of Type-II maize calli (Armstrong and Green, 1985) and immature zygotic embryo derived pearl millet calli (Chapter 3) from which large numbers of fertile plants were regenerated. They also found a significant linear relationship between L-proline concentration, up to 25 mM, and embryoid formation in maize cultures. However, the physiological role of L-proline is not clear. Many organisms, including higher plants, accumulate free L-proline in response to osmotic stress (Nanjo *et al.*, 1999a). Nanjo and co-workers (1999a) showed by generating antisense transgenic *Arabidopsis* plants inhibiting the production of L-proline, that the transgenic leaves were hypersensitive to osmotic stress, morphological alterations in the leaves occurred, a defect in elongation of inflorescences occurred, and the structural proteins of cell walls were negatively affected. In another set of experiments, Nanjo and co-workers (1999b), introduced an antisense gene for the suppression of L-proline degradation into the genome of Arabidopsis, and found that the antisense transgenic plants were more tolerant to freezing and high salinity stress than the wild type, showing a positive correlation between L-proline accumulation and stress tolerance. Thus, the addition of L-proline in tissue culture media might benefit cultured plant tissue and plants since it has a unique function in osmotolerance and morphogenesis as a major constituent of cell wall structural proteins. Furthermore, L-proline may lead to an enhanced tolerance to stress caused by the tissue culturing process. This can potentially lead to higher numbers of shoots being regenerated and improved overall health and fertility of tissue cultured plants.

It is known that accumulation of ethylene released during the growth of the tissue in the gaseous atmosphere of the culture vessel takes place and that it might contribute to the poor regeneration response. Silver ion is a potent inhibitor of ethylene action (Beyer, 1976) and Ag^+ inhibits the physiological action of ethylene, without interfering with ethylene biosynthesis (Lieberman, 1979). A number of reports have shown that $AgNO_3$ can promote *in vitro* embryogenic callus and shoot production in various crops (Carvalho *et al.*, 1997; Chi and Pau, 1989; Cruz de Carvalho *et al.*, 2000; Oldach *et al.*, 2001; Pius *et al.*, 1993; Purnhauser *et al.*, 1987; Songstad, 1991; Vain *et al.*, 1989). DeBlock and co-workers (1989) reported that 2-10 mg l^{-1} $AgNO_3$ was a prerequisite for efficient shoot regeneration of *Brassica* under selective conditions using *Agrobacterium*-mediated transformation. Pius *et al.* (1993) reported a three-fold increase in regeneration rates of pearl millet cultures when supplementing regeneration medium with 10 mg l^{-1} $AgNO_3$. Pius and co-workers (1993) concluded that inhibitors of ethylene, such as $AgNO_3$, made it possible to sustain regeneration in cultures that lose their ability to regenerate. In addition, Oldach *et al.* (2001) reported a significant increase in the regeneration rates of pearl millet cultures when supplementing induction medium with 5 mg l^{-1} $AgNO_3$.

2.5.2.2 Biolistic-mediated transformation

Despite the recent developments to establish a transformation protocol for pearl millet (Girgi et al., 2002; Goldman *et al.*, 2003; Lambé *et al.*, 2000), production of transgenic plants is not yet routine. Stable transformation of pearl millet callus was achieved for the first time during 1995 by microprojectile bombardment. Two plasmids carrying the hygromycin phosphotransferase (*hph*) selectable marker and the β-glucuronidase (*uid*A) reporter genes were stably integrated into the genome of pearl millet (Lambe *et al.*, 1995). The transgenic callus of pearl millet (Lambe *et al.*, 1995) could not be regenerated due to the long period of culture needed for the selection of transgenic callus (Lambe *et al.*, 1999) and no evidence of transgenic fertile regenerants from the transgenic callus was reported.

Recently, procedures for transformation based on the use of the helium-driven PDS-1000/He or the particle inflow gun (PIG) using the *bar* herbicide resistance gene, or hygromycin phosphotransferase (*hph*) as selectable marker genes and/or *uid*A (GUS) as reporter gene in transgenic pearl millet have been published (Girgi *et al.*, 2002; Goldman *et al.*, 2003; Lambé *et al.*, 2000). Girgi and co-workers (2002) demonstrated a transformation system using 2-5 days precultured immature zygotic embryos as explant, whereas Lambé and co-workers (2000) reported on the transformation of shoot tip derived embryogenic units obtained 7-8 weeks after isolation. Differerent particle bombardment devices, tissue culture procedures and pearl millet genotypes were used to produce these transgenic lines. Common in both systems is the multiple copy integration of transgenes, an undesirable feature that can lead

to gene silencing in progeny (Iyer *et al.*, 2000; Anand *et al.*, 2003) and especially post transcriptional gene silencing in plants transformed with a class I β-1,3-Glucanase (Kunz *et al.*, 2001).

Pearl millet transformation is still limited by relatively low and erratic stable transformation efficiencies. Pearl millet transformation efficiencies targeting scutellum cells of proliferating immature zygotic embryos (Biolistic transformation, 0.02-0.28%; Girgi *et al.*, 2002) are very low in comparison to other cereal crops such as maize, wheat, rice and barley, which are routinely transformed (transformation efficiencies exceeding 10%) with agricultural valuable genes of interest. Goldman and co-workers (2003) reported on a transformation efficiency of 5.5 herbicide-resistant plants per plate for 60% of their experiments targeting inflorescence derived embryogenic pearl millet tissue. However, the authors did not screen all the transgenic plants with southern blot analysis to determine unique integration events, and some of these transgenic plants might have been clones. Finally, published work to date report only on selectable marker genes stably integrated into the genome of pearl millet.

Inadequate knowledge of antibiotic or herbicide resistance genes' impact on the environment and on human health, especially in food crops, has caused widespread public concern (Wang *et al.*, 2000). The POSITECH system is a novel and attractive alternative. POSITECH, the mannose positive selection system employs the *man*A gene encoding phosphomannose isomerase gene as selectable marker gene and mannose, converted to mannose-6-phosphate by endogenous hexokinase, as selective agent (Joersbo *et al.*, 1999). The transgenic PMI-expressing cells have acquired the ability to convert mannose-6-phosphate to fructose-6-phosphate, while the non-transgenic cells accumulate mannose-6-phosphate with a concomitant consumption of the intracellular pools of inorganic phosphate and ATP. The structural gene (*man*A) from *Escherichia coli* were previously used to successfully produce transgenic maize (Negrotto *et al.*, 2000; Wang *et al.*, 2000), cassava (Zhang and Puonti-Kaerlas, 2000) and sugarbeet (Joersbo *et al.*, 1998 and 1999) via biolistic or *Agrobacterium*-mediated transformation, crops which generally are recalcitrant to transformation. In addition, *man*A was shown to be a superior selectable marker gene for plant transformation; improving transformation efficiencies in crops such as maize, wheat and sugar beet (Joersbo *et al.*, 1998; Reed *et al.*, 2001; Wright *et al.*, 2001) when compared to antibiotic or herbicide (*pat* or *bar*) selectable marker genes. Furthermore, a preliminary risk assessment done by Reed and co-workers (2001) indicated that the PMI protein in transgenic maize was 1) readily digested in simulated mammalian gastric and intestinal fluids, 2) there was no detectable changes in glycoprotein profiles and 3) no statistically significant differences were obtained in yield and nutritional composition compared to

untransformed maize. Furthermore, a database search revealed no significant homology of the *E. coli manA* gene product to any known toxin or allergen (Reed *et al.*, 2001).

There is no published work reporting on the successful production of transgenic pearl millet via *Agrobacterium*-mediated transformation to date.

In conclusion then, it would be of great benefit to establish a routine transformation system for pearl millet in order to enhance the genetic make up of this valuable crop, indigenous to Africa and to subsequently introduce a gene to confer resistance to *S. graminicola*, in order to combat downy mildew.

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Chapter 3

Improved regeneration efficiency of a pearl millet (*Pennisetum glaucum* [L.] R.Br.) breeding line

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3.1 ABSTRACT

This study reports the establishment of an efficient, reliable *in vitro* tissue culture system for the regeneration of fertile plants from immature zygotic embryo explants of pearl millet. Eight pearl millet genotypes were assessed for their amenability to tissue culture. The tissue culture responses obtained were strongly genotype-dependent. One of the most regenerable genotypes, 842B, was selected for further screening. The influence of the addition of different compounds such as silver nitrate, an ethylene inhibitor, L-proline and high carbohydrate pulses were examined with respect to callus induction, production of embryogenic tissue and somatic embryo regeneration to form shoots and roots. Supplementation of the callus induction medium with L-proline in combination with maltose, produced the most significant improvement in the regeneration capacity of genotype 842B resulting in, on average, eighty regenerants per cultured immature zygotic embryo.

3.2 INTRODUCTION

High frequency plant regeneration from cultured explant material is a prerequisite for the successful transformation of crops. The limiting step in the development of genetic engineering technology for the improvement of cereal crops by biolistic transformation is the *in vitro* culture step. *In vitro* culture of cereals show a strong genotype dependence and production of the appropriate culture is generally limited to selected genotypes (Lambé *et al.*, 1999). Furthermore, the majority of cereal cultures lose their morphogenic capacity after several sub-cultures, as was also reported for pearl millet (Lambé *et al.*, 1999; Pius *et al*, 1993).

Procedures for pearl millet plant regeneration via somatic embryogenesis have been described for a range of tissues such as immature zygotic embryos (IZE) (Lambé *et al.*, 1999, Oldach *et al.*, 2001; Vasil and Vasil, 1981), mature embryos (Botti and Vasil, 1983), immature inflorescences (Vasil and Vasil, 1981) and shoot apices (Devi *et al.*, 2000, Lambé *et al.*, 1999). In this study, eight pearl millet genotypes were screened *in vitro*, using IZE as explants, for consecutive induction of embryogenic calli, somatic embryogenesis and regeneration. Genotype

purity and cultivation in Africa, or potential use in African breeding programmes, were considered in the initial selection of the genotypes. One of the highly regenerable genotypes, 842B, was assessed further on various media. In particular, the effect of the addition of L-proline and $AgNO_3$ to the tissue culture medium was investigated.

A highly efficient regeneration system has led to a highly efficient transformation system for oat plants (Gless *et al.*, 1998a&b). Therefore, the aim of this study was to improve regeneration capacity of pearl millet in order to establish a routine transformation system.

3.3 MATERIALS AND METHODS

3.3.1 Seed material

Seed material was kindly provided by International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Zimbabwe (pearl millet genotypes 841B, 842B, 843B), ICRISAT, India (pearl millet genotypes ICMB88006 and ICMB87001) and Savanna Agricultural Research Institute (SARI), Ghana (pearl millet genotypes 7042, Bongo Nara and Manga Nara). Seedlings were planted in a soil mix consisting of red soil, rough sand and compost (1:1:1), and were watered three times per week with a soluble fertiliser (Hortichem N:P:K at 3:1:5, Ocean Agriculture) until flowering. Cross-pollination was prevented by covering the flowers with brown paper bags.

3.3.2 Excision of IZE

Greenhouse-grown florets of pearl millet containing IZE (10-14 days post-pollination) were rinsed in 70% (v/v) ethanol for one minute and sterilised for 15 minutes in a 2.5% (m/v) sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20, before being thoroughly rinsed with sterile distilled water. IZE (0.8 – 1 mm in size) were aseptically excised from the florets using a dissecting microscope and placed with their axes in contact with the callus induction medium and their scutella positioned upwards. All tissue culture was performed under aseptic conditions.

3.3.3 Tissue culture of IZEs

IZE were cultured on either MS (Murashige and Skoog, 1962) based medium as described by Pinard and Chandrapalaiah (1991) and designated PM1 medium, or on L3-based medium as previously described (Gless *et al.*, 1998a; Jähne *et al.*, 1991) and designated PM2 medium (Table 1). All PM1 medium (regime A - E) contained 88 mM sucrose as a carbon source, 9 µM in the induction medium and were solidified by 8 g l^{-1} agar. All PM2 media (regimes F - M) were solidified by 4 g l^{-1} Gelrite, contained 11 µM 2,4-D in the callus induction medium and 83 µM maltose (regimes F- L) or 88 µM sucrose (regime M). Embryo derived calli were transferred to

fresh callus induction medium after two weeks. Some one-month-old callus initiated on L-proline containing media D-E and J-M was transferred to maturation medium for a period of two weeks (Table 1). Maturation medium contained 6% of the carbohydrate and no L-proline (D, E, J, K and M) or a combination of $AgNO_3$ and hormones (medium L; Table 1). Thereafter, embryogenic tissue was transferred to regeneration medium, containing only 3% of the carbohydrate, and subcultured every three weeks until the development of plantlets (> 1 cm). Calli induced on media regimes A-C and F-I had no maturation phase, but were transferred directly to regeneration medium as indicated in Table 1. Developing plantlets on regeneration media A-C, E, G, H and K were transferred to rooting medium without $AgNO_3$ and hormones, and subcultured every three weeks. Rooted plantlets of >1cm were recorded as regenerants. A small selection of regenerants was hardened-off in the greenhouse as described by O'Kennedy *et al.* (1998). Cultures on callus induction, maturation and regeneration medium were incubated at 24-25°C, under low-light conditions (1.8 µmol $m^{-2}s^{-1}$), whereas regenerating shoots (≥1 cm) were incubated under dim light (18 µmol $m^{-2}s^{-1}$) at 24-25°C.

3.3.4 Tissue culture of shoot apices

Seed material was rinsed with 70% ethanol for one minute and incubated in sterility broth (autoclaved solution of half-strength MS salts, MS vitamins, 10 g l^{-1} yeast extract, 3 g l^{-1} beef extract and 5 g l^{-1} glucose) for four hours. Subsequently, the seeds were rinsed with sterile distilled water and sterilised for 30 min in a 3.5% sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20, before being thoroughly rinsed with sterile distilled water. Sterilised seeds were plated on half-strength MS medium without any hormones at 24-25°C under a light intensity of 80 µmol$m^{-2}s^{-1}$. Uncontaminated germinating seedlings with a leaf length of 0.5 cm – 7 cm were cut into 0.25 – 1 mm sections and placed with the cut surface on the culture medium. Shoot apex cultures were initiated and cultured on callus induction medium as described by Gless *et al.* (1998a) for oat plants. White compact callus produced on callus induction was transferred to maturation medium, as described by Gless *et al.* (1998a), and then transferred to regeneration medium for the development of plantlets. Regenerated plantlets were transferred to regeneration medium in Magenta boxes.

3.4 RESULTS AND DISCUSSION

Pearl millet IZE were cultured on PM1 or PM2 tissue culture media to induce embryogenic calli from which fertile plantlets were regenerated via somatic embryogenesis. The two media differ in their carbohydrate source, solidifying agent and basal salts, vitamins, ammonium nitrate, and amino acid contents. PM1 is a MS (Murashige and Skoog, 1962) basal medium containing sucrose and solidified by agar, whereas PM2 is a L3 (Jahne *et al.*, 1991) basal medium

containing maltose and solidified by Gelrite. PM1 includes media A-E tested in this study and PM2 includes media F-M tested in this study (Table 1).

IZEs at a critical stage of development were used as the most appropriate explant source. In this study, the frequency of embryogenic calli initiation was significantly higher in cultured IZE (95%, induction medium A) of pearl millet genotype 842B than in cultured shoot apices of this genotype (no more than 54%), whereas Lambé *et al.* (1999) found the opposite. In our laboratory, shoot apices of genotype 842B produced an average of only 6.1 regenerated plantlets per explant in 16 months. IZEs of 0.6-1 mm in length excised approximately 8-12 days post pollination, produced embryogenic calli that regenerated to form plantlets. The epidermal and subepidermal cells of the scutellum of the IZE at the coleorhizal end either formed somatic embryos (PM1 medium) or gave rise to embryogenic calli, which subsequently matured to form somatic embryos (PM2 callus induction and maturation medium; Fig. 1A-D). PM2 induction medium supplemented with 20mM L-proline (I-L induction media) gave rise to friable embryogenic calli (type II) within one month of culture (Fig. 1C). Numerous white cup-shaped somatic embryos developed on the surface of friable watery calli (Fig. 1D) within two weeks after transfer of the cultures to PM2 maturation medium (J-M maturation media). After two weeks of culture on the maturation medium, cultures were transferred to hormone-free PM2 regeneration medium and within 1.5 - 6.5 months, somatic embryos developed to form independent rooted plants ready for hardening-off under greenhouse conditions (Fig. 1E). Small selections of PM1-derived and PM2-derived plantlets were hardened-off under greenhouse conditions and matured to form fertile plants, which set seed (Fig. 1F).

Eight pearl millet genotypes were screened on the PM1 medium regime A (induction, regeneration and rooting media). White, compact callus, constituting mainly somatic embryos, developed within one month from the scutellum cells of cultured IZE. Almost 100% of the explants produced white compact callus, which regenerated to form clusters of plantlets that were difficult to separate. Plantlets that were obtained during regeneration rooted successfully. Results of the eight genotypes screened on medium regime A clearly show genotype dependence of regeneration of pearl millet (Fig. 2). The one-way ANOVA test shows significant difference ($P = <0.001$) between 1) genotypes 842B and Manga nara (MN) which produced the highest number of regenerants per ten cultured embryos (110 ± 43.7 and 146 ± 27.5, respectively) and 2) genotypes 7042 and ICMB 88006 (2 ± 2.2 and 1 ± 1.5, respectively) which produced the lowest number of regenerants per ten embryos. Therefore, $842B^{A} = MN^{A} > 7042^{B} = ICMB88006^{B}$; where the means followed by the same uppercase letters are not

Table 1: Composition of pearl millet tissue culture media regimes tested. Regime B and F are media as described by Pinard and Chandrapalaiah (1991) and Gless *et al.* (1998), respectively. Media regimes A, C-E are modified Pinard and Chandrapalaiah (1991) media with modifications as indicated. Media regimes G-M are modified Gless *et al.* (1998a) media, with modifications as indicated.

Media regime		Carbohydrate source for regime	Callus induction medium	Maturation medium				Regeneration medium		
			L-proline 20 mM	% carbohydrate	$AgNO_3$ 60 µM	IAA 1 µM	Kinetin 2.3 µM	$AgNO_3$ 60 µM	IAA 1 µM	Kinetin 2.3 µM
PM1	A	sucrose	-	na	na	Na	na	+	+	+
	B	sucrose	-	na	na	Na	na	-	+	+
	C	sucrose	+	na	na	Na	na	+	+	+
	D	sucrose	+	6	-	-	-	-	-	-
	E	sucrose	+	6	-	-	-	+	+	+
PM2	F	maltose	-	na	na	Na	na	-	-	-
	G	maltose	-	na	na	Na	na	+	-	-
	H	maltose	-	na	na	Na	na	+	+	+
	I	maltose	+	na	na	Na	na	-	-	-
	J	maltose	+	6	-	-	-	-	-	-
	K	maltose	+	6	-	-	-	+	-	-
	L	maltose	+	3	+	+	+	-	-	-
	M	sucrose	+	6	-	-	-	-	-	-

na = not applicable; media regimes A-C and F-I had no maturation phase

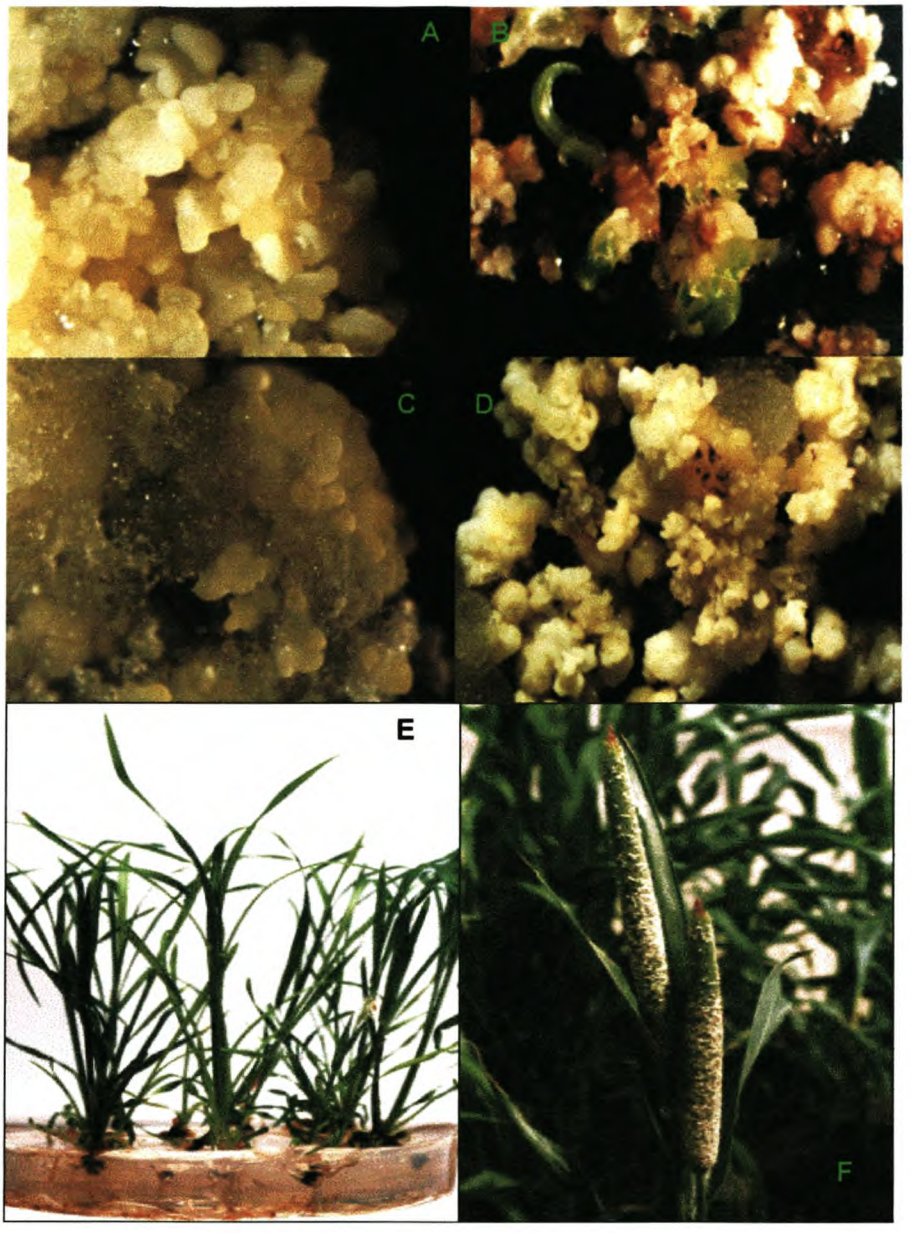

Figure 1: Tissue culture cycle of pearl millet genotype 842B. A, callus tissue after one month of culture of IZEs on C induction medium. B, callus tissue producing shoots after one month of culture of IZEs on C induction medium followed by two months of culture on C regeneration medium. C, callus tissue after one month of culture of IZEs on J induction medium. D, callus tissue after one month of culture of immature zygotic embryos on J induction medium followed by two weeks of culture on J maturation medium. E, rooted shoots via somatic embryogenesis on medium regime J, and ready for hardening-off in the greenhouse. F, a fertile regenerant produced on medium regime L and growing in the greenhouse after successful hardening-off.

significantly different at P =<0.005. There is no significant difference between the other treatments (P = <0.005). Bhaskaran and Smith (1990) hypothesised that the genetic basis of variability in tissue culture response and morphogenesis is most likely due to differences in hormone metabolism within the explant, which is established by the level of gene expression for individual hormones of the particular genotype. The authors stated that all genotypes are capable of producing embryogenic cultures, if the correct meristematic explant and initial exposure to the appropriate plant growth regulators are used. Hopefully, future advances will clarify the underlying source(s) of variavility in regeneration responses between genotypes.

Subsequently, genotype 842B, which produced a high number of regenerants on tissue culture medium regime A, was screened on thirteen different tissue culture media regimes designated A-M (Table 1, Fig. 3). The highest number of regenerants was obtained on medium regime J, where the induction medium was supplemented with L-proline and maltose was increased to 6% during the maturation phase. The one-way ANOVA test shows significant difference (P = <0.001) between the number of regenerants per ten cultured embryos cultured on tissue culture

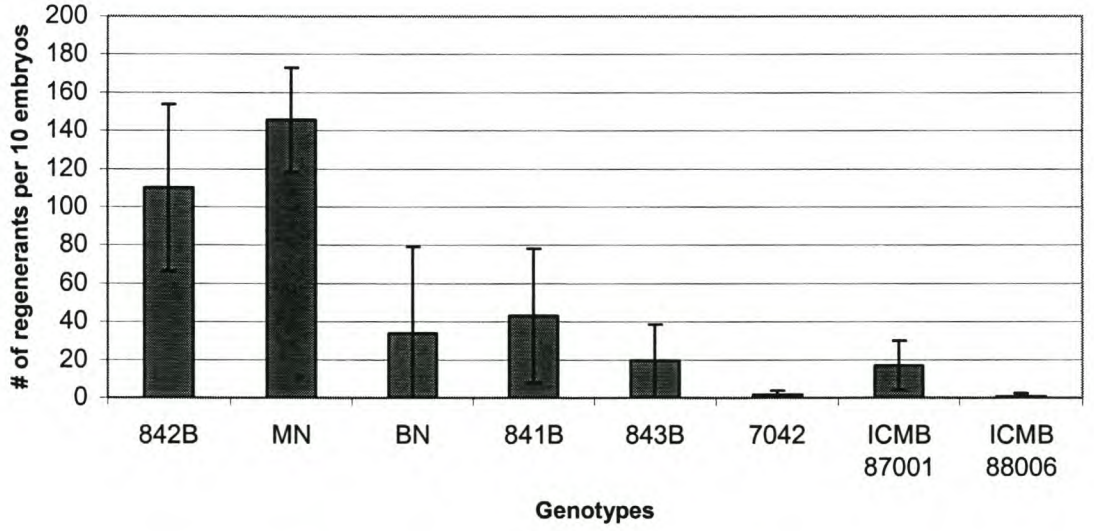

Figure 2: The number of regenerants obtained per ten embryos when eight pearl millet genotypes were screened on tissue culture medium regime A (see table 1). The standard deviation is indicated by the bars. One-way ANOVA test indicated no statistically significant difference (P = <0.001) between genotypes 842B and Manga nara (MN). Genotype Bongo nara was abbreviated as BN. Four independent replications were done per treatment.

medium regime J (807 ± 222.1) and A, B and G (110 ± 43.7, 146 ± 52.3 and 73 ± 43.2, respectively). Indicating that $J^A > A^B = B^B = G^B$; where the means followed by the same uppercase letters are not significantly different at P =<0.005.

There is no significant difference between the other treatments (P = <0.005). The highest number of regenerants produced per individual explant cultured on PM1 medium was produced on medium regime E where the regenerants produced ranged from 0 to 60 per individual explant. PM2 medium regime J produced the highest number of regenerants overall, ranging from a minimum of 31 per explant to a maximum of 282 per explant.

The addition of L-proline to the induction medium or $AgNO_3$ to the regeneration of PM1 media regimes did not give a statistically significant increase in the number of regenerants per cultured IZE. The addition of $AgNO_3$ and the hormones kinetin and IAA to the regeneration medium rather severely inhibited rooting of the shoots produced (previous study, data not shown). Therefore, both the hormones and $AgNO_3$ were omitted from rooting medium. In contrast, the addition of L-proline to the PM2 induction medium and the inclusion of a maturation medium step (culture for 2 weeks on auxin-free medium containing double the quantity of maltose, 6% instead of 3%) in medium regime J, resulted in a two-fold increase compared to the medium described by Gless *et al.*, (1998a) for oats (medium regime F). Once more, there was no significant effect of $AgNO_3$ on regeneration efficiency (Table 1, Fig. 3). Media regimes J and L had the identical induction medium (containing L-proline). However, maturation medium J was auxin-free and contained 6% maltose, whereas L maturation medium contained kinetin, IAA and $AgNO_3$, and the standard 3% maltose (Table 1). The doubling of the carbohydrate source (medium regime J) resulted in higher numbers of regeneration per ten explants when compared to medium regime L where maturation was induced by hormones and $AgNO_3$ (Fig. 3). The difference was however not statistically significant. It is however significant that medium regime J produced only 0.8% albino regenerants whereas regime L produced 3% albino regenerants. The production of albino plants might be the result of the addition of L-proline and/or the hormones to the tissue culture medium. Nevertheless, the final numbers of regenerants presented in the figures text reflects only green plantlets. In addition, medium regimes F and I (with or without the addition of L-proline in the induction medium, respectively) resulted in almost an identical number of regenerants per ten explants (408 ± 146.0 and 416 ± 58.0, respectively). This clearly indicates that the two weeks maturation phase is essential to increase (medium regime L, 552 ± 200) or even double (medium regime J, 807 ± 222.1) the number of regenerants per ten explants.

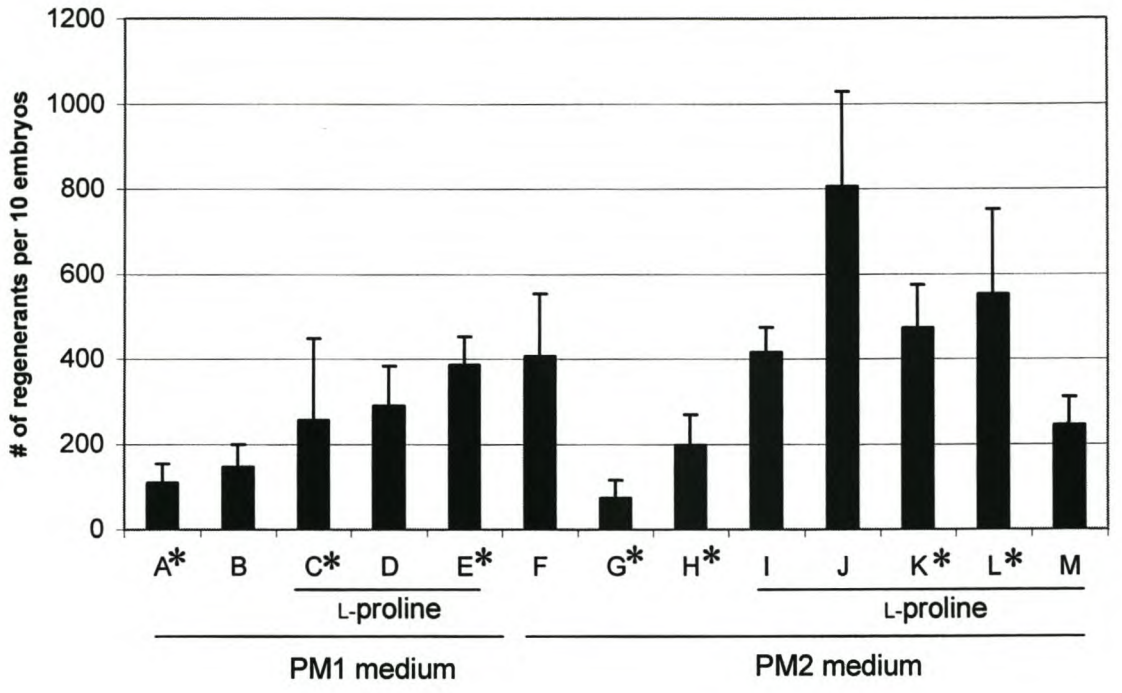

Figure 3: The influence of 13 independent callus induction and regeneration media A to M (see table 1) on regeneration rates from cultured immature pearl millet embryos of genotype 842B. The addition of AgNO3 (*) and/or L-proline (bars underneath) are indicated on the graph. PM1 or PM2 based media regimes are also indicated. Four independent replications were done per treatment. The standard deviation is indicated by the bars.

Finally, selected genotypes 842B, Manga nara (MN) and Bongo nara (BN) were tested on tissue culture media regimes A, J and K (Fig. 4) expecting an improved regeneration efficiency for Manga nara and Bonga nara cultured on especially medium regime J. However, significant differences were only obtained amongst genotypes, but no significant difference was obtained in the same genotype cultured on media regimes A, J or K.

In previous studies, accumulation of ethylene released during the growth of the tissue in the gaseous atmosphere of the culture vessel has been identified as one of the factors responsible for poor regeneration response. Silver ion is a potent inhibitor of ethylene action (Beyer, 1976) and Ag^+ inhibits the physiological action of ethylene, without interfering with ethylene biosynthesis (Lieberman, 1979). A number of reports have shown that $AgNO_3$ can promote *in*

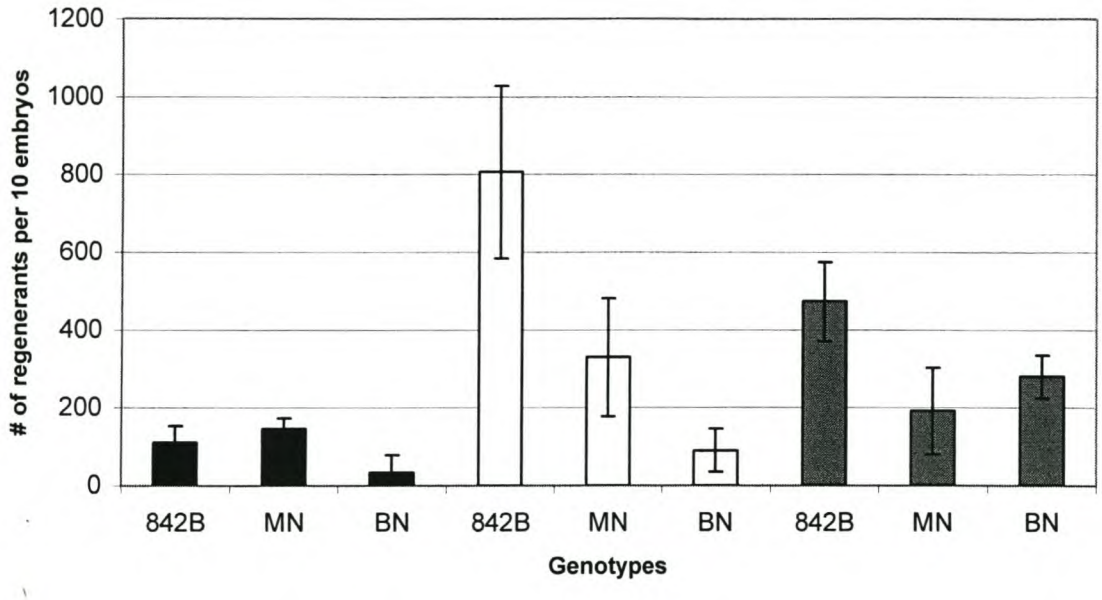

Figure 4: The influence of callus induction and regeneration media A■, J□ and K ▒ (see table 1) on regeneration rates from cultured immature pearl millet embryos of genotype 842B, Manga nara (MN) and Bongo nara (BN). The standard deviation is indicated by the bars. Four independent replications were done per treatment.

vitro embryogenic tissue and shoot production in various crops (Carvalho *et al.*, 1997; Chi and Pau, 1989; Cruz de Carvalho *et al.*, 2000; Oldach *et al.*, 2001; Pius *et al.*, 1993; Purnhauser *et al.*, 1987; Songstad, 1991; Vain *et al.*, 1989). DeBlock and co-workers (1989) reported that 2-10 mg l^{-1} $AgNO_3$ was a prerequisite for efficient shoot regeneration of *Brassica* under selective conditions using *Agrobacterium*-mediated transformation. Pius *et al.* (1993) reported a three-fold increase in regeneration rates of pearl millet cultures when supplementing regeneration medium with 60 µM $AgNO_3$. Pius and co-workers (1993) concluded that inhibitors of ethylene, such as $AgNO_3$, made it possible to sustain regeneration in cultures that lose their ability to regenerate. Oldach *et al.* (2001) reported a significant increase in the regeneration rates of pearl millet cultures when supplementing induction medium with 30 µM $AgNO_3$. In this study, however the effect of supplementing regeneration medium with 60 µM $AgNO_3$ to improve the regeneration rate of pearl millet was insignificant.

The addition of L-proline is commonly used in maize tissue culture media (Armstrong and Green, 1985; Pareddy and Petolino, 1990). Armstrong and Green (1985) found that L-proline was

essential for routine initiation of Type-II maize callus, from which large numbers of fertile plants were regenerated. They also found a significant linear relationship between L-proline concentration, up to 25 mM, and embryoid formation in maize cultures. However, the physiological role of L-proline is not clear. Many organisms, including higher plants, accumulate free L-proline in response to osmotic stress (Nanjo *et al.*, 1999a). Nanjo and co-workers (1999a) showed by generating antisense transgenic *Arabidopsis* plants inhibiting the production of L-proline, that the transgenic leaves were hypersensitive to osmotic stress, morphological alterations in the leaves occurred, a defect in elongation of inflorescences occurred, and the structural proteins of cell walls were negatively affected. In another set of experiments, Nanjo and co-workers (1999b), introduced an antisense gene for the suppression of L-proline degradation into the genome of Arabidopsis, and found that the antisense transgenic plants were more tolerant to freezing and high salinity stress than the wild type, showing a positive correlation between L-proline accumulation and stress tolerance. Thus, the addition of L-proline in tissue culture media might benefit cultured plant tissue and plants since it has a unique function in osmotolerance and morphogenesis as a major constituent of cell wall structural proteins. Furthermore, L-proline may lead to an enhanced tolerance to stress caused by the tissue culturing process. This can potentially lead to higher numbers of shoots being regenerated, which is the case in this study, and improved overall health and fertility of tissue cultured plants.

In conclusion, we report on a highly efficient regeneration system for pearl millet genotype 842B via somatic embryogenesis. IZE of genotype 842B cultured on medium J proved to be the most efficient regeneration system in this study. The significantly high regeneration rate obtained for genotype 842B on medium regime J can be attributed to the responsiveness of this genotype, as well as the nutrient composition of the J medium regime. An average of 80 regenerants per individual IZE were obtained, and roots appeared simultaneously with shoot production or shortly after shoot production. This coincides with the theory that somatic embryos are defined as bipolar structures that shoot and root simultaneously. The total period in tissue culture was 3 to 10 months. Similarly, Devi et al., (2000) obtained 80 green vegetative shoots from each originally cultured shoot apex, and roots formed two weeks after culture initiation. However, the total period in tissue culture and regenerant fertility is not reported. It is possible that secondary somatic embryo formation resulted in the high number of regenerants in our study and the study of Devi *et al.*, (2000), as Oldach *et al.* (2001) reported a total tissue culture period of no more than 5 months. Nevertheless, the regenerants hardened-off in this study were fertile which is an essential factor for the future production of transgenic plants.

The establishment of a highly efficient regeneration system for pearl millet genotype 842B will greatly benefit pearl millet breeding programmes as it is widely used in breeding programmes in Africa and India by ICRISAT. Genotype 842B has been of economic importance to the pearl millet hybrid seed industry in India over the past 15 years. It is one of the elite inbred lines, which are maintainer lines of seed parents of hybrid cultivars. This line has the potential to contribute to the production of early maturing grain-type hybrids for parts of southern and eastern Africa (Dr T. Hash, ICRISAT, India, personal communication, 1999). The authors are currently working on establishing an efficient routine transformation protocol for pearl millet genotype 842B. This would form the technological basis for the genetic enhancement of pearl millet and would provide the means to introduce agronomical significant genes into a cereal crop grown widely in both India and parts of Africa.

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Chapter 4

A protocol to produce transgenic fertile pearl millet (*Pennisetum glaucum* [L.] R.BR.) plants using the particle inflow gun

A shortened version of this work was incorporated with the work done at the University of Hamburg and published in collaboration with Dr Maram Girgi who obtained transformation using the PDS 1000/He gun. The title of the published paper is: "Transgenic and herbicide resistant pearl millet (P. glaucum [L.] R.Br.) via microprojectile bombardment of scutellar tissue", 2002, Molecular Breeding 10: 243-252. The work presented in this chapter however contains the full details and is work done solely by the authors. Furthermore, it only reports on the transformation of pearl millet using the particle inflow gun (PIG).

4.1 ABSTRACT

A pearl millet hybrid parental line, 842B, was transformed with a selectable marker gene (*bar*) and a reporter gene (*uid*A) both driven by the constitutive promoter ubiquitin. Pre-cultured immature zygotic embryos were bombarded using a simple and inexpensive particle inflow gun. The selectable marker gene *bar* from *Streptomyces hygroscopicus,* conferring herbicide resistance, was used to establish pearl millet transformation. Two fertile transgenic pearl millet plants were obtained as confirmed by resistance to application of the herbicide Basta®, *uid*A expression, PCR and southern blot analysis. Both transformation events, clones, had a multicopy integration pattern of the *bar* transgene.

4.2 INTRODUCTION

Pearl millet (*P. glaucum* formerly *P. americanum*) is a crop of vital importance to millions of African families living in semi-arid regions of the continent. Pearl millet is one of the world's most resilient crops. In many areas where millet is the staple food, nothing else will grow. Nevertheless, *S. graminicola*, an obligate oomycetous fungal phytopathogen, and the causal agent of downy mildew in pearl millet plants, is a major constraint in the production of pearl millet. The development of a reliable transformation protocol for pearl millet will form the basis for future genetic enhancement of this crop by complimenting classical breeding programmes for the benefit of India and sub-Saharan Africa.

Despite the recent development of a transformation protocol for pearl millet, production of transgenic plants is not yet routine. Stable transformation of pearl millet callus was achieved by microprojectile bombardment with two plasmids carrying the hygromycin phosphotransferase

(*hph*) selectable marker and the β-glucuronidase (*uid*A) reporter genes (Lambe *et al.*, 1995). The transgenic callus of pearl millet (Lambe *et al.*, 1995) could not be regenerated due to the long period of culture needed for the selection of transgenic callus (Lambe *et al.*, 1999), however no evidence of transgenic fertile regenerants from the transgenic callus was reported. Recently, Lambé and co-workers (2000) reported in a review paper on the production of transgenic pearl millet plants.

The aim of this study was to develop a reliable transformation system for pearl millet by optimising biolistic protocols using selected highly regenerable genotypes. We demonstrate stable transformation of a pearl millet breeding line by biolistic-mediated transformation using a simple and inexpensive device the particle inflow gun (PIG).

4.3 MATERIALS AND METHODS

4.3.1 Seed material

Seed material was kindly provided by ICRISAT, Zimbabwe (pearl millet genotype 842B), and Savanna Aricultural Research Institute, Ghana (pearl millet genotypes Bonga Nara and Manga Nara). Seedlings were planted in a soil mix consisting of red soil, rough sand and compost (1:1:1), and were watered three times per week with a soluble fertiliser (Hortichem N:P:K at 3:1:5, Ocean Chemicals) until flowering. Cross pollination was prevented by covering the flowers with brown paper bags.

4.3.2 Excision of IZEs

Florets from greenhouse-grown pearl millet containing IZEs (10-14 days post-pollination) were soaked in 70% (v/v) ethanol for one minute and sterilised for 15 min in a 2.5% sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20, before being thoroughly rinsed with sterile distilled water. IZEs (0.5 – 1 mm in size) were aseptically excised from the florets using a dissecting microscope and placed with their axes in contact with the callus induction medium. All tissue culture was performed under aseptic conditions.

4.3.3 Tissue culturing of IZEs

Callus induction medium A is described by Pinard and Chandrapalaiah (1991), and contains MS salts (Murashige and Skoog, 1962), 9 µM 2,4 D, 88 mM sucrose as carbon source and 8 g l^{-1} agar as solidifier. Cultures initiated on induction medium A were further cultured on regeneration medium as described by Pinard and Chandrapalaiah (1991), containing the hormones IAA (1 µM) and kinetin (2.3 µM $AgNO_3$), but the medium was modified by the addition 60 µM $AgNO_3$ (for genotype 842B) or 120 µM $AgNO_3$ (for genotypes Manga nara and Bongo nara). These cultures were subsequently transferred to rooting medium, which was identical to the

regeneration medium, except that both hormones and silver nitrate were omitted. Cultures initiated on callus induction medium A containing 20 mM L-proline, were transferred to the regeneration and rooting media described above.

Callus induction medium J is described by Gless and co-workers (1998) and contains L3 salts and vitamins as described by Jähne *et al.* (1991), 11 µM 2,4-D, 83 mM maltose as a carbon source, 4 g l^{-1} Gelrite as solidifier and was modified by supplementing the medium with 20 mM L-proline. Cultures initiated on callus induction medium J were regenerated and rooted on induction medium J from which the 2,4-D was omitted and containing 166 mM maltose instead of 83 mM maltose. The matured cultures were then regenerated and rooted on regeneration medium without 2,4-D and 83 mM maltose.

Cultures on callus induction and regeneration media were incubated at 24-25°C, under light (1.8 µmol $m^{-2}s^{-1}$), whereas regenerating shoots (≥1 cm) were incubated under light (18 µmol $m^{-2}s^{-1}$).

4.3.4 Transfer regime

Immature zygotic embryo derived calli were transferred to fresh medium every two weeks. White compact calli were produced within 4 weeks on callus induction medium A, but only after 6 weeks on medium J: callus induction (4 weeks) and maturation (2 weeks). After 4 or 6 weeks on A or J based medium, respectively, calli were transferred to the appropriate regeneration media as described above, and subcultured every three weeks to fresh media. Rooted plantlets of >1 cm, produced on regeneration medium A, were transferred to rooting medium. Plantlets grown to 8-10 cm, were hardened-off to a mist bed for approximately two weeks and then transferred to pots in the greenhouse. Addition of the bialaphos selection agent to tissue culture medium is described below (4.3.8)

4.3.5 Plasmids

Plasmid pAHC25 is a dual expression vector which contains the *uid*A reporter gene that encodes the β-glucuronidase (GUS) enzyme, and for selection, the *bar* gene, that encodes the enzyme phosphinothricin acetyl transferase (PAT), which confers herbicide resistance. PAT inactivates the herbicidal compound phosphinothricin (PPT) by acetylation. L-PPT is the active ingredient in several herbicide formulations, such as Basta® (Hoechst AG, Germany), containing glufosinate ammonium, the salt of a chemically synthesized racemic mixture D,L-PPT, and Herbiace® (Meiji Seika Kaisha Ltd., Japan) which contains bialaphos, a tripeptide consisting of L-PPT and two alanine residues. Both the *uid*A and *bar* genes are under the control of the maize *Ubi1* promoter, first exon and first intron, and the nopaline synthase terminator (Christensen and Quail, 1996). Plasmids pAHC25 DNA was extracted from overnight *E. coli* cultures using a

Qiagen (Southern Cross Biotechnologies) maxiprep kit according to the supplier's recommendation.

4.3.6 Microprojectile bombardment

Pre-cultured IZEs were placed in the middle (0 - 1 cm diameter) of a 9 cm petri dish, containing callus induction media supplemented with approximately 25% increased solidifier, plus 0.2 M D-Sorbitol and 0.2 M D-Mannitol described by Vain and co-workers (1993). A bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles using $CaCl_2$ and spermidine free base as described by O'Kennedy *et al* (1998). All experiments were conducted with the particle inflow gun (PIG). Sixteen hours after bombardment, bombarded tissue was transferred to the respective media omitting D-Sorbitol and D-Mannitol. DNA delivery parameters were as follows: 900-1200 kPa, 0.16 µg DNA per shot, 17 cm from the target tissue and a 500 µm nylon mesh screen placed 8 cm above the target. A vacuum of approximately -87 kPa was applied and the bombardment mix particles on the filter syringe were discharged when the helium was released following activation of the solenoid. The timer duration was 50 milliseconds.

4.3.7 Transient expression

Bombarded tissue was assayed for β-glucuronidase (GUS) activity 48h to 28 days, after bombardment by staining the tissue according to Jefferson *et al.* (1987). Blue foci showing GUS activity were counted using a dissecting microscope, each distinct spot was taken as one expression event and the results were recorded as the number of blue foci per explant. Small leaf pieces of putative transgenic pearl millet plants were subjected to GUS assaying to determine whether the *uid*A gene was expressed in this tissue.

4.3.8 Selection and regeneration of transformants

Selection for phosphinothricin (PPT)-resistant pearl millet tissue was initiated 1-7 days after bombardment by placing the cultured immature embryos on callus induction medium containing 1.5-6 µM of bialaphos (phosphinothricin based selective agent) (Tables 1-3). The bialaphos content in the medium was sometimes increased in increments ranging from 3-6 µM with 2 weekly transfers to fresh selection medium. After 4-6 weeks on selection medium, cultured embryos that produced white compact calli, presumably somatic embryos, were transferred to regeneration selection medium containing 0.3-1.5 µM bialaphos. Regenerating putative transgenic plants were subcultured at 2-3 weeks intervals until they reached 8-10 cm in height. These were then hardened-off as described by O'Kennedy *et al.* (1998).

4.3.9 Herbicide application

A 2% (v/v) Basta (200 g l^{-1} of the active ingredient, glufosinate ammonium), 0.01% (v/v) Tween 20 solution was applied to both surfaces of selected leaves of transgenic pearl millet plants as described previously (O'Kennedy *et al.*, 1998). Glufosinate and its commercial formulation, Basta® and the tripeptide, bialaphos, or its commercial formulation Herbiace® are both phosphinothricin (PPT)-based selective agents.

4.3.10 Germination of progeny on bialaphos containing medium

Progeny of transgenic plants were germinated on 0.5 MS medium containing half strength MS salts, 44 mM sucrose, 8 g l^{-1} agar and 3 µM bialaphos.

4.3.11 DNA extraction

Genomic DNA was extracted from putative transgenic pearl millet leaf material using a mini extraction procedure (Dellaporta *et al.* 1983).

4.3.12 PCR analysis

Bar specific primers (BAR_L: 5'-CATCGAGACAAGCACGGTCAACTTC-3' and BAR_R: 5'-CTCTTGAAGC CCTGTGCCTCCAG-3') were used to amplify a 0.28 kb fragment, from genomic DNA preparations of putative transgenic pearl millet plantlets.

4.3.13 Southern blot analysis

Five microgrammes of pearl millet genomic DNA either undigested or digested with restriction enzymes (*Sac* I, *Hind* III, *Pst* I or *Eco* RI) were separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy *et al.* (1998). The *uid*A and *bar* genes of pAHC25 were labeled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche Biochemicals).

4.4 RESULTS

4.4.1 Culture medium and genotypes

Pearl millet lines 842B, Bongo nara or Manga nara were used in this study. The pearl millet parental line 842B is used extensively in breeding programmes in Africa and India by ICRISAT, whereas Bongo nara and Manga nara are both breeder lines in the Sudan Savanna of Ghana. Callus media A and J were tested to identify the media and pearl millet genotype combination most suitable for pearl millet transformation (Tables 1-3).

4.4.2 Optimisation of selected bombardment conditions by transient and stable expression of the *uid*A gene

High levels of transient *uid*A expression were obtained following bombardment of cultured IZEs with pAHC25 (Fig. 1). On average, the number of blue foci obtained 48 hours after bombardment for genotype 842B ranged from 89-231 per embryo, pre-cultured for 4-11 days on induction medium A and bombarded at helium pressures ranging from 900-1200 kPa. When embryos of genotype 842B were pre-cultured for 6 or 8 days on medium J and bombarded at 700-1100 kPa, the number of blue foci obtained was in the range of 155-179 or 343-355, respectively (Data not shown).

In a similar way, embryos of genotype Manga nara pre-cultured for 4-7 days on induction medium A resulted on average in 14-61 blue foci per embryo, 48 hours after bombardment at 900-1000 kPa. Embryos of Manga nara pre-cultured for 7-8 days on medium J, resulted in an average number of blue foci per embryo of 81-141 at a helium pressure of 900 kPa. The number of blue foci obtained for genotype Bongo nara pre-cultured for 4-8 days on medium A ranged from 51-157 per embryo, bombarded at 800-1100 kPa. When IZEs of genotype Bongo nara were pre-cultured for 4-10 days prior to bombardment at 800-900 kPa on medium A, the number of blue foci ranged from 28-54 per embryo.

The transient expression results for all three genotypes, 842B, Manga nara and Bongo nara, indicate that the number of days of embryo pre-culture, as well as the different callus induction media, influence the number of blue foci per embryo. However, to avoid targeting multiple cell pre-embryoids in the scutellum of the pre-cultured IZEs (8-14 days), which could result in chimeric transgenic plants, we focused our transformation efforts mainly on embryos pre-cultured for 2-8 days prior to bombardment (Tables 1-3).

Evidence of stably transformed callus was obtained when *uid*A expression was followed over a period of one month on bialaphos free medium with expression determined 48h, as well as 7, 14, 21 and 28 days, post bombardment. As expected, the number of blue foci per embryo dropped dramatically from 48 hours to 28 days after bombardment. However, an immature zygotic embryo of line 842B pre-cultured on medium J for six days before bombardment at 1100 kPa gave rise to a single transgenic somatic embryo 28 days after bombardment (Fig. 2).

Table 1: Conditions for bombardment of pearl millet IZEs of genotype 842B on Medium A and J.

Callus induction medium	# embryos bombarded	Preculture (days)[a]	Helium pressure (kPa)	Selection initiated (days)[b]	Bialaphos selection[c]	Putative transgenics[d]	
						negative	positive
A	534	2-8	900	1-5	B0.5 → B1	148	0
	449	2-8	1000	1-5		46	0
	122	4-8	1100	1-5		38	0
	58	2-4	1200	5		2	0
Total	**1163**					**234**	**0**
A	209	5-6	900	1-4	B1 → B2	38	2
	272	5-6	1000	1-4		8	0
	77	5-6	1100	1-4		19	0
	7	5	1200	2		0	0
Total	**538**					**57**	**2**
A	28	6	1000	4	B2 → B2	0	0
	30	6	1100	4		0	0
	58					**0**	**0**
A*	51	8	900	1	B1 → B1	0	0
	58	8	1100	1		0	0
	109					**0**	**0**
J	157	6	900	1	B0.5 → B1	13	0
	129	6	1000	1		7	0
	32	6	1100	1		5	0
	65	6	1200	1		12	0
Total	**383**					**37**	**0**
J	127	6-7	900	2-4	B1 → B2	4	0
	94	6-7	1000	2-4		3	0
	61	6-7	1100	2-4		0	0
Total	**282**					**7**	**0**
J	48	8	900	1	B1 → B1	0	0
	83	5	1000	2		1	0
	52	8	1100	1		0	0
	22	5	1200	2		0	0
Total	**205**					**1**	**0**

[a] = pre-culture period of IZEs before bombardment
[b] = bialaphos selection initiated after bombardment, indicated by the number of days
[c] = bialaphos (B n mg l^{-1}) selection during the first 4-6 weeks of culture
[d] = number of regenerants screened by application of Basta to sections of leaves, PCR analysis and southern blot analysis for selected plants
* = addition of L-proline

Table 2: Conditions for bombardment of pearl millet IZEs of genotypes Manga nara bombarded on various tissue culture medium.

Callus induction media	# embryos bombarded	Preculture (days)[a]	Helium pressure (kPa)	Selection initiated (days)[b]	Bialaphos selection[c]	Putative transgenics[d]	
						neg	pos
A	119	4-6	900	2	B0.5 → B1	44	0
	97	4-6	1000	2		16	0
Total	**216**					**60**	**0**
A	233	6-7	900	1-7	B1 → B2	1	0
	166	6-7	1000	1-7		4	0
	35	7	1100	4		0	0
	50	6-7	1200	1		1	0
Total	**484**					**6**	**0**
J	55	7	900	1	B1 → B2	0	0
	20	7	1000	1		0	0
Total	**75**					**0**	**0**
J	150	6-7	900	1-4	B1 → B2	14	0
	194	6-7	1000	1-4		3	0
	114	6-7	1100	1-4		0	0
	99	6-7	1200	1		8	0
Total	**557**					**25**	**0**

[a] = pre-culture period of IZEs before bombardment
[b] = bialaphos selection initiated after bombardment, indicated by the number of days
[c] = bialaphos (B n mg l^{-1}) selection during the first 4-6 weeks of culture
[d] = number of regenerants screened by application of Basta to sections of leaves or PCR analysis of selected plants

Table 3: Conditions for bombardment of pearl millet IZEs of genotypes Bongo nara bombarded on various tissue culture medium.

Callus induction media	# embryos bombarded	Preculture (days)[a]	Helium pressure (kPa)	Selection initiated (days)[b]	Bialaphos selection[c]	Putative transgenics[d]	
						neg	pos
A	94	6	900	2	B0.5 → B1	4	0
	66	6	1000	2		6	0
Total	**160**					**10**	**0**
A	311	4-8	900	2-7	B1 → B2	9	0
	296	4-8	1000	2-7		3	0
	170	5-8	1100	2-6		1	0
Total	**777**					**13**	**0**
A	31	5	900	2	B2 → B2	1	0
	29	5	1000	2		0	0
Total	**60**					**1**	**0**
J	57	5	900	2-6	B1 → B2	0	0
	70	5	1000	2-6		2	0
	26	5	1100	6		0	0
Total	**153**					**2**	**0**

[a] = pre-culture period of IZEs before bombardment
[b] = bialaphos selection initiated after bombardment, indicated by the number of days
[c] = bialaphos (B n mg l^{-1}) selection during the first 4-6 weeks of culture
[d] = number of regenerants screened by application of Basta to sections of leaves or PCR analysis of selected plants

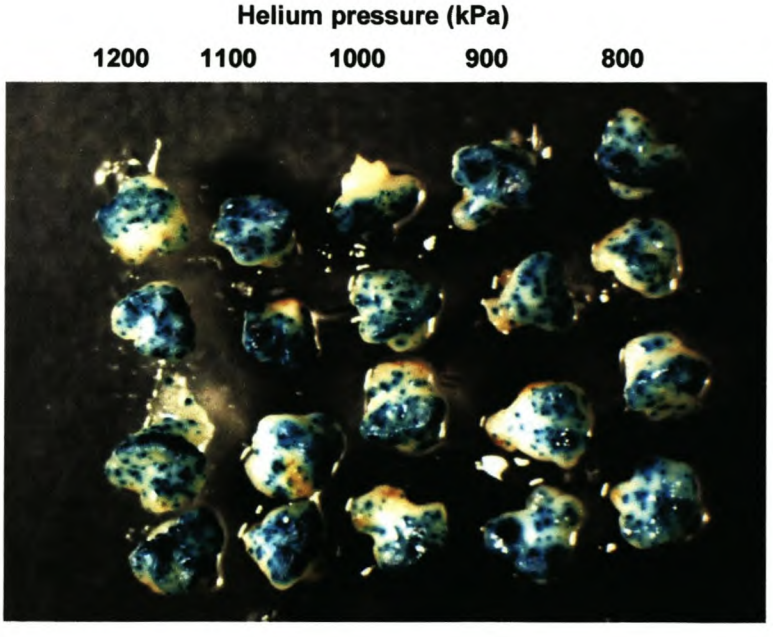

Figure 1: Transient GUS activity in scutellum cells of IZEs of line 842B, pre-cultured for 11 days on callus induction medium A prior to bombardment. The embryos were stained for GUS activity 48h after bombardment.

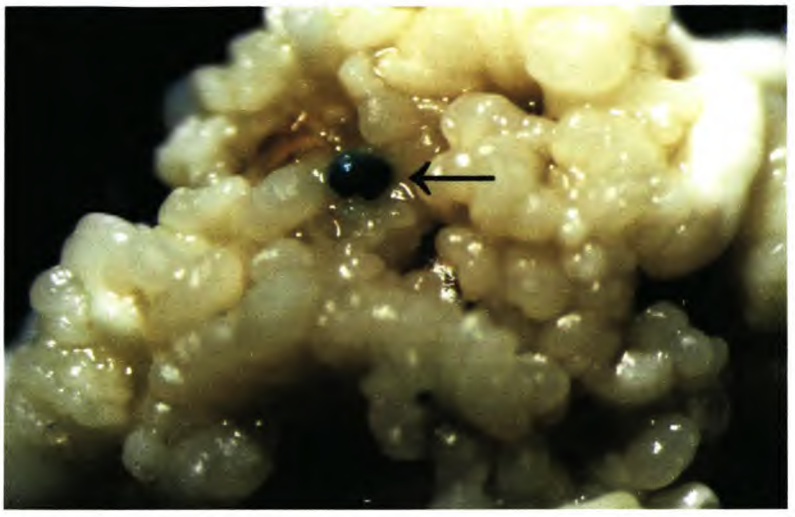

Figure 2: A transgenic somatic embryo expressing the *uid*A (GUS) gene (dark blue embryo, indicated by arrow) amongst non-transgenic white compact somatic embryos.

4.4.3 Selection of transgenic tissues and regeneration of T_0 plants

A range of pre-culture periods on various tissue culture media, bialaphos selection conditions and bombardment pressures were applied in attempts to obtain stable transformation for three different genotypes 842B, Bongo nara and Manga nara (Tables 1-3). A total number of 2738 IZEs of genotype 842B were bombarded as well as 1332 and 1150 of the Ghanaian genotypes Manga nara and Bongo nara, respectively. Putatively transformed callus tissue was identified by its vigorous growth on bialaphos-containing medium, an indication of the stable integration and functionality of the *bar* gene in the pearl millet genome. Non-transformed tissue turned brown and watery on selection media. Bialaphos-resistant cells or cell clumps developed embryogenic calli which matured to form somatic embryos on medium containing 1.5-6 µM (0.5-2.0 mg l^{-1}) bialaphos and a subsequent step-wise increase to 3-9 µM (1-3 mg l^{-1}) bialaphos (Tables 1-3). Subsequently, putative transgenic calli were transferred to regeneration and rooting medium containing 0.3-1.5 µM (0.1-0.5 mg l^{-1}) bialaphos. Regenerated putative transgenic plantlets were hardened-off in the greenhouse as described in materials and methods and screened for the presence or the absence of the transgenes.

The successful transformation events of pearl millet genotype 842B were obtained by the following protocol: IZEs were pre-cultured for five days on callus induction medium A, bombarded at 900 kPa, and transferred to selection medium 24 hours after bombardment. Transgenic tissue was selected on induction medium containing 3 µM (1 mg l^{-1}) bialaphos (weeks 1-4), followed by 6 µM (2 mg l^{-1}) (weeks 5-6). Thereafter, remaining tissue was transferred to regeneration medium supplemented with 0.5 mg l^{-1} (weeks 7-9) followed by 0.1 mg l^{-1} bialaphos (weeks 10-15). Subsequently, putative transgenic regenerants were transferred

to rooting medium supplemented with 0.3 µM (0.1 mg l^{-1}) bialaphos for an additional 8-10 weeks. Thereafter the plant was hardened-off to the greenhouse. The total duration in tissue culture after bombardment on bialaphos selection was therefore approximately five and a half months.

It is clear from the data presented that a high number of escapees were obtained for genotype 842B even at bialaphos concentrations of 3-6 µM (1-2 mg l^{-1}). However, the transgenic events BB206.10A and BB206.10I were obtained when a short pre-culture period, five days, proceeded bombardment and bombarded IZEs were transferred to 3 µM (1 mg l^{-1}) bialaphos 24h after bombardment. Furthermore, within two weeks the calli were transferred to callus induction medium containing 6 µM (2 mg l^{-1}) bialaphos. Subsequently, the bombardment experiments with genotypes Bongo nara and Manga nara were conducted with short pre-culture periods of four to eight days and bombarded IZEs were transferred to bialaphos containing medium within 1-7 days after bombardment (Tables 2-3).

4.4.4 Analysis of transgenic plants

To date, bombarded IZEs of genotype 842B cultured on callus induction medium A (1868) or J (870) gave rise to numerous putative transgenic pearl millet plants. However, only two, designated as transformation events BB206.10A and BB206.10I, of the 455 plants screened were positive for the *bar* transgene. *Uid*A gene expression was also present in leaves of these plants (Fig. 3).

Figure 3: GUS negative, untransformed (left) and GUS positive (stable integration and expression of the u*idA* gene) leaf segments of transgenic plant BB206.10A (right).

Digests of both transformation events BB206.10A and BB206.10I with *Sac* I, *Hind* III and *Pst* I appear identical in integration pattern of the *bar* transgene (Fig. 4), which hints that these two plants represent clones of the same transformation event. However, digestion with restriction enzyme *Eco* RI, a restriction enzyme that releases the *bar* gene, indicates that BB206.10A and BB206.10I were distinct transformation events. This might be due to a different methylation status in these transgenic plants. The transformation efficiency is then 0.04% if calculated only for genotype 842B, and 0.02% if results of Manga nara and Bongo nara are included.

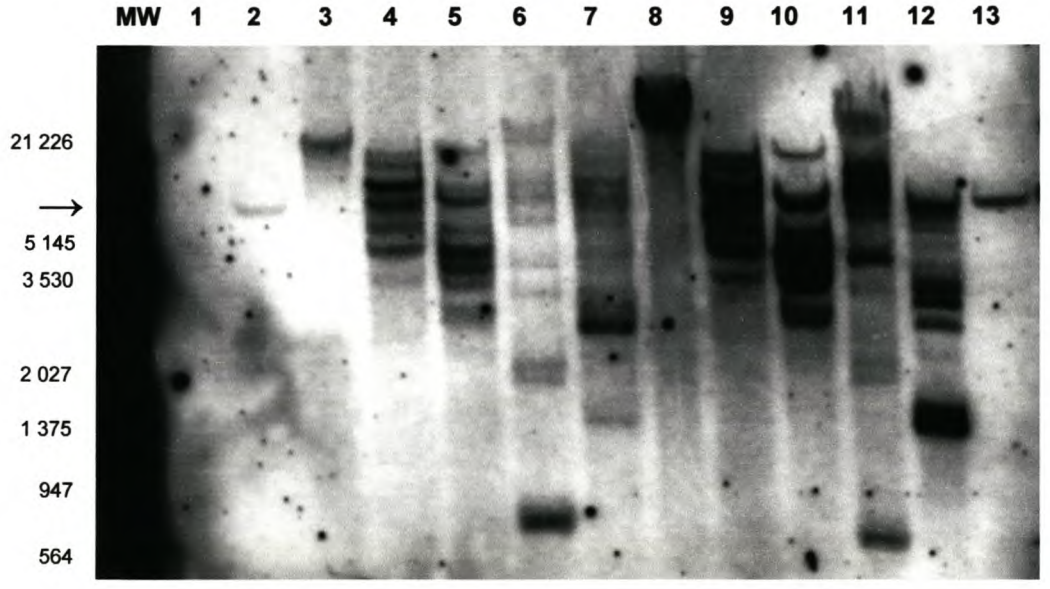

Figure 4: Southern blot analysis of two transgenic T_0 plants of pearl millet line 842B, transformation events BB206.10A and BB206.10I. Genomic DNA was purified from plant leaf material, restricted with *Sac* I, *Hind* III, *Pst* I or *Eco* RI and resolved in a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal *bar* fragment. MW, DIG labelled molecular weight marker III (Roche Biochemicals); lane 1, untransformed pearl millet DNA, line 842B; lanes 2 & 13, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lanes 3, 1 µg undigested DNA from event BB206.10A; lanes 4-7, BB206.10A DNA digested with *Sac* I, *Hind* III, *Pst* I and *Eco* RI; lane 8, 5 µg undigested DNA from event BB206.10I; lanes 9-12, BB206.10I DNA digested with *Sac* I, *Hind* III, *Pst* I and *Eco* RI. The arrow indicate the size of pAHC25 restricted once with *Sac* I, which is 9.7 kb.

4.4.5 Progeny of transformation events

The transgenic plants designated BB206.10A and BB206.10I were used as pollen recipients and pollinated with untransformed pearl millet line 842B. Transformation event BB206.10A produced only non-trangenic progeny (110 seeds). None of the progeny contained the transgenes of the primary transformant BB206.10A as determined by application of Basta to leaves, *uid*A staining of leaf material and selected plants screened by PCR and southern blot analysis. Transformation event BB206.10I produced in total 40 seeds. Once more both the *uid*A (GUS) and *bar* gene were absent in the progeny. This indicates that both pearl millet transgenic plants BB206.10A and BB206.10I were chimeric.

4.5 DISCUSSION

In this study, IZEs were used as explant material for stable integration of marker genes into the genome of pearl millet. Morphological and histological studies of cultured IZEs of pearl millet have shown that single subepidermal cells at the periphery of the scutellum in the coleorhizal end undergo internal segmenting divisions, resulting in discrete groups of richly cytoplasmic cells (Vasil and Vasil, 1981 & 1982). These cells, which were differentiated, and attained embryogenic and transformation competence during the early stages of culture, could be perpetuated by subculture, and gave rise to embryoids and plants. Furthermore, there is sufficient evidence in the literature (Lu and Vasil, 1985, Woodward, 1989) to show that grass and cereal somatic embryos in tissue culture either arise directly from single cells, or indirectly from a proembryonal complex which itself arises from a single cell. Somatic embryos of pearl millet fit the same pattern (Vasil and Vasil, 1982). The single cell origin of cereal somatic embryos is essential, since regenerated plants of multicellular origin or tissue culture complex may result in chimerism.

In the development of a transformation protocol for pearl millet, genotypes were selected on their regeneration potential and used in the following order of importance: 842B, Manga nara and Bongo nara. Scutellum cells of pre-cultured IZEs were bombarded with DNA coated tungsten particles, accelerated by the PIG. The pre-cultured IZEs were used in all biolistic experiments, as limited success was achieved with the culture of pearl millet leaf base explants in our laboratory (Chapter 3). However, the growth of mature donor plants for a regular supply of IZEs is labor intensive, time and space consuming.

High levels of transient expression were obtained following bombardment of pre-cultured IZEs using the expression levels of the *uid*A gene as reporter. In this study, transient expression studies were only done as a guide in the development of systems for stable pearl millet

transformation. In order to establish a protocol for stable integration of the transgenes into the genome of pearl millet, the following parameters were varied 1) tissue culture medium 2) pre-culture period 3) helium pressures for bombardment 4) number of days post bombardment before commencement of selection and 5) selection pressures (Tables 1-3).

The low selection pressures are most probably the reason for the numerous escapees obtained in this study. However, high selection pressures might result in the selection of transgenic plants with multicopy transgene inserts. This is undesirable, as multicopy complex inserts, abundant specifically during direct DNA transformation processes such as biolistics, is often a major cause of gene silencing (Iyer *et al.*, 2000).

Two pearl millet transformation events were obtained for genotype 842B when IZEs were cultured on medium A and bombarded with 160 ng plasmid DNA per shot. Southern blot analysis confirmed that the two transgenic plants were clones. Furthermore, a single transgenic somatic embryo was obtained when IZEs of genotype 842B were cultured and bombarded on the medium J. However, the *uid*A assay is lethal and therefore it was not possible to regenerate a transgenic plant from the single transgenic somatic embryo obtained.

Genetic engineering of pearl millet for the control of diseases such as *S. graminicola*, causal agent of downy mildew, will have a major impact on pearl millet production in the developing world. Therefore, the next goal will be to introduce antifungal genes conferring resistance to *S. graminicola* into the genome of the transformable parental pearl millet line 842B.

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Chapter 5

Pearl millet (*Pennisetum glaucum* [L.] R.Br.) transformation system using the positive selectable marker gene phosphomannose isomerase

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5.1 ABSTRACT

Fertile transgenic pearl millet plants expressing a phosphomannose isomerase (PMI) transgene under control of the maize ubiquitin constitutive promoter were obtained in this study. Proliferating immature zygotic embryos were used as target tissue for bombardment using a particle inflow gun. Different culture and selection strategies were assessed to optimise the mannose selection protocol. Stable integration of the *man*A gene into the genome of pearl millet was confirmed by PCR and southern blot analysis. Stable integration of the *man*A transgene into the genome of pearl millet was demonstrated in T_1 and T_2 progeny of two independent transformation events with no more than 4-10 copies of the transgene. Similar to previous studies with maize and wheat, the *man*A was shown to be a superior selectable marker gene for improving transformation efficiencies when compared to antibiotic or herbicide selectable marker genes.

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5.2 INTRODUCTION

Pearl millet (*P. glaucum* formerly *P. americanum*) is a staple food for the world's poorest and most food-insecure people in Africa and India. It is grown largely for its ability to produce grain under hot, dry conditions on infertile soils of low water-holding capacity, where other crops generally fail. Thus, it is produced mainly in outlying areas peripheral to the major production and population centres of the developing world and accounts for approximately 15 million tons. The development of a reliable transformation protocol for pearl millet will form the basis for future genetic enhancement of this crop by complimenting classical breeding programmes for the benefit of India and sub-Saharan Africa.

Procedures for pearl millet transformation based on the use of the helium-driven PDS-1000/He or the particle inflow gun (PIG) using the *bar* herbicide resistant gene, or hygromycin phosphotransferase (*hph*) as selectable marker genes and/or *uid*A (GUS) as reporter gene in transgenic pearl millet have been published (Girgi *et al.* 2002; Goldman *et al.* 2003; Lambé *et al.* 1995 and 1999). Apart from the work presented by Goldman and co-workers (2003), targeting inflorescence derived embryogenic tissue, pearl millet transformation is still limited by relatively low and erratic stable transformation efficiencies.

Furthermore, inadequate knowledge of antibiotic or herbicide resistant genes' impact on the environment (gene transfer to the wild relatives especially for pearl millet) and on human health, especially in food crops, has caused widespread public concern (Wang *et al.*, 2000).

It is well known, for genetic engineering of plants, that only a minor fraction of the treated cells become transgenic while the majority of the cells remain untransformed and need to be eliminated by selection (Joersbo and Okkels 1996). During negative selection, the majority of the cells in the cultured tissue die. These dying cells may release toxic substances (such as phenolics), which in turn may impair regeneration of the transformed cells. In addition, dying cells may form a barrier between the medium and the transgenic cells preventing or slowing uptake of essential nutrients (Joersbo and Okkels 1996). The selection system based on herbicide (Girgi *et al.* 2002) or antibiotics resistance (Zhang and Puonti-Kaerlas 2000) either allows regeneration of escapes, even at high selection pressure, or are deleterious to the regeneration process. In contrast, the mannose positive selection system favours regeneration and growth of the transgenic cells while the non-transgenic cells are starved but not killed. Therefore, untransformed tissue is separated from transgenic tissue by carbohydrate starvation of the untransformed cells.

The mannose selection system employs the phosphomannose isomerase (PMI) expressing gene (*man*A) as selectable marker gene and mannose, converted to mannose-6-phosphate by endogenous hexokinase, as selective agent (Joersbo *et al.* 1999). Transgenic PMI-expressing cells acquire the ability to convert mannose-6-phosphate to fructose-6-phosphate, while the non-transgenic cells accumulate mannose-6-phosphate. Phosphorylation of mannose triggers a signalling cascade resulting in the repression of genes needed for germination (Pego *et al.* 1999) and energy depletion during seed germination (Wang *et al.*, 2000). Mannose, readily taken up by the roots, inhibits the germination of seeds as a consequence of mannose's phosphorylation and not by the toxicity of the compound per se (Joersbo *et al.* 1998; Negrotto *et al.* 2000). Subsequently, the accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, causing a block in glycolysis, and inhibits respiration by competitive inhibition of phosphoglucose isomerase (Goldsworthy and Street 1965). Pego and co-workers (1999) showed that the absence of germination of Arabidopsis seed in the presence of mannose was not due to ATP or phosphate depletion. These authors reported that mannoheptulose, a hexokinase specific inhibitor, restores germination of these seeds and therefore concluded that mannose-mediated repression of germination is the result of a hexokinase mediated carbohydrate-induced gene regulation.

The structural gene (*man*A) from *Escherichia coli* was previously used to successfully produce transgenic maize (Negrotto *et al.* 2000; Wang *et al.* 2000), cassava (Zhang and Puonti-Kaerlas 2000) and sugarbeet (Joersbo *et al.* 1998 and 1999). The *man*A gene was superior to antibiotic or herbicide (*pat* or *bar*) selectable marker genes for plant transformation in maize, wheat and sugar beet (Joersbo *et al.* 1998; Reed *et al.* 2001; Wright *et al.* 2001). Furthermore, a preliminary risk assessment done by Reed and co-workers (2001) indicated that the PMI protein in transgenic maize was 1) readily digested in simulated mammalian gastric and intestinal fluids, 2) there was no detectable changes in glycoprotein profiles and 3) no statistically significant differences were obtained in yield and nutritional composition compared to untransformed maize. Furthermore, the database search revealed no significant homology of the *E. coli man*A gene product to any known toxin or allergen (Reed *et al.* 2001).

The aim of this study was to use the positive mannose selectable marker gene technology to 1) limit the number of escapes, 2) improve the transformation efficiency and 3) avoid using antibiotic or herbicide resistant genes as selectable marker genes in pearl millet transformation.

5.3 MATERIALS AND METHODS

5.3.1 Seed material

Pearl millet seed, genotype 842B, was kindly provided by ICRISAT, Zimbabwe. Seedlings were planted in a soil mix consisting of red soil, rough sand and compost (1:1:1), and were watered three times per week with a soluble fertiliser (Hortichem N:P:K at 3:1:5, Ocean Agriculture South Africa) until flowering. Covering the flowers with brown paper bags prevented cross-pollination.

5.3.2 Excision of IZEs

Greenhouse-grown florets of pearl millet containing IZEs (10-14 days post-pollination) were soaked in 70% (v/v) ethanol for one minute and sterilised for 15 minutes in a 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20, before being thoroughly rinsed with sterile distilled water. IZEs (0.5 – 1 mm in size) were aseptically excised from the florets using a dissecting microscope and placed with their axes in contact with the callus induction medium. All tissue culture was performed under aseptic conditions.

5.3.3 Tissue culturing of IZEs

Callus induction medium A is described by Pinard and Chandrapalaiah (1991), and contains MS salts (Murashige and Skoog 1962), 9 µM 2,4 D, 88 mM sucrose as carbon source and 8g l^{-1} agar as solidifier. Cultures initiated on medium A induction medium were further cultured

on regeneration medium as described by Pinard and Chandrapalaiah (1991), containing the hormones IAA (1 µM) and kinetin (2 µM), but the medium was modified by the addition 60 µM $AgNO_3$. These cultures were subsequently transferred to rooting medium, which was identical to the regeneration medium, except that both hormones and silver nitrate were omitted.

Callus induction medium, designated medium J in this study, is described by Gless and co-workers (1998) and contains L3 salts and vitamins as described by Jähne *et al.* (1991), 11 µM 2,4-D, 83 mM maltose as a carbon source, 4 g l^{-1} Gelrite as solidifier and modified by supplementing the medium with 2.3 g l^{-1} (20 mM) L-Proline. L-Proline and 2,4-D were omitted from regeneration and rooting medium. Cultures initiated on medium J, were matured on regeneration medium containing double the amount of carbohydrates for a period of two weeks. The matured cultures were then regenerated and rooted on regeneration and rooting medium as described above.

Cultures on callus induction and regeneration media were incubated at 24-25°C, under low-light conditions (1.8 µmolm^{-2} s^{-1}), whereas regenerating shoots (≥1 cm) were incubated under dim light (18 µmol m^{-2} s^{-1}).

5.3.4 Transfer regime

Immature zygotic embryo derived calli were transferred to fresh medium every two weeks. White compact calli were produced within 4 weeks on callus induction medium A, but only after 6 weeks on medium J: callus induction (4 weeks) and maturation (2 weeks) medium. After 4 or 6 weeks on A or J based medium, respectively, calli were transferred to the appropriate regeneration media as described above, and subcultured every three weeks to fresh media. Plantlets of >1 cm, produced on medium A, was transferred to rooting medium. Rooted plantlets grown to 8-10 cm were hardened-off to a mist bed for approximately two weeks and then transferred to pots in the greenhouse. Rooted plantlets of >1 cm, produced on medium J, remained on the regeneration medium described for this regime until they were hardened-off.

5.3.5 Plasmids

The construct pNOV3604 was obtained from Syngenta, and the ubiquitin promoter inserted upstream of the *man*A gene as a *Hind* III/*Bam* HI insert (Fig. 1A). Plasmid pNOV3604ubi DNA was extracted from overnight *E. coli* cultures using a Qiagen (Southern Cross Biotechnologies) maxiprep kit according to the supplier's recommendation.

5.3.6 Microprojectile bombardment

Pre-cultured IZEs were placed in the middle (0 - 1 cm diameter) of a 9 cm petri dish, containing A or J induction media supplemented with approximately 25% (m/v) increased solidifier, plus 0.2 M D-Sorbitol and 0.2 M D-Mannitol described by Vain and co-workers (1993). A bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles with $CaCl_2$ and spermidine free base as described by O'Kennedy and co-workers (1998). All experiments were conducted with the particle inflow gun (PIG). Sixteen hours after bombardment, unless otherwise stated, bombarded tissue was transferred to the respective media omitting D-Sorbitol and D-Mannitol. DNA delivery parameters were as follows: 900-1000 kPa, 0.16 µg DNA per shot, 17 cm from the target tissue and a 500 µm nylon mesh screen placed 8 cm above the target. A vacuum of approximately -87 kPa was applied and the bombardment mix particles on the filter syringe were discharged when the helium was released following activation of the solenoid. The timer duration was 50 milliseconds.

5.3.7 Selection and regeneration of transformants

Selection for *man*A containing and expressing pearl millet tissue was initiated 2-7 days after bombardment by placing the cultured IZE on selection medium. Selection medium containing maltose (medium J) or sucrose (medium A) supplemented with mannose is described under results and discussion and indicated in tables 1-2 for each individual experiment. The mannose content in the medium was kept constant throughout the selection period or stepwise increased ranging from 30-83 µM (5-15 g l^{-1}) whilst lowering the content of sucrose or maltose. After 4-6 weeks on selection medium, cultured embryos that produced white compact calli, presumably somatic embryos, were transferred to regeneration selection medium. Regenerating putative transgenic plants were subcultured at 2-3 weeks intervals until they reached 8-10 cm in height. These were then hardened-off as described by O'Kennedy and co-workers (1998).

5.3.8 Germination of progeny on mannose containing medium

Progeny of transgenic plants were germinated on 0.5MS medium containing half strength MS salts, 4.5 mM (1 g l^{-1}) sucrose, 83 mM (15 g l^{-1}) mannose and solidified by 8 g l^{-1}agar.

5.3.9 DNA extraction

Genomic DNA was extracted from putative transgenic pearl millet leaf material using the mini extraction procedure of Dellaporta and co-workers (1983).

5.3.10 PCR analysis

Primers *manA* (PMI fwd: 5'-CGT TGA CTG AAC TTT ATG GTA TGG-3' and PMI as: CAC TCT GCT GGC TAA TGG TG-3') specific primers were used to amplify a 965 Kb fragment, from genomic DNA preparations of putative transgenic pearl millet plantlets.

5.3.11 Southern blot analysis

Five micrograms of pearl millet genomic DNA digested with *Sac* I was separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy *et al.* (1998). The *man*A gene of pNOV3604ubi was labeled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche Diagnostics South Africa).

5.4 RESULTS AND DISCUSSION

In this study, the mannose containing medium was supplemented with sucrose, glucose or maltose, in order to alleviate the toxic effect of mannose-6-phosphate. A dose-response curve was designed for pearl millet, testing increasing mannose content and decreasing maltose content (Table 1). The results showed that mannose inhibited callus production in a dose-dependent manner up to 110 mM (20 g l^{-1}) where growth was 95% suppressed, even when supplemented with 6 mM (2 g l^{-1}) maltose (Table 1). In previous studies, it was shown that the indirect toxic effect of mannose caused by the conversion to mannose-6-phosphate by endogenous hexokinase increases with decreasing sucrose concentration in the medium indicating an interaction between mannose and sucrose (Joersbo *et al.*, 1998). The addition of sucrose, therefore, can alleviate the effect of mannose on growth and germination. Pego and co-workers (1999) reported that the addition of metabolisable sugars reversed the mannose-6-phosphate mediated inhibition of germination of Arabidopsis seed. Joersbo and co-workers (1999) reported that the phytotoxic effect of mannose-6-phosphate was strongly dependent on the nature and concentration of the added saccharides. In particular, glucose interacts strongly with the mannose-6-phosphate caused phytotoxic effect (Goldsworthy and Street 1965; Joersbo *et al.* 1999). Glucose was found to be 4-7 fold more potent than the other saccharides (sucrose, maltose and fructose) tested and was able to eliminate the mannose-6-phosphate toxicity at concentrations 15-25 fold higher than mannose (Joersbo *et al.* 1999).

In this study, various parameters were applied to optimise pearl millet transformation using the *man*A transgene: 1) tissue culture media A or J, 2) preculture of IZEs of 4-10 days, 3) bombardment at a helium pressure of 900 or 1000 kPa, 4) selection initiated 2-7 days after bombardment, 5) single (160 ng per shot) or double bombardment (320 ng plasmid DNA introduced in total) and 6) selection pressure ranging from 44 mM (15 g l^{-1}) sucrose or maltose supplemented with 30 mM (5 g l^{-1}) mannose to 110 µM (20 mg l^{-1}) glucose or

maltose supplemented with 83 mM (15 g l^{-1}) mannose, for media regimes A or J, respectively (Table 2). However, the selection pressure was subsequently set for both media A and J on 83 µM (15 g l^{-1}) mannose, the osmotic equivalent of 88 µM (30 g l^{-1}) sucrose, supplemented with 2 g l^{-1} sucrose/maltose (60/6 mM) or 20 mg l^{-1} glucose/maltose (110/56 µM), for medium A/J respectively.

Table 1: Response of IZEs cultured on mannose-containing medium J, supplemented with maltose. Twelve embryos per plate were cultured, three replicates per treatment.

Mannose g l^{-1}	Maltose g l^{-1}	Average fresh weight g	% inhibition
0	30	3.65 ± 0.74	0
2.5	20	2.41 ± 0.38	34
2.5	12	2.26 ± 0.49	38
5	10	1.75 ±0.26	52
5	5	1.10 ± 0.15	70
10	2	0.57 ± 0.20	84
20	2	0.17 ± 0.02	95

Transgenic selection was carried out on mannose for the full period as indicated in Table 2, until regenerated plantlets were hardened-off to the greenhouse. Thirteen transformation experiments using the full pNOV3604ubi construct containing the *man*A gene were performed (Table 2). Several combinations of mannose and sucrose or mannose and maltose in tissue culture media regime A and J, respectively, were assessed using predominantly mannose. A constant high selection pressure of mannose was applied for experiments 1-2, 7-8 and 13, whereas a stepwise increase of the selection pressure was applied for experiments 3-6 and 9-12 (Table 2), until pearl millet plantlets were hardened-off to the greenhouse. Callus tissue, within the first two months, encompassing four transfers, failed to proliferate further and became watery and browning when cultured on high selection pressure of 2/15 (2 g l^{-1} sucrose or maltose and 15 g l^{-1} mannose) or 0.02/15 (20 mg l^{-1} glucose or maltose and 15 g l^{-1} mannose). Nevertheless, seven out of the eight independent transformation events arise on the high selection pressure (Table 2). The total period in tissue culture, from the excision of IZE until plantlets are hardened-off to the greenhouse, was 12 weeks.

Table 2: Conditions for particle bombardment of pearl millet IZEs (genotype 842B). A sorbitol/mannitol osmoticum treatment or 120 g l^{-1} sucrose (medium A) or maltose (medium J) osmotic treatment was used (sucrose or maltose osmotic treatment in italics) before bombardment with plasmid DNA of pNOV3604ubi (160 ng per shot). Parameters that lead to the production of stable transgenic plants are underlined.

Exp number[a]	# embryos	Preculture (days)[b]	Helium pressure (kPa)	Osmoticum treatment (h) +osm/-osm	Selection[c]	Mannose selection history[d]	# of putative regenerants	Transgenic T_0 plants designated
1	27	6 or 7	900	16/56	3	ind 15/5 → reg 15/5	0	
	34					ind 5/15 → reg 5/15	0	
2	48	6 or 7	1000	16/56	3	Ind 15/5 → reg 15/5	0	
	38					ind 5/15 → reg 5/15	0	
	36					ind 2/15 → reg 2/15	1	Man 2.24
3	80	4 or 5	900	16/56	3	Ind 15/5 → reg 1/15	0	
4	25	10	900	16/32	2	Ind 15/5 → reg 15/5 → reg 1/15	0	
5	27	6	900	16/56	3	Ind 5/15 → reg 5/15 → reg 1/15	0	
	27		1000				0	
6	40	7	1000	16/152	7		0	
	40			168/0		Init 0.02/15 → reg 0/15	0	
	40			***168/0***			0	
	20	5		16/152			0	
	20			168/0			0	
	20			***168/0***			0	
	522						**1**	**1**
7	19	6 or 7	900	16/56	3	ind 15/5 → reg 40/10 → reg 15/5	0	
	58					ind 2-5/15 → reg 4/20 → reg 2-5/15	1	Man 1.31
8	44	6 or 7	1000	16/56	3	Ind 15/5 → reg 40/10 → reg 15/5	0	
	49					Ind 5/15 → reg 20/20 → reg 5/15	0	
	44					Ind 2/15 → reg 4/20 → reg 2/15	0	
9	96	4 or 5	900	16/56	3	Ind 15/5 → reg 40/10 → reg 1/15	1	Man 4.3
10	68	10	900	16/32	2	15/5 → 40/10 → 1/15	0	
11	31	6	900	16/56	3	Ind 5/15 → reg 5/25 → reg 5/15 → reg 1/15	2	Man 7.1 J1 & J3
	36		1000				3	Man 7.1 KK1, 2, 4
							6	Man 7.1 M1-4, O1
12	60	7	1000	16/152	7		0	
	40			168/0		Ind 0.02/15 → reg 0.02/30 → reg 0/15	1	
	40			***168/0***			0	
	20	5		16/152			0	
	20			168/0			0	
	20			***168/0***			0	
13	58*	7	900	120/48	7		0	
	88			120/48		Ind 0.02/15 → reg 0.02/30 → reg 0.02/15	2	Man 11.6 A & B
	57			16/152			3	Man 11.7 A - C
	57			***168/0***			0	
	45			120/48			1	
	26	5		16/152			1	
	976						**22**	**17**

[a] = Experiment (Exp) number of cultures on medium A (experiments 1-6) or medium J (experiments 7-13).

[b] = Pre-culture period of IZEs before bombardment

[c] = Mannose selection initiated after bombardment, indicated by the number of days

[d] = Sucrose/mannose (n mg l^{-1}) (medium A) or maltose/mannose (n mg l^{-1}) (medium J) selection during callus induction (ind, month 1) and regeneration (reg, months 2-6); glucose was used instead of sucrose for low selection (0.02 mg l^{-1}) for medium A

* = Double bombardment, 160 ng per shot, therefore 320 ng introduced in total

Primary transgenic plantlets were identified by PCR and subsequently Southern blot analysis of primary transformants and their progeny (Figures 1 and 2). Genomic DNA was digested with *Sac* I a unique restriction site in the construct. Eighteen of the twenty-three putative transgenic plants contained the transgene *man*A (Table 2). Escapees were therefore limited to approximately twenty percent, which resulted in a major reduction in molecular analysis of untransformed plants. Only eight of the eighteen transformation events had a unique integration pattern of the transgene *man*A (Figures 1 and 2). Although occasionally high copy number integration were obtained (Fig 1), the transgenic plants producing T_1 and T_2 progeny had a low copy number integration of the *man*A transgene (utmost 4-10 copies in tandem, Fig. 2). Furthermore, the integration pattern was maintained from one generation to the next. The southern blot of transformation event clones Mann 7.1 KK1 and KK2 clearly showed an intact band of 6.21 Kb, which corresponded to the linear pNOV3604ubi construct of an identical size and indicate the intactness of the *man*A gene (Fig. 2).

The transformation efficiency using the particle inflow gun and the herbicide resistance gene, *bar,* was improved from 0.02% on medium regime A (Girgi *et al.*, 2002), to 0.19 and 0.72% with *man*A as selectable marker gene on media regime A and J, respectively. However, an individual experiment gave a frequency of 3% (Table 2, experiment 11). The highest percentage of transformation efficiency was obtained for experiment eleven, where a stepwise increase of the selection pressure was applied. This translated to one transformation event per plate, which contains on average 31-35 pre-cultured IZE per plate. Eighteen of the 23 *man*A putative transgenic plants, surviving the mannose selection pressure were transgenic, as confirmed by southern blot analysis (Table 2). Eight of the eighteen transgenic plants obtained had a unique integration pattern, whereas the other ten were clones. Although the transformation efficiency is still low, the mannose positive system is effective in selecting almost only transgenic tissue and eventually transgenic plants. In contrast, 455 putative transgenic plants were regenerated on the bialaphos (1-2 mg l^{-1} bialaphos, active ingredient of the herbicide Basta®) selection system of which only two stable transgenic plants, clones, contained the *bar* transgene (Girgi *et al.* 2002). Therefore, using the *man*A selectable marker gene for pearl millet transformation does not only increase the transformation efficiency (highest ever recorded was 0.28% using the PDS 1000/He particle gun; Girgi *et al.*, 2002), but also provide an effective selection system, which eliminates the labour intensive tissue culture selection and molecular analysis of putative transgenics.

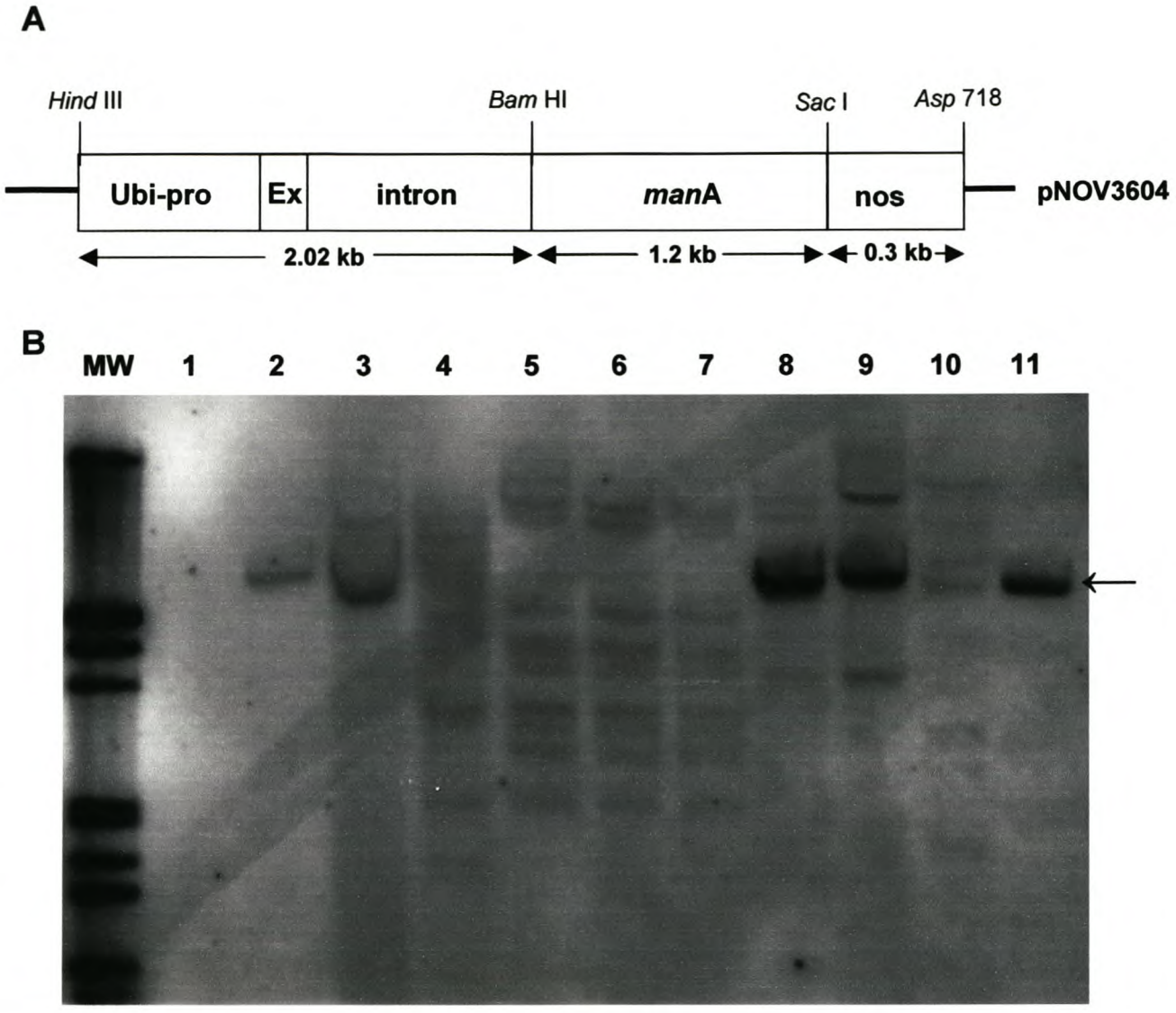

Figure 1: A. Diagram of construct pNOV3604ubi used for pearl millet transformation. Plasmid pNOV3604ubi (6210 bp) contains the *man*A selectable marker gene, under the control of the maize *Ubi1* promoter, first exon (Ex) and first intron, and the nopaline synthase terminator (nos). B. Southern blot analysis of independent T_0 transformation events of pearl millet genotype 842B. Genomic DNA was purified from plant leaf material, restricted with *Sac* I and resolved in a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal fragment of the *man*A transgene. MW, DIG labelled molecular weight marker III (Roche Diagnostics South Africa); lane 1, untransformed pearl millet DNA, line 842B; lanes 2 and 11, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lane 3, Mann 1.31, lane 4, Mann 4.3; lanes 5-7, clones of Mann 7.1 designated J1, M2 and M4; lane 8, Mann 11.6A; lane 9, Mann 11.6B; lane 10, Mann 11.7C. The arrow indicates the size (6.21 kb) of pNOV3604ubi linearised with *Sac* I.

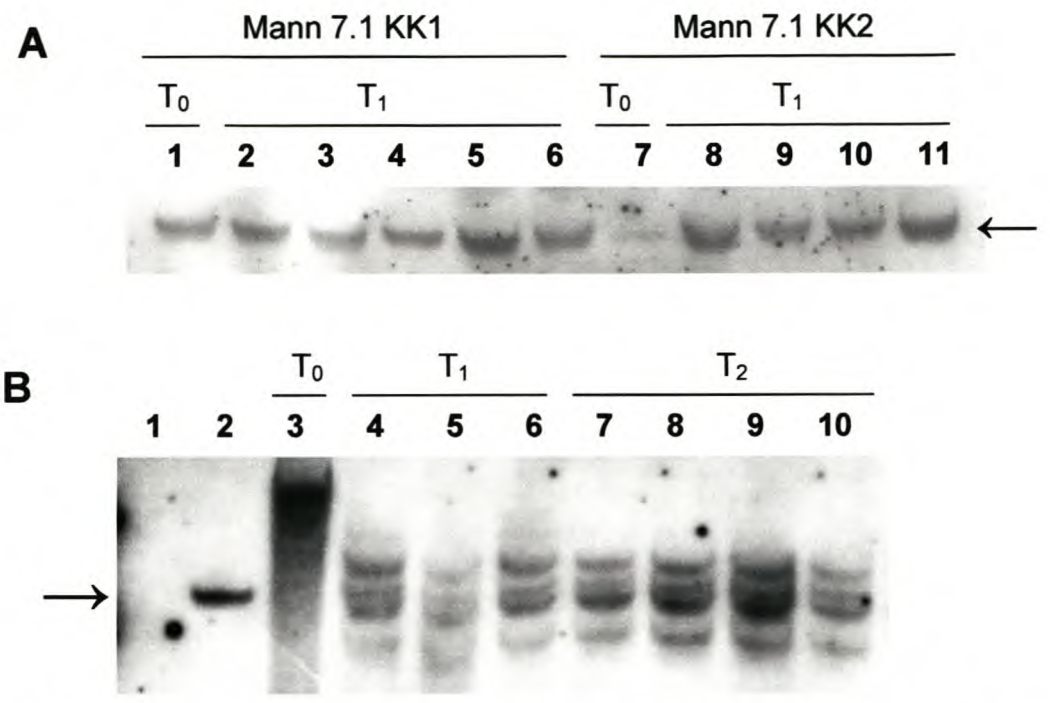

Figure 2: Southern blot analysis of progeny of transgenic pearl millet genotype 842B: A) two clones of transformation event designated Mann 7.1 KK1 and KK2 or B) transformation event designated Mann 2.24. Genomic DNA was purified from plant leaf material, restricted with *Sac* I and resolved in a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal fragment of the *man*A transgene. A) lane 1, Mann 7.1 KK1 T_0, lane 2-6, individual Mann 7.1 KK1 T_1 progeny; lane 7, Mann 7.1 KK2 T_0 ; lanes 8-11, individual Mann 7.1 KK2 T_1 progeny. B) Lane 1, untransformed pearl millet DNA, genotype 842B; lane 2, represents untransformed pearl millet spiked with 2 transgene copies; lane 3, undigested genomic DNA of Mann 2.24 T_0, lane 4-6, individual Mann 2.24 T_1 progeny; lanes 7-10, individual Mann 2.24 T_2 progeny. The arrow indicates the size (6.21 kb) of pNOV3604ubi linearised with *Sac* I.

The germination of progeny of transgenic pearl millet seeds on mannose containing medium provided a rapid and easy screen for elimination of non-transgenic material since T_0 and T_1 plants often produced numerous seeds (Table 3). Although T_0 plants were often stunted and produced a limited number of T_1 seeds (sometimes only 5 seeds), T_1 plants were phenotypically normal and produced numerous seeds (up to 453 seeds per spike).

Mendelian segregation was determined by germinating some seed from transgenic plants on media containing 3 mM (1 g l^{-1}) sucrose and 83 mM (15 g l^{-1}) mannose as described in materials and methods (Table 3). Although stable integration up to the first or second generation were obtained for transformation events Mann 7.1 KK1 and KK2 and Mann 2.24, a Mendelian segregation was not obtained for any of the transformation events. The reason

for the lack of a Mendelian segregation of seed produced by the transgenic pearl millet can most possibly be contributed to the small population size germinated on mannose selection medium, as genotype 842B produces on average 1000 seeds per inflorescence.

Table 3: Segregation of the *man*A gene activity in T_1 and T_2 progeny of transgenic pearl millet plants germinating on medium containing 3 mM (1 g l^{-1}) sucrose and 83 mM (15 g l^{-1}) mannose as described in materials and methods. The plants were self-pollinated.

Transformation event	T_1 germination	T_2 germination
	+ : -	+ : -
Man 2.24	29 : 37	22 : 47
Man 7.1 J1	1 :132	
Man 7.1 J3	0 : 5	
Man 7.1 M1	4 : 54	
Man 7.1 M3	0 : 48	
Man 7.1 M5	2 : 91	
Man 7.1 KK1	137: 146	
Man 7.1 KK2	65 : 173	50 : 403
Man 7.1 KK4	0 : 707	
Man 11.6 B	0 : 579	
Man 11.7 A	0 : 140	
Man 11.7 B	2 : 105	
Man 11.7 C	0 : 147	

Finally, in order for *man*A to be a useful, selection system the protocol should be applicable to a range of genotypes. It would be interesting to assess the transformability of other pearl millet genotypes using the *man*A gene as selectable marker gene.

5.5 CONCLUSION

In this study, an effective transformation system has been established to routinely produce transgenic pearl millet plants. The protocol provides an efficient transformation system to introduce into the genome of pearl millet the genes of agricultural interest combined with the *man*A selectable marker gene. In addition, particle bombardment of pearl millet gave rise to transformation events with relatively low copy integration of the transgene *man*A. Finally, the mannose selection system has no potential risk to animals, humans or environmental safety.

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Chapter 6

Genetic enhancement of pearl millet (*Pennisetum glaucum* [L.] R.BR.) for downy mildew resistance

6.1 ABSTRACT

Sclerospora graminicola, an obligate oomycetous fungal phytopathogen, and the causal agent of downy mildew in pearl millet plants, is a major constraint in the production of pearl millet. In this study a hydrolytic enzyme, β-1,3-glucanase, from the biocontrol fungus *Trichoderma atroviride* was introduced into the genome of pearl millet by particle bombardment. Constructs were prepared containing the *gluc*78 gene, encoding the 78 kDa β-1,3-glucanase protein, driven either by a strong constitutive promoter (ubiquitin promoter, exon and intron) or a wound inducible promoter, the potato proteinase inhibitor IIK gene promoter (PinII). The positive selectable marker gene, *man*A, encoding mannose-6-phosphate isomerase (phosphomannose isomerase) driven by the ubiquitin promoter, was used in co-transformation experiments. Transgenic plants were obtained harbouring the *man*A selectable marker gene and the antifungal gene *gluc*78 driven either by the ubiquitin or *pin*2 promoter. Progeny of stably transformed plants, harbouring the *gluc*78 transgene driven by the *pin*2 promoter followed by the rice *Act*1 intron sequences, were subjected to pathogenicity trials. Transgenic plants of one transformation event reduced fungal occurrence by 58%. The transgenic plants conferring decreased susceptibility to *S. graminicola* had high levels of the glucanase transcript and even higher in infected transgenic plants.

6.2 INTRODUCTION

T. atroviride is a well-characterised biocontrol soil-borne filamentous fungus, capable of parasitising several plant pathogenic fungi (Ait-Lahsen *et al.*, 2001; Emani *et al.*, 2003). *T. atroviride* is therefore a potential source of powerful resistance genes against fungal induced plant diseases. Secretion of lytic enzymes, mainly glucanases and chitinases, is considered the most crucial step of the mycoparasitic process (Cohen-Kupiec *et al.*, 1999). The lytic enzymes degrade the cell walls of the pathogenic fungi, enabling *Trichoderma* to utilize both their cell walls and cellular contents for nutritional purposes.

It is well documented that acidic and basic plant β-1,3-glucanases are associated with fungal infection (Gheong *et al.* 2000; Ji *et al.* 2000; Jung and Hwang, 2000). According to Kini *et al.* (2000a) there is an induction of β-1,3-glucanase in seedlings of pearl millet in response to infection by *S. graminicola*. High levels of a 30 kDa β-1,3-glucanase is present in downy mildew resistant pearl millet cultivars and absent in susceptible cultivars (Kini *et al.*, 2000b).

These studies indicate that β-1,3-glucanases play an important role in the defence mechanism of pearl millet plants against *S. graminicola*. Transgenic plants expressing β-1,3-glucanases show decreased susceptibility against oomycetes such as *Phytophthora* e.g. transgenic potato plants expressing a soybean β-1,3-endoglucanase gene, exhibited increased resistance to *Phytophthora infestans* (Borkowska *et al.*, 1998).

Lorito and co-workers (Lorito *et al.*, 1998) introduced an antifungal endochitinase gene from *T. atroviride* into the genome of tobacco and potato plants and this resulted in broad spectrum resistance against soil-borne and foliar pathogens. The high level and broad spectrum of resistance obtained against these pathogens with a single constitutively expressed endochitinase gene from *T. atroviride* (*chit*42) overcome the limited efficacy of transgenic expression in plants of chitinase genes from plants and bacteria (Lorito *et al.*, 1998). Furthermore, a 78 kDa β-1,3-glucanase (EC 3.2.1.58) from *T. atroviride* exhibits antifungal activity (ED_{50} of 35-75 μg ml^{-1}) against the oomycetous pathogen *Phytophthora*, also when applied in combination with microbial toxins and chemical fungicides (Fogliano *et al.*, 2002). Therefore, the aim of this study was to introduce the gene, designated *gluc*78, encoding the 78 kDa β-1,3-glucanase enzyme from *T. atroviride* into the genome of pearl millet to improve resistance against the oomycetous phytopathogen *S. graminicola*. Since the gene originates from *T. atroviride* and not from plant or bacteria, it is anticipated that stable integration and expression of this single glucanse gene from *T. atroviride* in pearl millet, could significantly contribute to the improvement of resistance against downy mildew.

The potato proteinase inhibitor II gene (*pin*2) family is probably the best-studied system of systemic gene activation in plant defence response (Xu *et al.*, 1993). Xu and co-workers (1993) showed that the expression of the potato proteinase inhibitor II gene (*pin*2) promoter, fused with the *uid*A (*Gus*) gene, displays a systemic wound response in the monocotyledonous plant rice, and even higher expression levels (6 fold) was obtained incorporating the intron of the rice actin 1 gene (*Act*1) into the 5'-untranslated region of the *pin*2-*Gus* construct. In addition, wound induced *Gus* mRNA levels in transgenic rice plants transformed with the *pin*2-*Gus* construct was much lower than that in transgenic plants transformed with the *pin*2-*Act*1-intron-*Gus* fusion construct. In directly wounded leaves, GUS activity was detected in whole leaf tissues including blades and leaf sheaths with the highest intensity of GUS staining in the leaf vascular tissue, mainly in the phloem and vascular bundles. Roots that were directly wounded induced *Gus* gene expression in the whole root, whereas roots from leaf-wounded plants displayed, like unwounded roots, activity in the root tip only. The authors concluded that the wound-inducible *pin*2 promoter in combination with the rice *Act*1 intron 1 might be used as an efficient regulator for foreign gene expression in transgenic monocotyledonous plants. Furthermore, the expression of the *pin*2-*Act*1-intron-

Gus fusion gene in transgenic rice plants can be systemically induced by both methyl jasmonate (MJ) and the phytohormone abscisic acid (ABA), but not to the levels obtained with direct wounding (Xu *et al.*, 1993). Proteinase inhibitors are common components of plant-derived food for humans and animals, and they are easily inactivated by cooking, and therefore the introduction of the potato proteinase inhibitor gene (*pin*2) into new host crops can be regarded as a safe strategy for insect and pathogen control from the food-safety standpoint (Duan *et al.*, 1996). In addition, transgenic rice plants harbouring an introduced potato proteinase inhibitor II gene, driven by the *pin*2 promoter plus rice actin intron insert, conferred resistance to the rice pink stem borer. It is therefore anticipated that the *pin*2 promoter would be safe in the food crop pearl millet and followed by the rice *Act*1 intron sequence (pPA*gluc*78), be effective in driving selected genes for fungal resistance in transgenic monocotyledonous pearl millet.

The use of heterologous leader sequences, to increase expression of engineered genes or cDNAs without changing the antigenic or biological properties of the encoded protein, might be an advantage. Eukaryotic messenger RNAs are translated with unequal efficiencies *in vivo* and *in vitro* and the molecular basis of this phenomenon is not understood (Jobling and Gehrke, 1987). Jobling and Gehrke (1987) used the untranslated leader from the coat protein RNA of alfalfa mosaic virus (AMV RNA4), a well-translated, highly competitive RNA, to replace the leader sequence of barley alpha-amylase (B alpha A) and human interleukin 1 beta (IL-1 beta) cDNAs. They reported that the RNA 4 leader resulted in as much as a 35-fold increase in mRNA translation efficiency. These results suggest that the chimaeric AMV-RNA have either a higher relative affinity or a diminished requirement for a limiting component(s) or the translational machinery. In addition, Cihlar and Cherrington (1997) found that both the catalytic subunit and accessory protein of the Human Cytomegalovirus polymerase, a truncated form of the alfalfa mosaic virus (AMV) RNA 4 leader sequence, was superior over either the full-length AMV leader sequence or the original HCMV leader sequence of different lengths. Therefore, in this study, a construct were also prepared containing the *gluc*78 gene driven by the *pin*2 promoter followed by an AMV leader sequence (pGEM*gluc*78) to potentially obtain higher expression levels in the monocotyledonous plant.

In this study, pearl millet were co-transformed by constructs containing the *gluc*78 gene and the positive selectable marker gene, *man*A. Transgenic T_2 pearl millet of selected transformation events was tested for disease resistance against *S. graminicola*.

6.3 MATERIAL AND METHODS

6.3.1 Preparation of vectors containing *gluc*78 and *man*A

6.3.1.1 pUBI*gluc*78

pAHC25 (Christensen and Quail, 1996) was subjected to restriction with *Sma* I, purified with the QIAquick PCR purification kit and the DNA concentration determined. Subsequently, the *uid*A (GUS) gene was removed from the restricted pAHC25, by an *Eco* RI partial digest. Partial digests reaction mixes were prepared containing 500 ng of DNA per restriction reaction with 2.5 units *Eco* RI. The DNA was digested at 37°C for 35 min. The fragments were separated on an agarose gel and the expected 7559 bp pAHC25 fragment excised and purified using the QIAquick gel extraction kit. The *gluc*78 gene/nos terminator fragment was isolated from pBIN-GLUC as a *Nco* I klenow blunted (Roche Biochemicals) and *Eco* RI sticky end fragment. This *gluc*78/nos terminator fragment was ligated with the pAHC25 fragment in a 6:1 ratio. Aliquots of both the fragments were subjected to agarose gel electrophoresis in order to estimate the amounts of DNA present before ligation. The ligation mix was incubated at room temperature overnight using the Fast-link DNA ligation kit from Epicentre. The product was designated pUBI*gluc*78 (Fig. 1; Appendix A, Fig. 1). The terminator of a gene for nopaline synthase (nos) was used for all the constructs prepared in this study.

6.3.1.2 pGEM*gluc*78

The *Hind* III/*Eco* RI fragment from pBIN-GLUC containing the Pin II promoter, AMV leader sequence, *gluc*78 gene and nos terminator was isolated in a double digest. This fragment was inserted in the *Hind* III/*Eco* RI restriction sites of the multiple cloning region of pGEM-7Zf(-) (Promega). The construct prepared was designated pGEM*gluc*78 and consisted of only 6 584 bp DNA instead of the 16 000 bp of pBIN-GLUC. This was done to simplify further vector manipulations (Fig. 1; Appendix A, Fig. 2).

6.3.1.3 pNOV3604ubi

The *Hind* III/*Bam* HI ubiquitin promoter, exon and intron fragment from pAHC25 was isolated in a double digest. This fragment was inserted as sticky ends in the *Hind* III/*Bam* HI restriction sites of the multiple cloning region of pNOV3604 (Syngenta Seeds AG). The prepared construct was designated pNOV3604ubi and consists of 6210 bp DNA (Fig. 1; Appendix A, Fig. 3).

6.3.1.4 pPA*gluc*78

pDX109 (Xu *et al.*, 1993) contains the Pin II promoter, rice actin intron, *uid* A (GUS) gene and pin II terminator region. A fragment containing the Pin II promoter and rice actin intron was isolated from pDX109. This was done by a *Hind* III/*Sma* I double restriction digest, followed by gel purification and determination of DNA concentration. pGEM*gluc*78 was restricted with *Nco* I, QIAquick gel extraction kit purified, and Klenow treated to blunt. Subsequently, the restricted plasmid was digested with *Hind* III to remove the Pin II promoter and AMV leader sequence. The Pin II promoter and rice actin intron fragment from pDX109

was then inserted in position by ligating the *Hind* III/*Hind* III sticky ends and the *Nco* I/*Sma* I blunt ends. The final construct, designated pPA*gluc*78, contains the Pin II promoter, rice actin intron, *gluc*78 gene and nos terminator (Fig. 1; Appendix A, Fig. 4).

6.3.1.5 Confirmation of sequence integrity of expression vectors

The prepared DNA plasmids were inserted into *Escherichia coli* (JM83 competent cells, Promega). Two micro-litres of the ligation reaction and 50µl of competent cells were used in the transformation reaction. The mix was electroporated at 1.8 kV, 200Ω and 25µF in a glass cuvette. Luria broth was added to each transformation reaction and the cells were incubated in a shaking incubator at 37°C for 1 hour. The cells were spread onto Luria agar plates containing either kanamycin or ampicillin. The plates were incubated at 37°C overnight. Single colonies were screened by mini-preparation of plasmid DNA from *E.coli* transformants using a modification of the Nucleobond kit miniprep method. Plasmid DNA was digested by selected restriction enzymes to confirm that the vectors and inserts ligated and that the insert was in the desired orientation.

One to two colonies containing constructs with the expected digestion pattern were used to prepare a maxi-preparation of plasmid DNA using the Qiagen maxiprep kit (Southern Cross Biotechnology). This plasmid DNA was restricted with various restriction enzymes individually and in combinations to verify that the expected fragment sizes were obtained for the three constructs prepared.

Sequence integrity of the vectors and inserts across ligation regions were verified using primers ubi S, *gluc*78 S, *gluc*78 AS, PinII S and PinII AS to sequence recombinant plasmids designated pUBI*gluc*78, pGEM*gluc*78, pPA*gluc*78 and pNOV3604ubi as indicated in figure 1 (Appendix A, Figs 5 and 6). Sequence analysis was performed (ABI PRISM™ dye terminator cycle sequencing) using the ready reaction kit with AmpliTaq® DNA polymerase (The Perkin Elmer Corporation, Norwalk, USA), by the Core Sequencing Facility at the University of Stellenbosch, South Africa.

The following primer sequences were used to analyse the prepared constructs: Gluc78 sense (*gluc*78 S), 5'-CCGTCAAGCTCTTTGGTA-3'; Gluc78 antisense (*gluc*78 AS), 5'-AATAGACGCAAGGCCAGG-3'; Ubiquitin sense (Ubi S), 5'–TATGCTCTAACCTTGAGTA CCTATC–3'; Ubiquitin antisense (Ubi AS), 5' ACTTAGACATGCAATGCTCATTATC-3'; Pin II sense (PinII S), 5'-TGATCACTCGTTTGCTATAA-3'; Pin II antisense (PinII AS), 5'-CATTTCTGCTTCTAACACATCATAC–3', *man*A sense (*man*A S), 5'- GGGTGAATCAGCG TTTATTG –3' and *man*A antisense (*man*A AS), 5'-CATTTCTGCTTCTAACACATCATAC–3'.

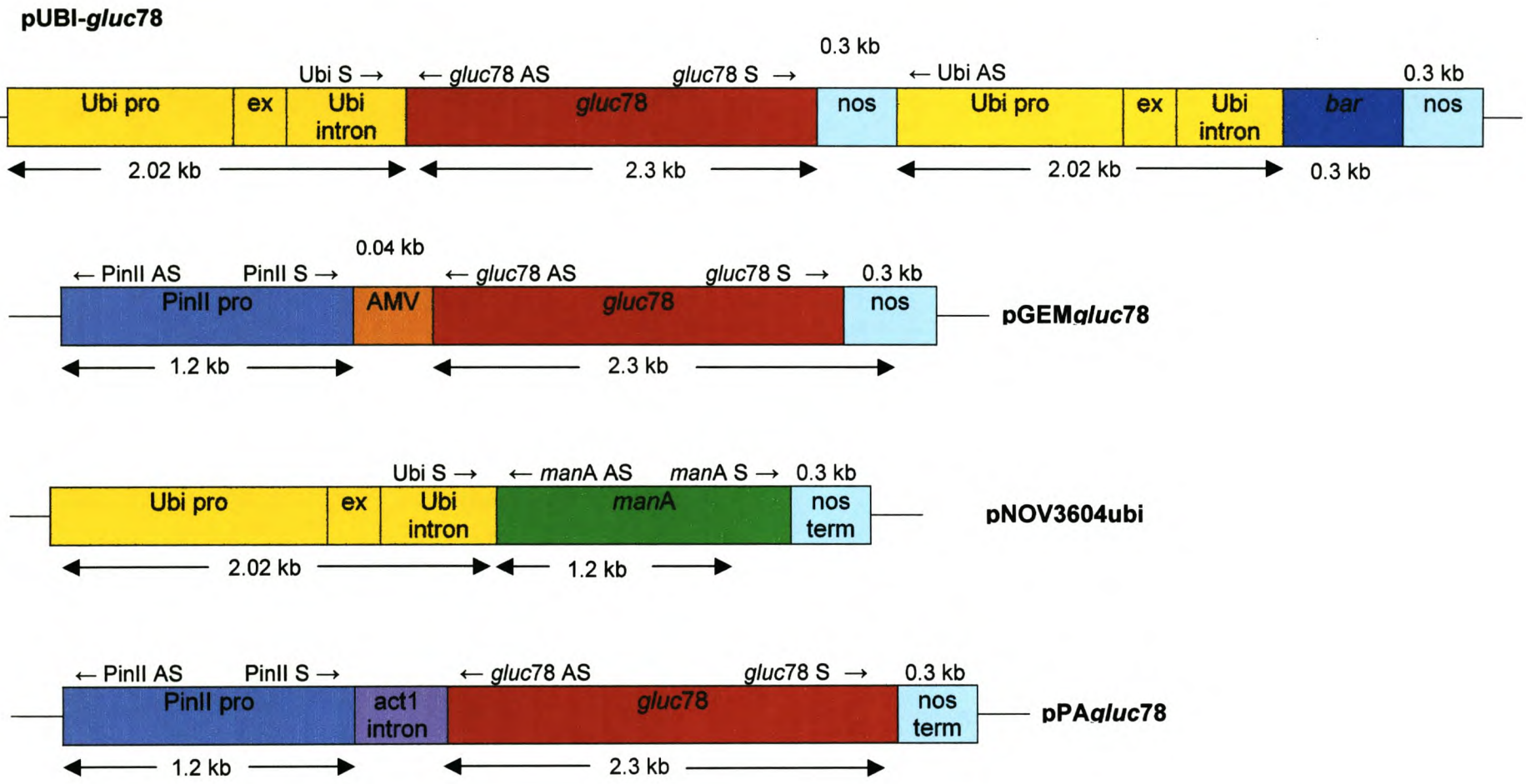

Figure 1: Constructs containing either the selectable marker genes *man*A or *bar*, or the antifungal gene *gluc*78. One and two direction sequence analysis was done to confirm that sequence integrity was maintained across ligation sites in the constructs during the cloning process using antisense (AS) primers and sense primers (S).

6.3.2 Pearl millet plant transformation procedures

6.3.2.1 Tissue culturing of IZEs

Seed material supply and excision of IZEs was described before (Chapter 3). Callus induction medium A is described by Pinard and Chandrapalaiah (1991), and contains MS salts (Murashige and Skoog, 1962), 9 µM 2,4 D, 88 mM sucrose as carbon source and 8g l^{-1} agar as solidifier. Cultures, initiated on induction medium A for a period of one month, were transferred to regeneration medium as described by Pinard and Chandrapalaiah (1991), containing the hormones IAA (11 µM) and kinetin (2.3 µM), but the medium was modified by the addition 60 mM $AgNO_3$. These cultures were subsequently transferred to rooting medium, which was identical to the regeneration medium, except that both hormones and $AgNO_3$ were omitted.

Callus induction medium J is described by Gless and co-workers (1998) and contains L3 salts and vitamins as described by Jähne *et al.* (1991), 1 µM 2,4-D, 83 mM maltose, 4 g l^{-1} Gelrite as solidifier and supplemented with 20 mM L-Proline as described (Chapter 3). Cultures initiated on this medium were matured on callus induction medium, omitting the 2,4-D, increasing the maltose concentration to 6% and designated maturation medium. The matured cultures were then regenerated and rooted on the maturation medium with only 3% maltose, and designated regeneration medium.

Cultures on callus induction and regeneration media were incubated at 24-25°C, under low-light conditions (1.8 µmol $m^{-2}s^{-1}$), whereas regenerating shoots (≥1 cm) were incubated under dim light (18 µmol $m^{-2}s^{-1}$).

6.3.2.2 Transfer regime

Immature zygotic embryo derived calli were transferred to fresh medium every two weeks. White compact calli were produced within 4 weeks on callus induction medium A, but only after 6 weeks on medium J: callus induction (4 weeks) and maturation (2 weeks) medium. After 4 or 6 weeks on A or J based medium, respectively, calli were transferred to the appropriate regeneration media as described above, and subcultured every three weeks to fresh media. Rooted plantlets of >1 cm, produced on medium A, were transferred to rooting medium. Plantlets grown to 8-10 cm, were hardened-off to a mist bed for approximately two weeks and then transferred to pots in the greenhouse. Rooted plantlets of >1 cm, produced on medium J remained on the regeneration medium described for this regime until they were hardened-off. Addition of mannose to tissue culture medium as selection agent, and the corresponding concentrations of sucrose or maltose or glucose in the medium, is described in 6.3.2.5.

6.3.2.3 Plasmid maxipreparations

Plasmid DNA for particle bombardment of constructs pUBI*gluc*78, pGEM*gluc*78, pNOV3604ubi and pPA*gluc*78 were isolated from overnight *E. coli* cultures using a Qiagen (Southern Cross Biotechnologies) maxiprep kit according to the supplier's recommendation.

6.3.2.4 Microprojectile bombardment

Pre-cultured IZEs were placed in the middle (0 - 1 cm diameter) of a 9 cm petri dish containing induction media A or J. The tissue culture medium was supplemented with approximately 25% increased solidifier, plus 0.2 M D-Sorbitol and 0.2 M D-Mannitol as described by Vain and co-workers (1993). A bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles with $CaCl_2$ and spermidine free base as described by O'Kennedy *et al* (1998). Experiments were conducted with the particle inflow gun (PIG) as previsouly described for maize (O'Kennedy *et al.*, 2001) and specifically for pearl millet (Girgi *et al.*, 2001). Bombardment with the PIG was done at 17 cm from the target tissue and a 500 µm nylon mesh screen placed 8 cm above the target. A vacuum of approximately -87 kPa was applied and the bombardment mix particles on the filter syringe were discharged when the helium was released following activation of the solenoid. The timer duration was 50 milliseconds.

6.3.2.5 Selection and regeneration of transgenic pearl millet

Cells containing and expressing the *man*A transgene, encoding phosphomannose isomerase, were selected for by various concentrations of mannose in combination with maltose, sucrose or glucose (Table 1). Selection was initiated 3-4 days after bombardment by placing the embryos on callus induction medium containing mainly 2/15 (2 g l^{-1} sucrose [6 µM; medium A] or maltose [6 µM; medium J], and 83 µM [15 g l^{-1}] mannose) or 0.02/15 (20 mg l^{-1} glucose or maltose [110 or 56 µM], respectively, and 15 g l^{-1} mannose).

Regenerating putative transgenic plants were subcultured at 2-3 weeks intervals until they reached 8-10 cm in height. These were then hardened-off to a mist bed and eventually the greenhouse as described by O'Kennedy *et al.* (1998).

6.3.3 Molecular analysis of putative transgenic plants

6.3.3.1 Wound inducible expression of the glucanase gene

Plants forty centimetres in height were wounded by making small 1 mm cuts along both edges of the leaf blade without damaging the middle vein. RNA extractions were made at 0, 2, 6, 18, 24 and 48h after wounding using the Qiagen Rneasy Plant mini kit (Southern Cross

Table 1: Conditions for bombardment of pearl millet IZE of genotype 842B, co-bombarded with pNOV3604ubi and one of the following full constructs, pUBI*gluc*78, pGEM*gluc*78 or pPA*gluc*78. Parameters which led to production of transgenic plants are printed in bold.

Exp. #	Callus induction media	# IZEs	Precult. (days)[a]	Helium pressure (kPa)	Selection initiated (+osm/-osm)[b]	Combinatorial expression	Mannose selection history[c]	DNA introduced (ng per shot)	Transgnenic plants designated	Transgenic plants
1	A	164					Init 2/15 → reg 2/15 Init 0.02/15 → reg 0.02/15			
	J	147	5 or 7	1000	4/0	*man*A:pGEM*gluc*	Init 2/15 → reg 2/28 → reg 2/15 Init 0.02/15 → reg 0.02/30 → reg 0.02/15	64:130		
2	A	108			4/0		Init 2/15 → reg 2/15		7.7.2	1
			6	**1000**		*man*A:pPA*gluc*	Init 0.02/15 → reg 0.02/15	**64:104**	7.9	1
	J	146					Init 2/15 → reg 2/28 → reg 2/15		7.10 (1-3)	3
				1200			**Init 0.02/15 → reg 0.02/30 → reg 0.02/15**	2X 64:104	7.10 (A-C)	3
3	A	120		900			Init 0.02/15 → reg 0.02/15			
	J	90	7	1000	3/0	*man*A:pGEM*gluc*	Init 0.02/15 → reg 0.02/30 → reg 0.02/15	64:109		0
				900			**Init 2/15 → reg 2/15**			
4	**A**	276	**5**	1000	3/0	*man*A:pUBI*gluc*	**Init 0.02/15 → reg 0.02/15**	64:118	9.13 B1	**1**
				1100			Init 2/15 → reg 2/28 → reg 2/15		9.13 B2	**1**
	J	257	7	1200			Init 0.02/15 → reg 0.02/30 → reg 0.02/15			
5	A	114		900 to			Init 0.02/15 → reg 0.02/15			
	J	142	7	1200	3/0	*man*A:pUBI*gluc*	Init 0.02/15 → reg 0.02/30 → reg 0.02/15	64:118		0
	A	90		900			Init 2/15 → reg 2/15			
6			7	1000	3/0	*man*A:pUBI*gluc*	Init 0.02/15 → reg 0.02/15	64:118		**0**
	J	120		1100			Init 2/15 → reg 2/28 → reg 2/15			
				1200			Init 0.02/15 → reg 0.02/30 → reg 0.02/15			
	A	203		900			**Init 2/15 → reg 2/15**			
7			6	**1000**	3/0	***man*A:pUBI*gluc***	Init 0.02/15 → reg 0.02/15	64:115	12.10A(1-3)	**3**
	J	187	**7**	1100			**Init 2/15 → reg 2/28 → reg 2/15**			
				1200		*man*A:pGEM*gluc*	Init 0.02/15 → reg 0.02/30 → reg 0.02/15	64:109		
8	A	68		900			Init 2/15 → reg 2/15			
			6	1000	3/0	*man*A:pPA*gluc*	Init 0.02/15 → reg 0.02/15	64:117	0	
	J	47	7	1100			Init 2/15 → reg 2/28 → reg 2/15 Init 0.02/15 → reg 0.02/30 → reg 0.02/15			
Total # of embryos		**2279**					**Total number of transgenic plants**			**13**

[a] = pre-culture period of IZEs before bombardment

[b] = mannose selection initiated after bombardment, indicated by the number of days on mannitol and sorbitol containing osmoticum medium(+osm) and without osmoticum (-osm)

Init = initiation medium

reg = regeneration medium

2/15 = 2 g l^{-1} sucrose for medium A or 2 g l^{-1} maltose for medium J and 15 g l^{-1} mannose for both media

0.02/15 = 0.02 g l^{-1} glucose for medium A or 0.02 g l^{-1} maltose for medium J and 15 g l^{-1} mannose for both media

2/28 = regeneration medium supplemented with 28 g l^{-1} mannose instead of 15 g l^{-1}

0.02/30 = regeneration medium supplemented with 30 g l^{-1} mannose instead of 15 g l^{-1}

[c] A = sucrose/mannose selection during callus induction (month 1) and regeneration (month 2- 6)

[c] J = maltose/mannose selection during callus induction (month 1), maturation (2 weeks of month 2) and regeneration (month 2.5 - 6)

Biotechnology). Plant material was harvested and immediately flash frozen in liquid nitrogen and RNA was isolated directly or material was stored at -80°C until analysis.

6.3.3.2 PCR analysis

The *manA* specific primers (PMI fwd: 5'-CGT TGA CTG AAC TTT ATG GTA TGG-3' and PMI as: CAC TCT GCT GGC TAA TGG TG-3') were used to amplify a 965 Kb fragment, from genomic DNA preparations of putative transgenic pearl millet plantlets. The *gluc*78 specific primers (*gluc*78 fwd: 5'-ATG ATG GGT CTC TCA ACC GTC-3' and *gluc*78 as: 5'-TAG CAC CGC CAT TGA GAA TGG-3') were used to amplify a 1100 Kb fragment, from genomic DNA preparations.

6.3.3.3 Southern blot analysis using the non-radioactive DIG system

Genomic DNA was extracted from putative transgenic pearl millet leaf material using the mini extraction procedure of Dellaporta *et al.* (1983). Five micrograms of pearl millet genomic DNA digested with *Sac* I was separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy *et al.* (1998). An internal fragment of the *man*A gene of pNOV3604ubi and the *gluc*78 gene of pPA*gluc*78 were labeled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche).

6.3.3.4 Southern and northern blot analysis using ^{32}P radioactive labelling

Total RNA was extracted using the Qiagen RNeasy plant mini kit (Southern Cross Biotechnology) according to the manufacturer's recommendation. The RNA was separated on a formaldehyde gel as described in the booklet of the Qiagen RNeasy plant mini kit. The RNA were transferred to a nylon membrane using 10 X SSC (Sambrook *et al.*, 1989) overnight and fixed to the dried membrane for 30 min at 80°C and UV fixing face down for three minutes.

An internal DNA fragment probe to detect the *gluc*78 DNA and mRNA was prepared using redivue™ ^{32}P-dCTP labelling and the mega prime™ labelling kit (RPN1605) (Amersham Biosciences). Unincorporated nucleotides were removed by the Qiagen PCR clean up kit and denatured before addition to the blots. Prehybridisation was done for 4 hours in prehybridisation solution (100 mg l^{-1} denatured Salmon sperm DNA, 50 mM Tris pH 8.0, 10 mM EDTA, 5 X SSC, 20 mg each of BSA, Ficoll 400 and PVPP, 0.2% SDS [m/v]) and hybridised overnight in hybridisation solution (100 mg l^{-1} denatured Salmon sperm DNA, 500 g l^{-1} [m/v] dextran sulfate, 50 mM Tris pH 8.0, 10 mM EDTA, 5 X SSC, 20 mg l^{-1} each of BSA, Ficoll 400 and PVPP, 0.2% SDS [m/v]) both at 65°C in a Techne Hybridiser HB-1D. Washes were done as follows: 5 min at room temperature (2 X SSC, 0.5% SDS [m/v]), another 5 min at room temperature (0.1 X SSC, 0.1% SDS) and 10-15 min at 65°C (0.1 X SSC, 0.1% SDS).

Blots were exposed to X-ray film (Hyperfilm™ MP, Amersham Pharmacia Biotech) in the presence of intensifier screens at -80°C for 3-5 days.

6.3.3.5 Western blot analysis

Protein extractions were done according to Kini and co-workers (2000a) with the addition of PVPP and the protease inhibitor cocktail (Sigma P9599) for protection against protease activity. In short, leaf material were homogenised with a mortar and pestle in extraction buffer (50 mM sodium acetate buffer pH 5.2, PVPP and protease inhibitor cocktail according to the manufacturers recommendation. The extracts were centrifuged at 4°C for 10 minutes at 12 000 rpm. The supernatant was frozen at -20°C until SDS-PAGE analysis. The protein concentration in the resulting soluble protein extract of each sample was determined by the BioRad protein dye reagent with bovine serum albumin as the standard. For each sample, 2 µg aliquots of protein were separated by polyacrylamide gel electrophoresis (PAGE) on a 12% separating and 6% stacking gel according to the method of Laemmli (1970). Proteins were transferred to MSI PVDF-plus transfer membranes (0.45 micron) by means of a Hoefer TE22 blot system. Western blots were probed with antiserum (1:750) to the glucanase protein using the ECL RPN2108 western blotting kit (Amersham BioSciences). For biotinylated molecular marker detection (ECL RPN2107) streptavidin-horseradish peroxidase conjugate (ECL RPN1231) was used.

6.3.4 Pathogenicity trials

Transgenic pearl millet seedlings containing the antifungal gene, *gluc78*, from the biocontrol fungus *T. atroviride* (Donzelli *et al.*, 2001), and selectable marker gene, *manA* (Syngenta Crop Protection AG), were identified by germination on mannose containing medium. The transgenic germinating plantlets were transplanted to pots at the greenhouse facility at Pen-y-Ffridd Experimental Station, Bangor, to be challenged by the pathogen *S. graminicola*.

S. graminicola isolates from Niger, Senegal and Mali were maintained in the greenhouse on the highly susceptible pearl millet host-genotype 7042. The following numbers of plants were screened: 24 seedlings (non-transgenic, non-infected control 842B plants), 41 (non-transgenic, infected control 842B plants), 34 seedlings (transgenic BB gluc/mann 7.10.B, infected), five seedlings (transgenic BB gluc/mann 7.10.1, infected) and 57 seedlings (transgenic BB gluc/mann 7.10. 2, infected). All the transgenic plants are from the parental line 842B. The seedlings were planted in low-nutrient peat and sharp sand compost (Chempak Seed Base, Chempak Products, UK: NPK 25-39-30 mg l^{-1}). Pots were placed on flood benching in a controlled environment glasshouse providing a 16-h day length with light intensity between 500 and 1200 µmol Em^{-2} s^{-1} and a temperature of 25-30°C from 06:00-18:00 and 20°C from 18:00-06:00. The benches were flooded daily with Vitax Vitafeed 214

(Owen Street, Coalville, Leicester LE67 3DE, UK) to an approximate depth of 1 cm for 30 min and then drained. When the seedlings were at the coleoptile to one-leaf stage the inoculum was prepared. Leaves from 2-3 month old infected plants were wiped clean of old sporangia using moist laboratory roll (Kimwipes Roll, Kimberly Clark, Kent, UK) and incubated in sealed plastic boxes lined with moist laboratory roll for 6-8h at 20°C in the dark. The resulting sporangia were collected into chilled distilled water (below 2°C) and adjusted to an appropriate concentration after assessing the concentration using a haemocytometer. Each pot of seedlings was sprayed with approximately 4 ml of inoculum using a compressed-air cylinder-fed sprayer (Kestrel Eqpt. Ltd., London). The inoculum was maintained on ice throughout inoculation to prevent zoospore release and so ensure a uniform sporangial concentration over time (Jones *et al.*, 2001). The pots were covered with a polythene sheet to maintain a high level of humidity and incubated in the glasshouse at 20°C for 15h.

Protein and RNA extractions were prepared of selected non-transgenic control plants, infected and uninfected transgenic plants to determine expression levels of the *gluc78* transgene as described above.

6.4 RESULTS AND DISCUSSION

6.4.1 Preparation of constructs

Three constructs containing the β-1,3-glucanase from *T. atroviride* (*gluc78*) and designated pUBI*gluc*78, pGEM*gluc*78 and pPA*gluc*78, were prepared (Fig. 1; Appendix A, Figs 1-4) . Constructs pGEM*gluc*78 and pPA*gluc*78 were ligated upstream of the wound inducible promoter, the potato proteinase inhibitor IIK gene promoter. Theoretically, the protein, encoded by the *gluc78* gene in these constructs, should be expressed only in response to pathogen attack and minimise interference with the functions important for maintaining the agronomic qualities of the engineered crop. Furthermore, the ubiquitin constitutive promoter, exon and intron fragment was ligated in front of the *man*A gene to complete the minimal transgene cassette that requires promoter, gene and terminator sequences. This construct was designated pNOV3604ubi. Various restriction enzyme digestions confirmed the ligation of inserts and the correct orientation of inserts in all the constructs. Furthermore, two direction sequence analysis was done to confirm that sequence integrity was maintained across ligation sites in the constructs during the cloning process (Fig. 1; Appendix A, Figs 5 and 6).

Sequence information for the ubiquitin intron terminal end and *gluc*78 start region of construct pUBI*gluc*78 was obtained using the ubi sense (S) and the *gluc*78 antisense (AS) primers (Fig. 1). The *Sma* I blunt end ligated with the *Nco* I Klenow blunted site was present in construct pUBI*gluc*78. The *Nco* I site includes the ATG start codon of *gluc*78 (Appendix A,

Fig. 5A). The ATG indicated in the ubiquitin intron region would be spliced out and therefore the *gluc*78 ATG start codon would act as the start codon during translation. Furthermore, construct pUBI*gluc*78 was sequenced with the *gluc*78 S primer, which provided sequence information for the *gluc*78 terminal end, nos terminator and the start of the ubiquitin promoter driving the *bar* gene in the construct (Appendix A, Fig. 5C). The *Eco* RI sticky end nos terminator/ubiquitin promoter ligation site is present as indicated in Fig 5C.

The *Eco* RI sticky end nos terminator end/pGEM7Zf(-) vector ligation site for construct pGEM*gluc* 78 was confirmed. Furthermore, the *Hind* III sticky end pGEM vector end/Pin II promoter start region was confirmed by using the Pin II AS primer to sequence the junction point (Fig. 1). pGEM*gluc*78 was sequenced with the PinII S and *gluc*78 AS to determine the AMV sequence of 39 bp (Appendix A, Fig. 6B).

Construct pPA*gluc*78 was sequenced with PinII S and *gluc*78 AS primers, which provided sequence information for the terminal end of the rice actin intron and *gluc*78 start regions (Appendix A, Fig. 5B). Although two ATG start codons were identified in the rice actin exon, translation from the first ATG would place the *gluc*78 gene in the proper reading frame. It is expected that 95% of the protein would be translated from this first start codon in the rice actin exon.

Construct pNOV3604ubi was sequenced with ubi sense and *man*A antisense primers, which provided sequence information for the terminal end of the ubiquitin intron and *man*A start regions (Appendix A, Fig. 6A). Although two ATG start codons were identified in the ubiquitin intron, they will be spliced out as part of the intron, and translation from the first ATG, in the *man*A sequence, would place the *man*A gene in the proper reading frame. The *Hind* III vector end/ubiquitin start region ligation was also confirmed by using the Ubi AS primer to sequence the junction site.

6.4.2 Production of transgenic pearl millet

Proliferating IZEs were co-bombarded with pNOV3604ubi and one of the constructs containing the *gluc*78 gene (Table 1). The co-transformation ratio was approximately 1:2. IZEs were precultured for 5-7 days, bombarded at a helium pressure of 900-1200 kPa and then cultured for 3-4 days on osmoticum medium after bombardment, before being transferred to the mannose containing selection medium of 2/15 or 0.02/15.

Thirteen transgenic plants containing the *gluc*78 transgene from *T. atroviride* gene were obtained. Genomic Southern blot analysis of T_0 and T_1 transformation events of pearl millet

genotype 842B was done (Figs 2 and 3). It appears that transgenic plants BB gluc/mann 7.10 1-3 and A-C are clones, whereas BB gluc/mann 7.7-2 and BB gluc/mann 7.9 are unique transformation events. Identical integration patterns for specific transformation events were obtained for two independent southern blot analysis hybridising with the DIG or radioactive labelled probe (Figs 2 and 3). The first T_2 seed available for pathogenicity trials were events BB gluc/mann 7.10B, 7.10.1 and 7.10.2. T_2 progeny of transformation events BB gluc/mann 7.7.2 and 7.9 will be assessed in the near future.

6.4.3 Pathogenicity trials

T_2 transgenic pearl millet plants of transformation events BB gluc/mann 7.10.B, BB gluc/mann 7.10.1 and BB gluc/mann 7.10.2 were challenged by *S. graminicola* to screen for downy mildew resistance. Downy mildew disease was assessed 20 days after inoculation based on the percentage of infected plants within each pot (Table 2). The disease score for each genotype was the mean of infection percentage for individual pot replicates. This meant that the disease score for each entry could range from 0-100% and was a continuous variable. Symptoms were seen as distinct chlorosis of infected leaves, zoospore growth underneath the leaf and stunted growth which always leads to green ears, where panicles seed are replaced by leaf-like structures (phylloidy). Since most of the plants screened in this experiment were transgenic, they were incinerated before flowering.

A disease score reduction of almost 58% was obtained for T_2 progeny of transgenic plant BB gluc/mann 7.10.2 when infected with *S. graminicola* when compared to infected control 842B plants. Progeny of transgenic plants BB gluc/mann 7.10B and 7.10.1 gave an even higher disease incidence when compared to control plants. A small number of plants were screened (Table 2) and therefore these results are regarded as preliminary results only. It is known, that if heterologous glucanase is silenced (Sanders *et al.*, 2002), it may lead to diminishing levels of the plant's endogenous glucanase activity, and increased disease susceptibility which might explain the results obtained. A disease index were not determined as infected plants, whether a 1 or 100% infection ratio were obtained, will lead to green ears and the lack of production of any seed (Dr Wendy Breese, personal communication).

6.4.4 Molecular analysis of the expression of the *gluc*78 transgene

RNA and protein analyses were conducted to determine if a correlation existed between the expression levels of *gluc78*, and decreased disease susceptibility. Expression of the *gluc*78 transgene in leaves was analysed by RNA gel northern blotting and by protein gel western blotting for selected control and transgenic plants (Fig. 4 and 5). Wound induced expression of the glucanase transcript of transformation event BB gluc/mann 7.10.2 were 2-4 times the

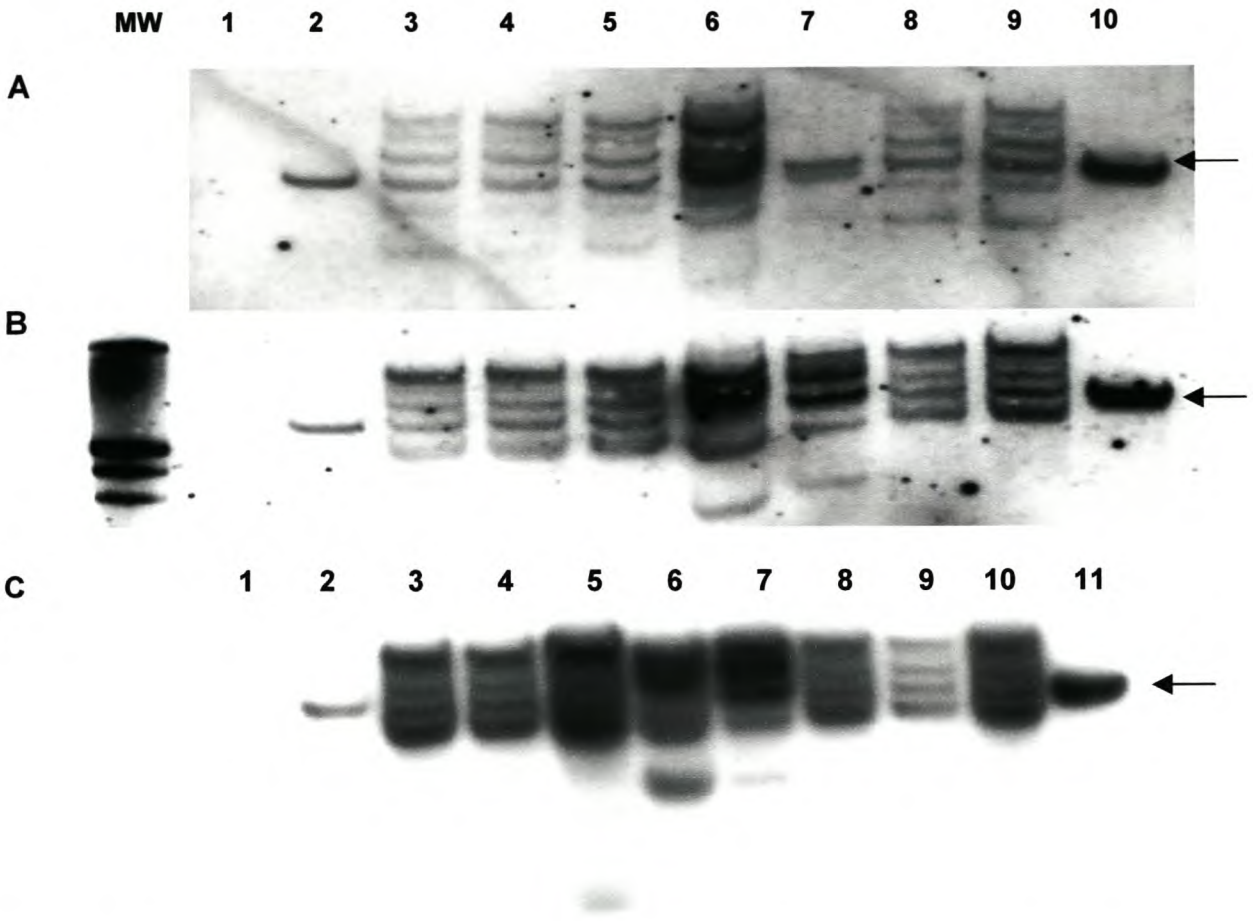

Figure 2: Southern blot analysis of independent T_0 transformation events of pearl millet genotype 842B. Genomic DNA was purified from plant leaf material, restricted with *Hind* III and resolved in a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal DNA fragment of the *man*A (blot A) or *gluc*78 (blot B and C) transgene, either DIG labelled (blots A and B) or radioactive labelling (blot C). Blots A and B: MW, DIG labelled molecular weight marker III (Roche Diagnostics South Africa); lane 1, untransformed pearl millet leaf material of genotype 842B; lane 2 and 10, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lane 3, BB gluc/mann 7.10A plant; lane 4, BB gluc/mann 7.10B plant; lane 5, BB gluc/mann 7.10C plant; lane 6, BB gluc/mann 7.7.2 plant; lane 7, BB gluc/mann 7.9 plant; lane 8, BB gluc/mann 7.10.1 plant; lane 9, BB gluc/mann 7.10.3 plant. Blot C: lane 1, untransformed control; lanes 2 and 11, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lane 3, BB gluc/mann 7.10A plant; lane 4, BB gluc/mann 7.10B plant; lane 5, BB gluc/mann 7.10C plant; lane 6, BB gluc/mann 7.7.2 plant; lane 7, BB gluc/mann 7.9 plant; lane 8, BB gluc/mann 7.10.1 plant; lane 9, BB gluc/mann 7.10.2 plant; lane 10, BB gluc/mann 7.10.3 plant. The arrows indicate the size of pNOV3604ubi (6.21 kb) or pPA*gluc*78 (7.15 kb) linearised with *Hind* III.

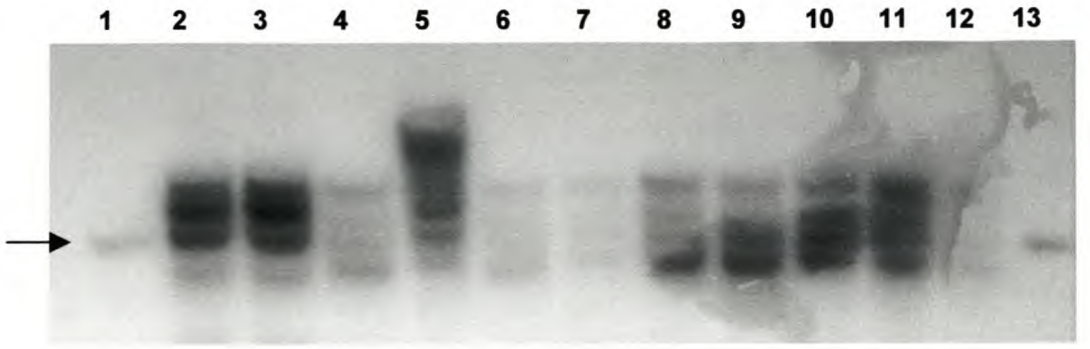

Figure 3: Southern blot analysis of independent T_0 and T_1 transformation events of pearl millet genotype 842B. Genomic DNA was purified from plant leaf material, restricted with *Hind* III and resolved in a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal fragment of the *gluc*78 transgene. Lane 1 and 13, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lane 2, BB gluc/mann 7.9 T_0 plant; lane 3, BB gluc/mann 7.9 T_1 plant; lane 4, BB gluc/mann 7.10.1 T_0 plant; lane 5, BB gluc/mann 7.10.1 T_1 plant, DNA only partially digested; lane 6, BB gluc/mann 7.10.2 T_0 plant; lane 7, BB gluc/mann 7.10.2-2 T_1 plant; lane 8, BB gluc/mann 7.10.2-3; lane 9, BB gluc/mann 7.10.3 T_0 plant; lane 10, BB gluc/mann 7.10A T_0 plant; lane 11, BB gluc/mann 7.10A T_1 plant; lane 12, BB gluc/mann 7.10C T_1 plant. The arrow indicates the size (7.15 kb) of pPA*gluc*78 linearised with *Hind* III.

intensity of the unwounded control leaf material 2, 6, 18, 24 and 48h after wounding (Data not shown). All the transgenic plants in this trial were challenged with *S. graminicola*. Under the stated assay conditions, very faint signals of the glucanase transcript were detected in non-transgenic plants for both challenged or non-challenged scenarios (Fig. 4A, B and C; lanes 1 and 2). An estimated 100-fold higher level of expression, was obtained in infected transgenic plants and 10 to 40-fold higher levels of expression in uninfected transgenic plants, when compared to control plants (Fig. 4). In addition, the protein encoded by *gluc*78 is clearly expressed in transgenic plants challenged with *S. graminicola* (Fig. 5), whereas the protein is absent in non-transgenic control plants whether the plants were challenged with the pathogen or not. The reason for the increased occurrence of diseased plants in comparison to the control plants for transformation events BB gluc/mann 7.10.B and 7.10.1 are not clear. Further pathogenicity trials with larger numbers of transgenic plants may explain this result.

This is the first report of an antifungal glucanase from *T. atroviride* stably expressed in a transgenic cereal crop. Preliminary pathogenicity trials and molecular analysis indicated that

the expression of this protein, encoded by the *gluc*78 transgene driven by a wound inducible promoter, during wounding and pathogen infection, increased the glucanase transcript.

Table 2: Transgenic and non-transgenic control plants screened for downy mildew susceptibility.

experimental plants	# of plants per pot	# plants infected verses non infected	Average (%)
non-transgenic, non-infected	12	0:12	0.0
control 842B plants	12	0:12	
	12	0:12	
non-transgenic, infected	22	10:22	41.5
control 842B plants	19	7:19	
transgenic BB gluc/mann	13	7:13	70.5
7.10.B, infected	8	6:8	
	13	11:13	
transgenic BB gluc/mann	2	1:2	60.0
7.10.1, infected	3	2:3	
transgenic BB gluc/mann	18	2:18	17.5
7.10.2, infected	9	2:9	
	17	6:17	
	13	0:13	

= number

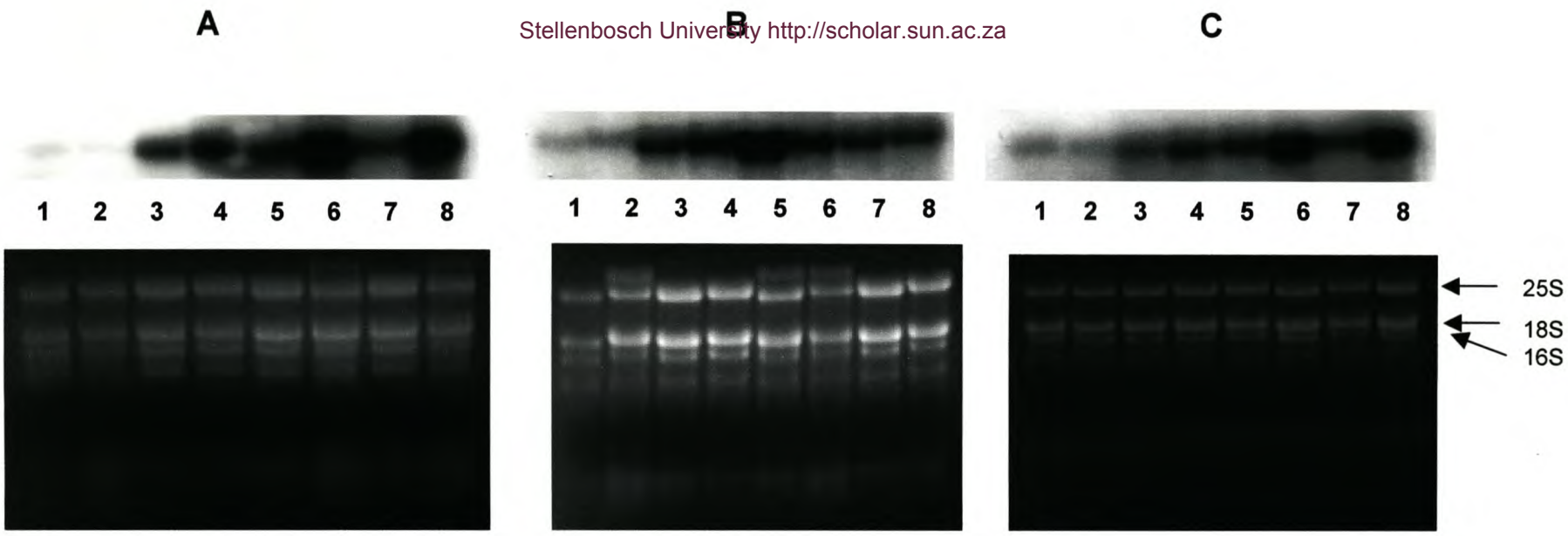

Figure 4: Detection of the *gluc*78 *T. atroviride* glucanase transcript in infected and non-infected selfed progeny T_2 generation transgenic and control untransformed pearl millet genotype 842B. Total RNA was extracted from leaf material and 8ug of RNA loaded per lane and blotted to a nylon membrane. The RNA gel blot was hybridised with a *gluc*78 internal DNA fragment probe. Northern blot analysis was performed on **A:** lane 1, non-infected non-challenged with *S. graminicola* control plant; lane 2, infected control plant; lanes 3 and 4, two individual infected plants from transformation event BB gluc/mann 7.10.1; lanes 5 and 7, non-infected BB gluc/mann 7.10B; lanes 6 and 8, infected BB gluc/mann 7.10B; **B:** lane 1, non-infected control plant; lane 2, infected control plant; lanes 3, 4, 7 and 8, non-infected challenged transgenic plants of event BB gluc/mann 7.10.2; lanes 5 and 6, infected challenged transgenic plants of event BB gluc/mann 7.10.2; **C:** lane 1, non-infected non-challenged control plant; lane 2, non-infected challenged control plant; lanes 3-8, non-infected challenged transgenic plants of event BB gluc/mann 7.10.2. Ethidium bromide stained gels show that equal amounts of total RNA were loaded in each lane, in each set A, B and C.

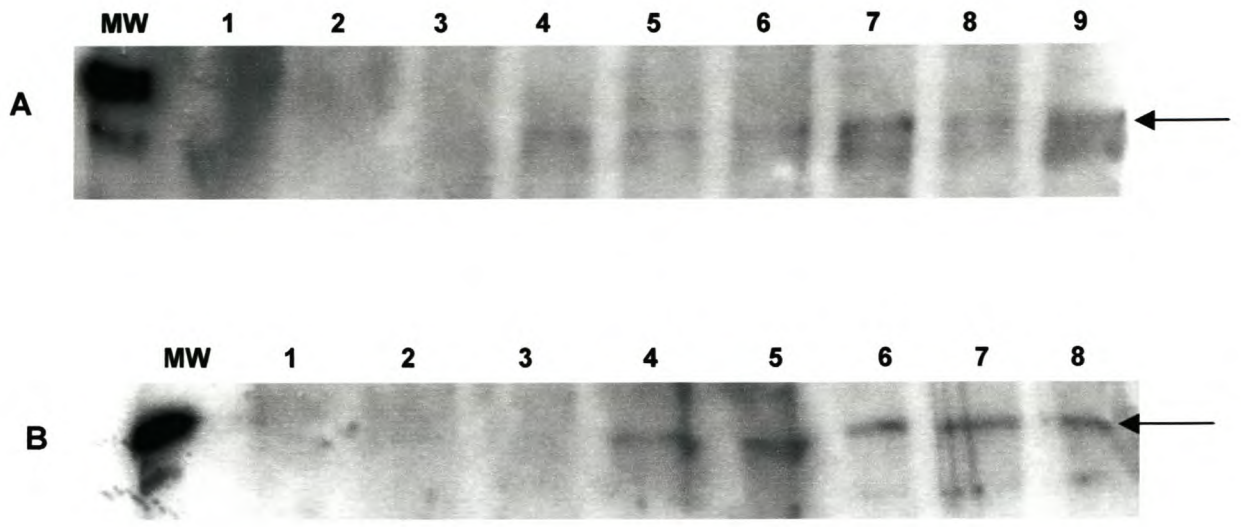

Figure 5: Western blot analysis of total soluble proteins from leaves of transgenic and non-transgenic control plants. A two microgram aliquot of protein was separated in each lane, the blot immunoprobed with polyclonal anti-*gluc*78 antibodies (dilution 1:750) and visualised with anti-rabbit secondary antibodies (dilution 1:5000). Blot **A** and **B**: MW, ECL molecular weight marker; lane 1, non-infected non-challenged with *S. graminicola* control plant; lane 2, non-infected *S. graminicola* challenged plant; lane 3, infected control plant. Blot **A**: lanes 4 and 5, two individual infected plants from transformation event BB gluc/mann 7.10.1; lanes 6 and 8, non-infected BB gluc/mann 7.10B; lanes 7 and 9, infected BB gluc/mann 7.10B. Blot **B**: lanes 4, 5 and 8, non-infected challenged transgenic plants of event BB gluc/mann 7.10.2; lanes 6 and 7, infected challenged transgenic plants of event BB gluc/mann 7.10.2. The arrows indicate the protein size of approximately 78 kDa.

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Chapter 7

Concluding remarks and future prospects

7.1 Concluding remarks

There is an urgent need for an increased focus on crops relevant to the small farm holders and poor consumers in the developing countries of the humid and semi-arid tropics (Sharma *et al*, 2002). Pearl millet is the only major staple cereal that reliably produces both grain and forage on poor, sandy soils under hot, dry conditions of Africa and Asia (Goldman *et al.*, 2003) yet fungal phytopathogens such as *Sclerospora graminicola*, has a devastating effect on grain production. Until very recently, plant breeding relied solely on sexual transfer of genes between plant species, whereas developments in plant molecular biology and genomics now give us access to the knowledge and understanding of plant genomes and genetic engineering (Job, 2002). Establishing an efficient routine transformation protocol for pearl millet would form the technological basis for the genetic enhancement of this crop and would provide the means to introduce agronomical significant genes into a cereal crop grown widely in both India and parts of Africa.

In order to establish an efficient transformation protocol for pearl millet to genetically enhance this crop and to produce transgenic pearl millet conferring improved resistance to *S. graminicola* the following parameters were optimised: 1) systematic screening of selected genotypes and explant tissues to obtain efficient transformation and regeneration systems, 2) identification of environmentally friendly selectable marker genes resulting in high transformation efficiencies and low numbers of escapes, 3) selection of constitutive and wound/fungal elicited promoter sequences, 4) optimisation of transcriptional signals suitable for this monocotyledonous system and 5) stable expression of a transgene from a bio-control fungi conferring improved resistance to the oomycete phytopathogen *S. graminicola*.

Since high frequency plant regeneration from cultured explant material is a prerequisite for the successful transformation of crops, identifying highly regenerable genotypes and optimising tissue culture media was the first step in establishing a reliable transformation protocol for pearl millet. In this study, a highly efficient L3 based tissue culture system, was established for pearl millet by the addition of the osmoprotectant L-proline. On average 80 regenerants were obtained per immature zygotic embryo explant of 842B, a genotype widely used in breeding programmes in Africa and India by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

Subsequently, pearl millet transformation protocols were established for a cereal crop recalcitrant to transformation. Initially, the selectable marker *bar* gene from *Streptomyces hygroscopicus,* conferring herbicide resistance, and a reporter gene (*uidA*) both driven by the constitutive promoter ubiquitin were used to establish pearl millet transformation. Pre-cultured immature zygotic embryos (455) of parental line 842B were bombarded using the simple and inexpensive particle inflow gun and resulted in two fertile transgenic pearl millet plants as confirmed by resistance to application of the herbicide Basta®, *uidA* expression, PCR and southern blot analysis. Both transformation events, clones, had a multicopy integration pattern of the *bar* transgene. The transformation efficiency of 0.02% using the *bar* gene (Girgi *et al.* 2002) was improved to 0.19 and 0.72% on two independent tissue culture media with *man*A as positive selectable marker gene. Although the L-proline containing media often produced clones of transformation events, it resulted in a 10-36 percent improvement on the transformation efficiency in combination with *man*A, had less than 20 percent escapes and utmost 3-4 copies of the transgene.

Constructs were prepared containing the gene, encoding 78 kDa β-1,3-glucanase, driven either by a strong constitutive promoter (ubiquitin promoter, exon and intron) or a wound inducible promoter, the potato proteinase inhibitor IIK gene promoter. The wound inducible promoter includes either the AMV leader sequence or the rice actin1 intron to obtain higher expression levels in the monocotyledonous pearl millet. Transgenic pearl millet containing the glucanase gene, driven by the wound inducible promoter, rice actin intron, was produced in this study. This is the first report on wound inducible expression of a single β-1,3-glucanase from a biocontrol fungi in a cereal crop. Preliminary studies indicate a positive correlation between reduced occurrence of fungal infection and expression of the transgene *gluc*78. This genetically improved pearl millet breeding line might be a powerful tool in improving resistance to *S. graminicola*, once thoroughly assessed under field conditions.

In conclusion, traditional breeding has been for many years the main avenue for crop improvement in pearl millet, whereas genetic engineering offers direct access to a vast pool of useful genes. This study opens the way to incorporate otherwise inaccessible traits, such as the gene from the bio-control fungi *Trichoderma atroviride* (formerly *T. harzianum*), into the genome of pearl millet to confer broad-spectrum resistance to the widespread and destructive oomycete pathogen *S. graminicola*.

7.2 Future prospects

Pearl millet lines developed by biotechnology will need to be tested as stringently as any other cultivars. A thorough assessment to the allergenic potential and unintended alterations of novel foods produced through biotechnology provides a solid basis for food safety assessment. The majority of known plant food allergens belong to seed storage proteins, protease and amylase-inhibitors, profilins or pathogenesis-related (PR) proteins, but the likelihood that the proteins derived from viruses, bacteria or non food plants will be allergens is low, since most proteins in nature are not allergens (Halsberger, 2003). The Food and Agricultural Organisation/World Health Organisation (WHO/FAO) have depicted decision trees for a rigorous assessment and testing for GM foods (Halsberger, 2003) which would be applicable to transgenic pearl millet expressing this fungal β-1,3-glucanase.

Furthermore, it is essential to eliminate gene flow from genetically modified (GM) pearl millet to non-GM pearl millet, as pearl millet is indigenous to Africa. The pollen-mediated flow of transgenes can be controlled by cytoplasmic-nuclear male sterility (Feil and Stamp, 2002; Budar and Pelletier, 2001). This can minimise the possibility of gene flow to non-GM pearl millet, which might represent a threat to contaminate the gene pool of pearl millet as cytoplasmic male sterility, is a maternally inherited trait (Budar and Pelletier, 2001). Furthermore, cytoplasmic-nuclear male-sterility (CMS) systems already contribute significantly to increasing productivity of pearl millet (Thakur *et al.*, 2001). The transgenic cytoplasmic-nuclear male sterile line can function as pollen recipient and 20% untransformed plants as pollen donors as previously proposed by Feil and Stamp (2002) to control pollen-mediated flow of transgenes in maize.

Finally, the gains in food production provided by the Green Revolution have reached their ceiling while the world population continues to rise (Wisniewski *et al.*, 2002). A new Green Revolution will necessitate application of recent advances in plant breeding, including new tissue culture techniques, marker-aided selection and genetic modification (Wisniewski *et al.*, 2002) to aid our staggering requirement for food, with cereal grains playing a pivotal role (Hoisington *et al.*, 1999). The affluent nations can afford to adopt elitist positions and pay more for food produced by the so-called natural methods; the one billion chronically poor and hungry people of this world cannot (Wisniewski *et al.*, 2002). Therefore, despite the diverse and widespread beneficial applications of biotechnology products, there remains a critical need to present these benefits to the general public in a real and understandable way that stimulates an unbiased and responsible public debate (Sharma *et al*, 2002) and pro-GMO government policies.

7.3 References

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Appendix A

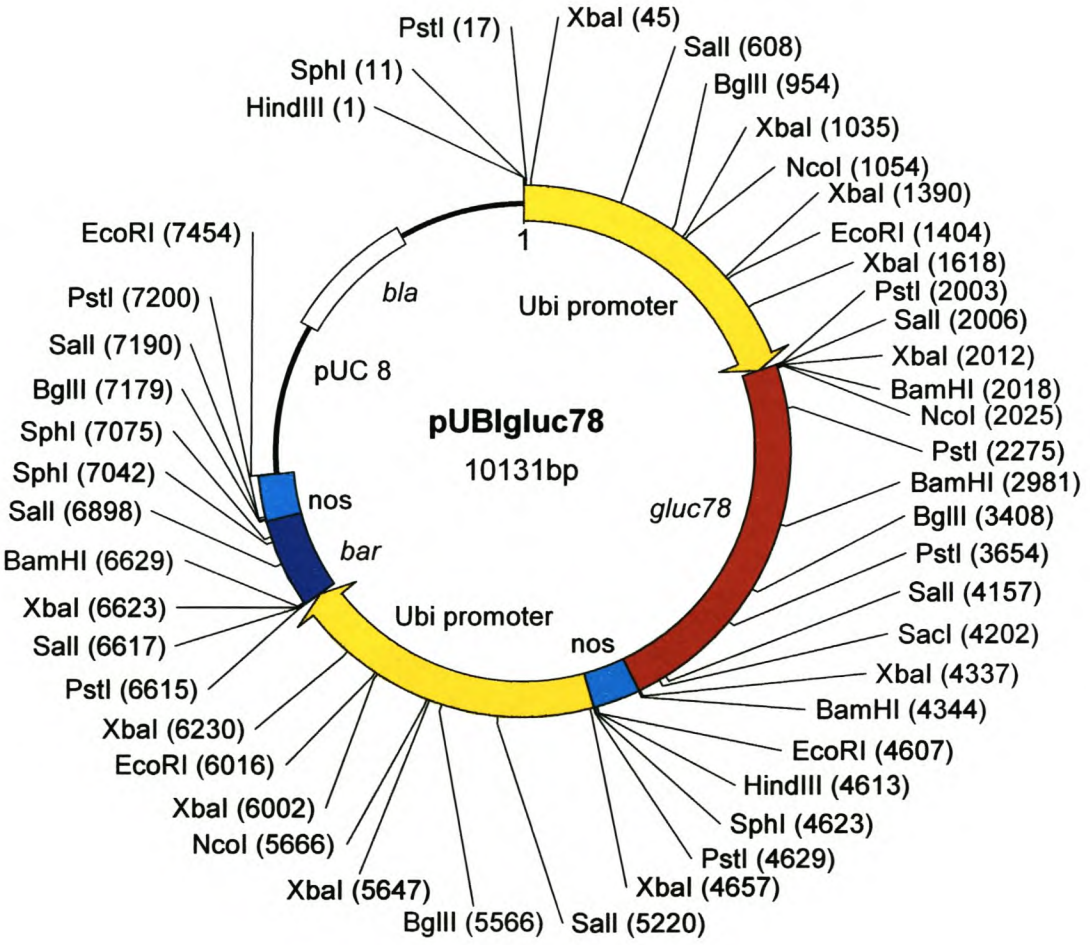

Figure 1: Construct pUBI*gluc*78 with selected restriction sites indicated. pUBI*gluc*78 contains the *gluc*78 and *bar* genes, both driven by the ubiquitin promoter, exon and intron and terminated by nos.

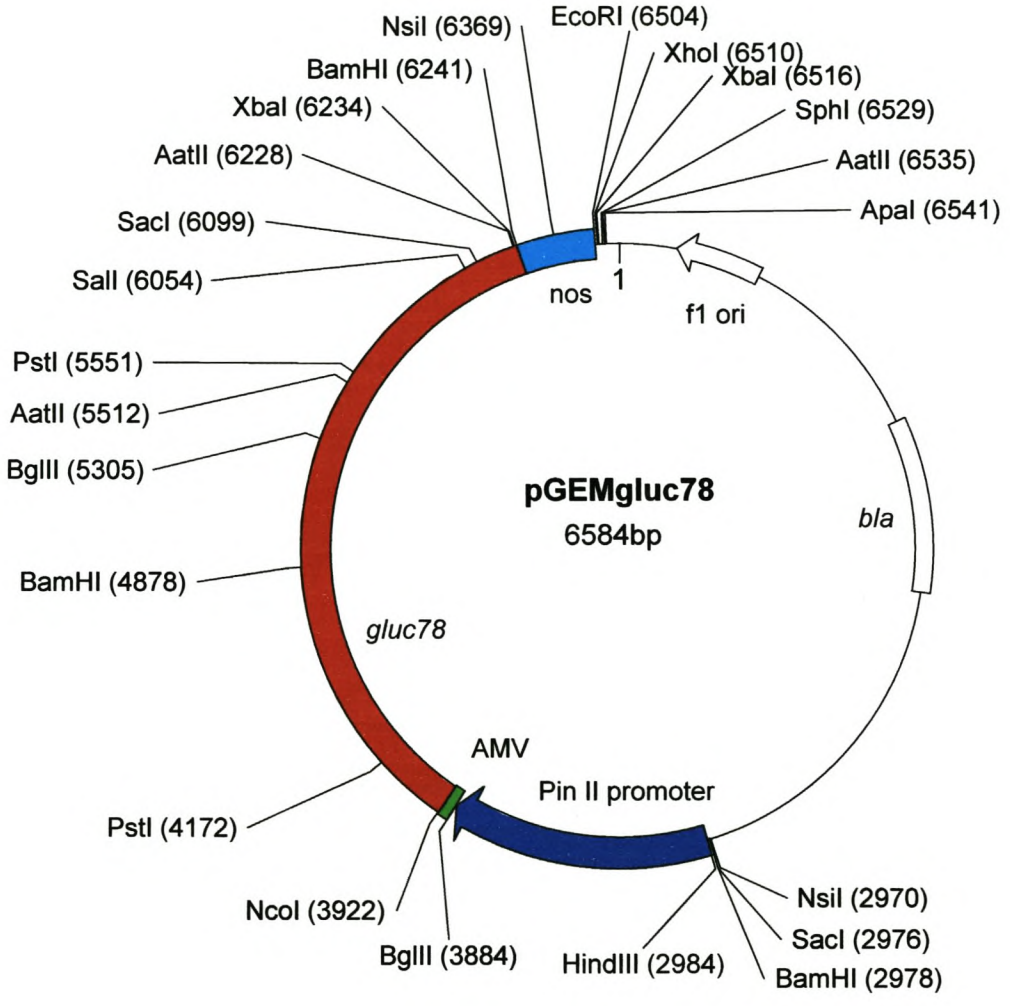

Figure 2: Construct pGEM*gluc*78 with selected restriction sites indicated. pGEMgluc78 contains the *gluc*78 gene driven by the potato proteinase inhibitor IIK gene promoter (PinII) and containing the AMV leader sequence.

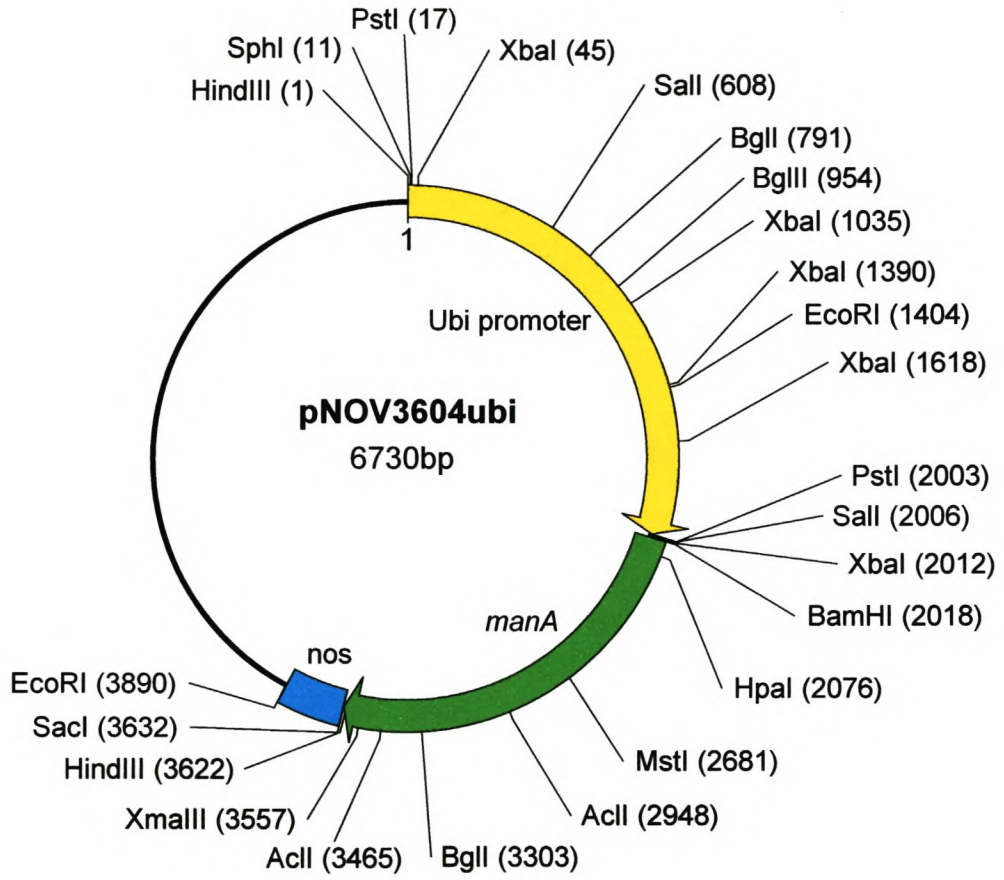

Figure 3: Construct pNOV3604ubi with selected restriction sites indicated. *Hind* III was used to digest genomic DNA for southern blot analysis. pNOV3604 contains the *man*A gene driven by the ubiquitin promoter, exon and intron and terminated by nos.

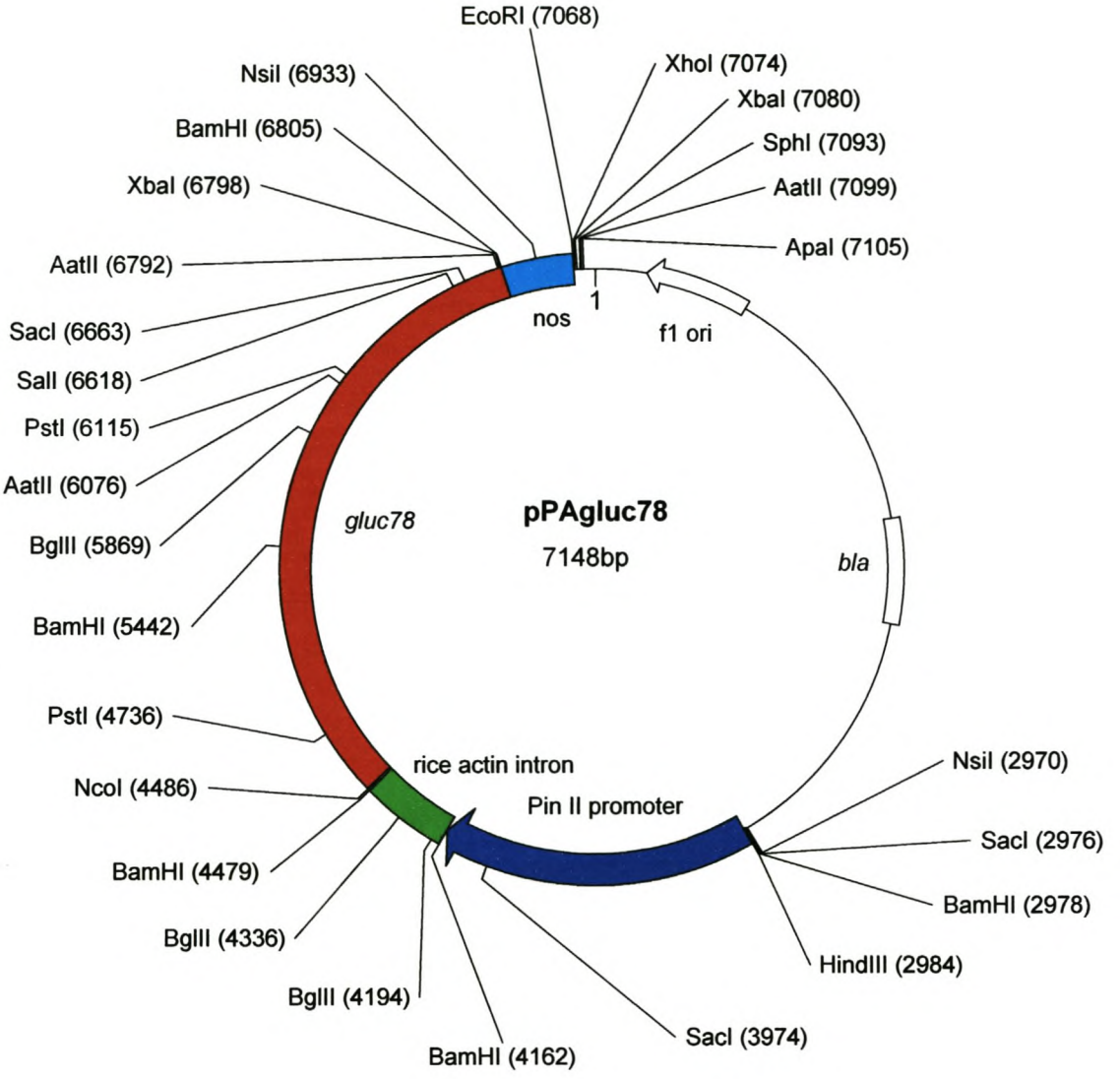

Figure 4: Construct pPA*gluc*78 with selected restriction sites indicated. *Hind* III is a unique restriction site and was used to digest genomic DNA for southern blot analysis. pPA*gluc*78 contains the *gluc*78 gene driven by the potato proteinase inhibitor IIK gene promoter (PinII) and containing the rice actin1 intron.

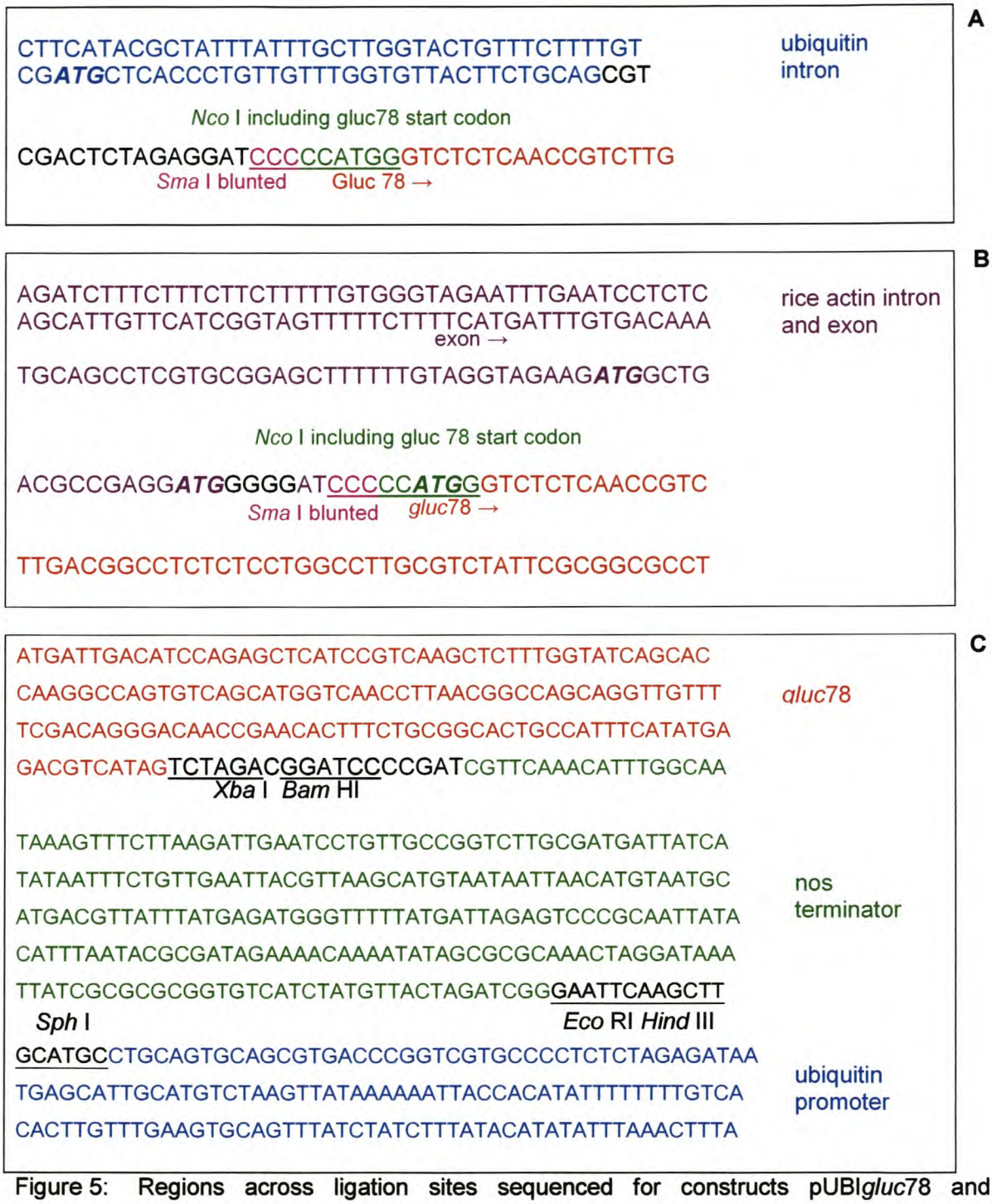

Figure 5: Regions across ligation sites sequenced for constructs pUBI*gluc*78 and pPA*gluc*78. A) pUBI*gluc*78 sequenced with the *gluc*78 antisense (AS) and Ubi S primers and B) pPA*gluc*78 sequenced with the *gluc*78 AS and PinII sense (S) primers and C) pUBI*gluc*78 sequenced with the *gluc*78 S and Ubi AS primers. Underlined sequence indicates restriction enzyme sites. Sequence in bold italics indicates an ATG triplet. Vector sequence is indicated in black print, and the gene sequence encoding for the *T. harzianum* 78 kD β 1,3- glucanase (*gluc*78 sequence) is indicated in red print. Other sequence is as shown below.

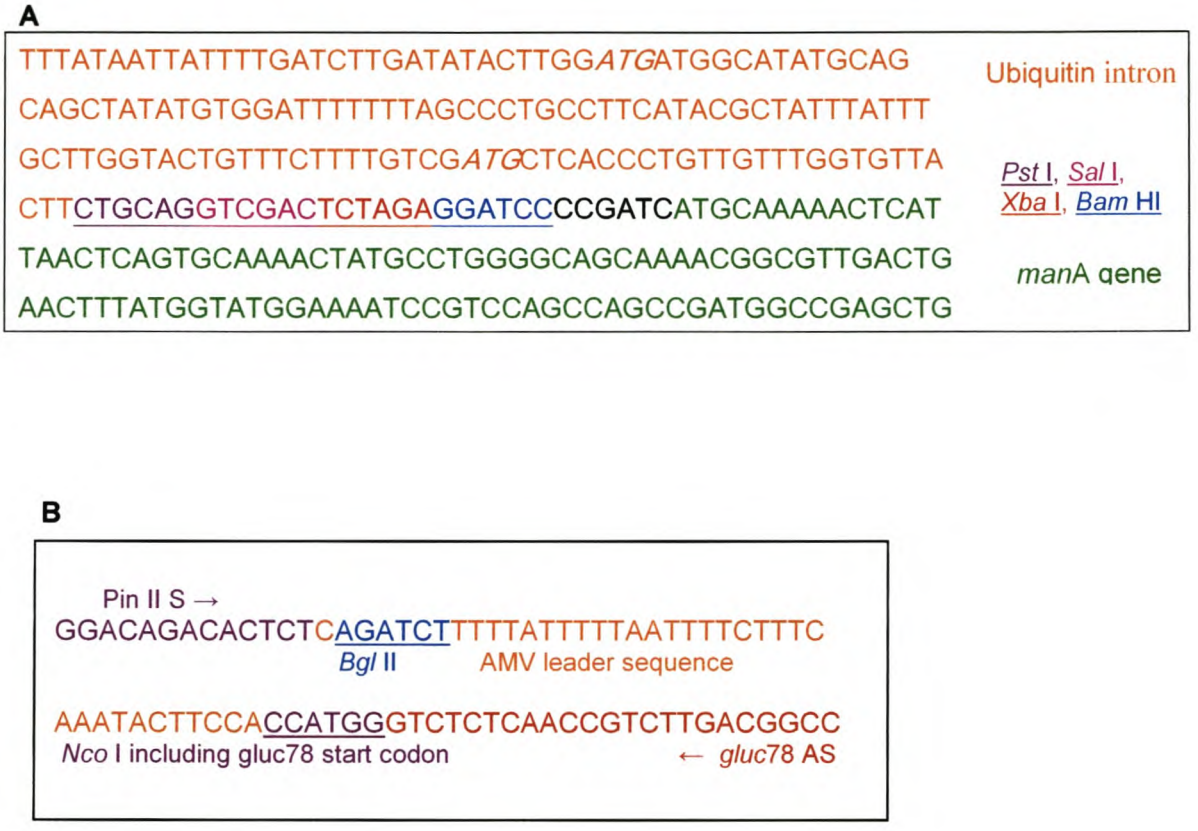

Figure 6: Regions across ligation sites sequenced for construct pNOV3604ubi and pGEM*gluc*78. A: pNOV3604ubi was sequenced with the *man*A antisense (AS) and Ubi Sense (S) primers as indicated in Figure 1. Sequence in bold italics indicates an ATG doublet in the ubiquitin intron. Vector sequence is indicated in black print, and the gene sequence *Escherichia coli man*A gene encoding phosphomannose isomerase, is indicated in green print. B: pGEM*gluc*78 was sequenced with the PinII S and *gluc*78 AS. In both regions the underlined sequences indicate restriction enzyme sites as indicated.