GENETIC MODIFICATION OF MAIZE

BY INTRODUCTION OF ANTIFUNGAL GENES

TO CONFER RESISTANCE TO

FUSARIUM VERTICILLIOIDES

by

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SUMMARY

Fusarium verticillioides causes ear- and stalk-rot infection of maize and, apart from decreasing the yield, this fungus produces mycotoxins, which accumulate when the harvested grain is stored for long time periods. Mycotoxins are toxic to selected animals and have recently been implicated in oesophageal cancer of humans. Thus, this study aimed at preventing fungal infection by introducing antifungal transgenes into selected maize genotypes via particle bombardment methods. Six elite white maize genotypes were initially screened on three different callus induction media (MS-, G2- and $N6_{10}$-based) for tissue culture amenability. Pre-cultured immature zygotic embryos (IZEs) of genotypes identified to be highly regenerable were used for bombardment experiments. The $N6_{10}$ callus induction media proved to be the best media for achieving high regeneration. Additionally, type-II callus tissue of a laboratory strain of maize (Hi-II) was used for bombardment.

The antifungal genes used in bombarded experiments were the maize *b32* gene (encoding a ribosome inhibiting protein) and the bean *pgip2* gene (encoding a polygalacturonase inhibiting protein). These two genes (under constitutive control of CAMV35S) in plasmid vectors pSC1b32 and pGEMpgip2, were co-bombarded targeting Hi-II callus tissues, as well as IZEs of selected elite maize genotypes. The *bar* selectable marker was used, thus conferring resistance to bialaphos-containing selection media in transgenic tissue. Putative transgenics identified were subjected to molecular analysis. A transformation efficiency of 0.17% was obtained for bombardment of elite maize genotype A483-4, with stable integration (1-2 copies) of the *b32* gene occurring in two events (BBE13.2.3 and BBE 16.1.1). Five fertile events (containing at least one of the antifungal genes) were obtained with Hi-II callus bombardment experiments. A transformation efficiency of 7.5% (per Hi-II callus plate bombarded) was obtained. Low copy integration (2-10 copies) events were confirmed by Southern blotting techniques of selected Hi-II maize transgenics, while northern blotting confirmed mRNA transcript production.

Fungal infection studies indicated a significant (up to 50%) reduction in *Fusarium verticillioides* (MRC826) infection of the transgenic seed surface, when compared to untransformed Hi-II seeds. This proves that the transgenic seeds do exhibit some resistance to fungal infection.

Further bombardment experiments with linear DNA minimal transgene cassettes (promoter, open reading frame and terminator only) were also performed on Hi-II callus tissues. Co-bombardment with the *bar-b32* and *pgip2* minimal cassettes (lacking vector backbone sequences) produced only one fertile event (BBF 1.1A, *b32* and *bar* PCR positive), however the T_1 progeny tested negative for all introduced genes.

Co-bombardment of the improved constructs, pSC1-mpib32 (containing the *b32* gene under control of a *Fusarium* inducible maize protease inhibitor (mpi) promoter) and pRTL2-improvedPGIP (encoding an oat cell wall-bound thionin signal peptide fused to PGIP2), did not yield any transgenics.

Additional bombardment experiments of Hi-II callus tissues with strawberry terpene synthase gene constructs (*inors* and *tnors*) were performed. The pAHCinors contains the *inors* gene and with no plastid targeting, is expected to produce nerolidol (a sesquiterpene), while the pAHCtnors (containing the *tnors* gene with chloroplast targeting), is expected to produce linalool (a monoterpene). These terpenes were previously reported to show inhibition of fungal mycelial growth of several *Fusarium* isolates, and therefore, these terpene synthase genes were used in this study. Bombardment experiments with pAHCinors did not produce any surviving transgenics, however those with pAHCtnors produced only one fertile event (BBT 1.1B). Plants of this event displayed a distinct linalool scent when regenerating under laboratory conditions, but lost this scent upon transferral to greenhouse conditions. T_0 plants were PCR positive for the *bar* and *tnors* gene, however these genes were not detected in the T_1 generation plants.

ACKNOWLEDGEMENTS

I can do all things through the Lord who strengthens me

(Phillipians 4:13)

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CHAPTER ONE:

AIMS AND OBJECTIVES

Maize is the world's third most important crop, however most of the maize produced and stored worldwide is considered to be below food safety levels due to the mycotoxin contamination. Mycotoxins are produced by the fungus *Fusarium verticillioides* which causes ear- and stalk-rot infection of maize. This fungus continues to pose a threat to farmers by destroying crops or dramatically reducing yields and to animal and human health by production of mycotoxins. Current research into use of fungicides and biological control is being conducted but no cost-effective way is available to date, to prevent fungal infection of crops in the field. The only real prospect of preventing fungal infection of crops is to develop plant varieties that are resistant to fungal infection, either through conventional plant breeding or through genetic modification.

Thus, this study aims at preventing fungal *infection* by genetically engineering fungal resistance into selected maize genotypes. Combinations of antifungal genes (antifungal properties explained in the literature review) will be introduced for constitutive expression into maize, via particle bombardment methods. The antifungal transgenes to be used in this study include the maize *b32* (encoding a ribosome inhibiting protein), bean *pgip2* (encoding a polygalacturonase inhibiting protein) and the strawberry terpene synthase gene.

The Hi-II and elite white maize genotypes will be used in this study. Each genotype has extremely diverse tissue culture and transformability characteristics, and thus bombardment of the Hi-II and elite maize genotypes will be reported in two separate chapters.

OBJECTIVES

- To screen six elite white maize genotypes on three different callus induction and regeneration media for regeneration ability, with the aim of identifying at least one highly regenerable elite maize genotype
- To identify a method for selection and regeneration of transgenics on Basta® herbicide
- To transform selected maize genotypes with the antifungal and *bar* selectable marker genes using microprojectile bombardment techniques
- To regenerate and screen putative transgenic plants
- To confirm the presence of antifungal genes in these transgenics
- To test resistance of transgenic seeds to the fungus *in vitro*

CHAPTER 2:

LITERATURE REVIEW

2.1 MAIZE PRODUCTION IN SOUTH AFRICA

Maize (known as corn in the US and Canada) is the world's third most important crop after rice and wheat. Introduced into Africa by the Portuguese at the beginning of the 16th century (Reader, 1998), maize has since become Africa's second most important food crop behind cassava. It is the single most important staple food crop in countries of the Southern African Development Community (SADC) (Angola, Botswana, Lesotho, Malawi, Mauritius, Mozambique, Namibia, South Africa, Swaziland, Tanzania, Zambia and Zimbabwe). Maize is mainly consumed in African households as a thick porridge, produced by hand pounding (usually of flint-type varieties preceded by soaking), or grinding in a hammer mill, followed by boiling (usually of dent varieties). A considerable amount of maize is consumed fresh as a food snack (either roasted or boiled). Maize also serves as a major source of stock feed in certain African and most European countries, and it is also used in the food processing and pharmaceutical industries.

Yellow maize forms the bulk of maize grown worldwide. The amount of maize used for livestock feed is three times higher than that used for direct human consumption. In developing countries where it remains an important part of the human diet, white maize tends to assume much greater importance than yellow varieties (FAO, 1996). White maize is biologically and genetically very similar to yellow maize. However, a difference in appearance due to the lack of carotin oil pigments in the kernel occurs, which normally gives yellow maize its colour (reviewed on the FAO website, 1996).

In South Africa, the National Crop Estimates Committee (NCEC) reported that commercial farmers planted an estimated 2.7 million hectares of maize in 2000/2001, down 18% from the previous year. This total included 1.6 million hectares of white maize (59%) and 1.1 million hectares of yellow maize (41%). The area planted in the non-commercial sector (subsistence farmers), was reported to have decreased by a commensurate amount (South African Corn Update, 2001). The reduced planting area and yield per hectare of maize (outlined in the graphs below) is increasingly becoming of great concern, especially since the consumption of maize in SADC countries is slowly exceeding the maize production.

South African Maize Area Planted (1969-2001)
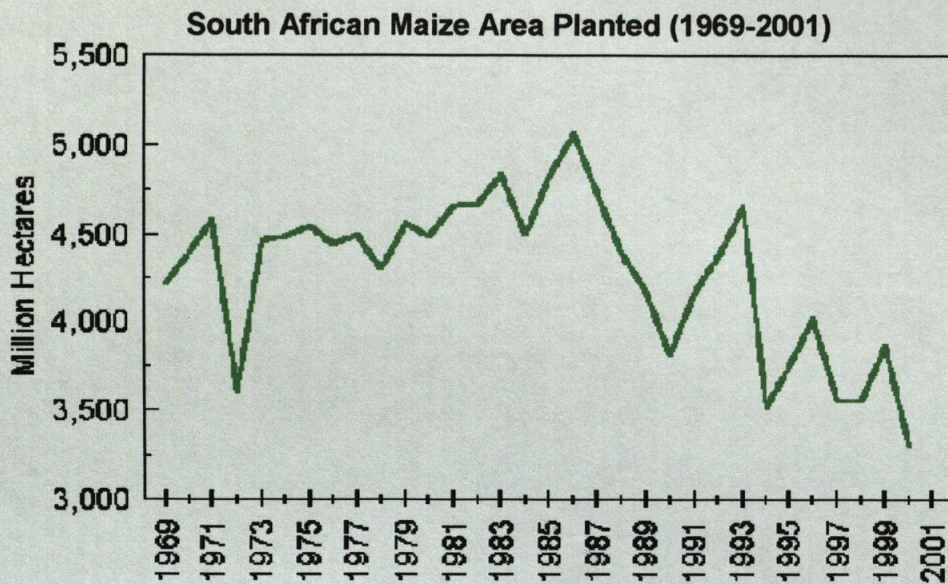
mp

Fig 2.1 The total estimated area (in million hectares) of maize planted by Commercial farmers in South Africa over the period 1969-2001. This total includes yellow and white maize planted.
Graph adapted from the South African Corn Update (12 Feb 2001)

South African Maize Yield (1969-2001)
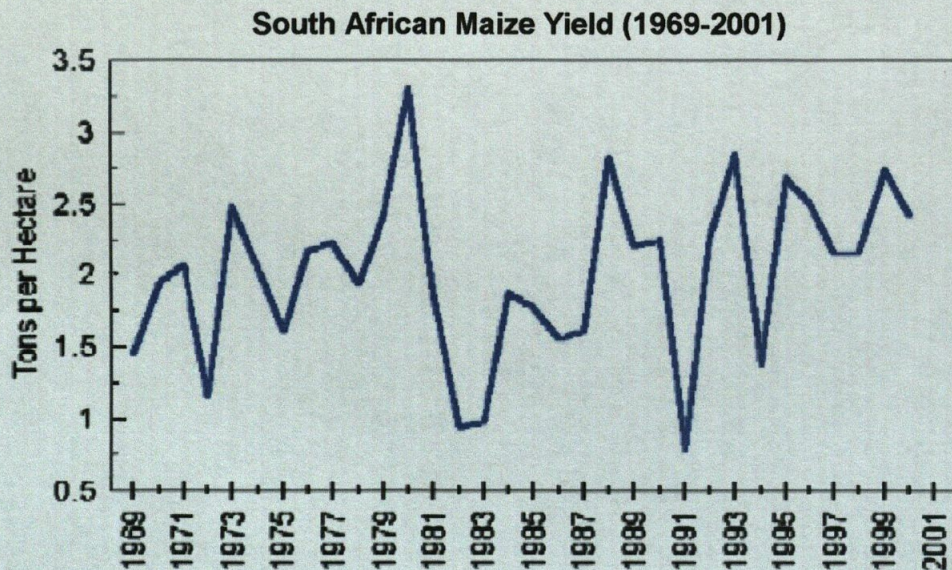
Fig 2.2 The total estimated maize yield (in tons per hectare) in South Africa over the period 1969-2001. This total includes yellow and white maize planted.
Graph adapted from the South African Corn Update (12 Feb 2001)

SADC Maize Production, Consumption and Requirements (1995-2002)
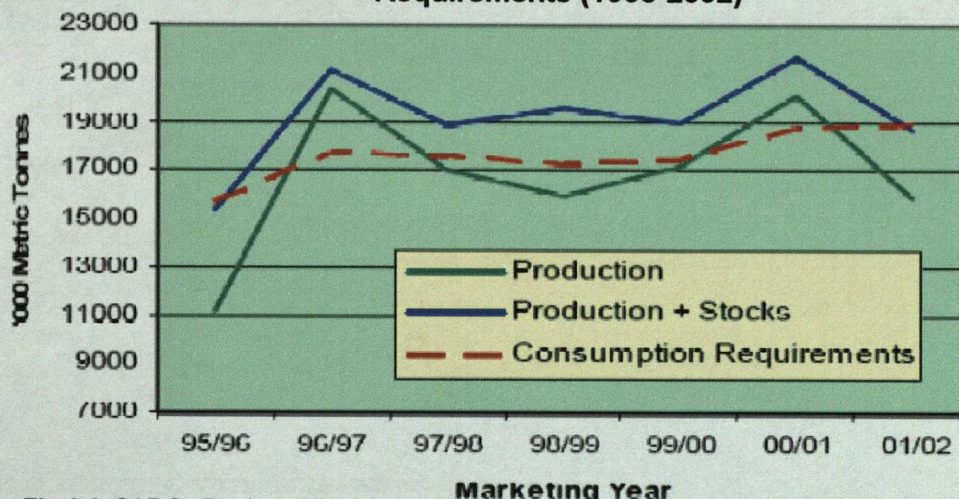
Fig 2.3 SADC Regional Maize production, consumption requirements and stocks (adapted from the SADC Food Security Network: Ministerial Brief, 5 Feb 2002). The average annual maize production for the 2001/2002 period in South Africa was approximately 8 million tonnes as outlined in the table below.

Table 2.1 Maize Availability ('000 tonnes) in South Africa: 1 May 1997 – 31 April 2002)

Local	1997/98	1998/99	1999/2000	2000/2001	2001/2002
White	4,614	4,383	4,141	6,154	4,109
Yellow	3,874	2,699	2,574	3,986	3,114
Developing Agriculture:					
White Maize	0,524	0,323	0,319	0,297	0,189
Yellow Maize	0	0,139	0,138	0,125	0,069
Imports	0,109	0,098	0,527	0	0,395
Total	**9,121**	**7,642**	**7,699**	**10,562**	**7,876**
Exports	1,921	1,388	0,627	1,423	1,281

Adapted from the website www.afma.co.za/AFMA_Template/feed_industry.htm

This varying in maize yields from year to year is attributed to the amount and distribution of rainfall, as well as outbreaks of pests and diseases in the region. Priority biotic constraints of maize include insect pests (stem borers, grain borers and weevils), foliar diseases (downy mildews, rusts leaf blights and leaf spots), fungal diseases (ear rots, stalk rots, smuts and kernel rots), bacterial diseases (stalk rot, wilting and leaf stripe), viral diseases and the parasitic weed, *Striga* (reviewed by the CIMMYT Maize Program, 2003). In Africa, the Maize Streak Virus (MSV) (indigenous to South Africa) ranks as the most widespread biotic constraint to maize production.

2.2 *FUSARIUM VERTICILLIOIDES* INFECTION OF MAIZE

Much of the maize produced and stored worldwide, especially in SADC countries such as Zambia, Zimbabwe and South Africa, is often at risk of being below acceptable food safety levels according to the FAO (Doko *et al.*, 1996). This is due to mycotoxins produced by the fungus *Fusarium verticillioides* (*F. monoliforme*), which is the most commonly reported species infecting maize (Nelson, 1992; Nelson *et al.*, 1993). This fungus causes ear- and stalk-rot infection in maize while growing in the field as well as in the harvested grain. Other Fusarium species causing ear-rot diseases include *Gibberella zeae*, *F. graminaearum* and *F. subglutinans*. *Stenocarpella maydis (Diplodia)* and other species of *Helminthosporium*, *Penicillium*, *Aspergillus* and *Nigrospora*, are other fungi causing ear-rot in maize. Stalk-rot fungi include *Colletotrichum graminicola* (anthracnose stalk rot), *Stenocarpella maydis* (diplodia stalk rot) and *Gibberella* species (reviewed in Flett, 1996).

F. verticillioides can be found in plant residues in almost every maize field at harvest, yet the disease symptoms vary widely and range from asymptomatic infection to severe rotting of all plant parts. In many cases, diseased and asymptomatic plants occur in the same field planted with a genetically uniform maize variety. Environmental conditions, water availability (Magan *et* al., 1984) and the genetic background of the plant and the pathogen (Leslie *et* al., 1990; Yates *et* al., 1997), may all be important factors in disease development. However, it is not known why disease does not occur during asymptomatic infections or what causes rotting and wilting of the diseased tissues.

2.2.1 Routes of infection

The infection of maize by *F. verticillioides* can occur via several routes: through wounds caused by insects or birds (Sutton *et al.*, 1980; Attwater *et al.*, 1983) or by growth of mycelium down silks to the kernels and cob (Koehler, 1942; Hesseltine *et al.*, 1977). However, the most commonly reported method of kernel infection is through airborne conidia that infect the silks (Headrick *et* al., 1991). After invasion through silks, the fungus infects the kernels but usually only a small percentage of infected kernels become symptomatic (Munkvold *et* al., 1997). Another proposed infection pathway is through the seed (Foley, 1962): this infection can start from fungal conidia or mycelia that are either carried inside seeds, or on the seed surface. The fungus develops inside the young plant, moving from the roots to the stalk and finally to the cob and kernels (Kedera *et al.*, 1992). Infection may also result from inoculum that survives in crop residues in the soil, however the relative importance of soilborne and seed-borne inocula as the cause of systematic infections is unknown (Leslie *et al.*, 1990; Rheeder *et al.*, 1998). Temperature and moisture conditions during the growth season and insect infestations are critical factors affecting fungal infection.

Peak levels of kernel infection occur 9 - 10 weeks after corn has silked and grain moisture has dropped below 25% (Carson *et al.*, 2002).

2.2.2 Mycotoxins

F. verticillioides produces several mycotoxins that have potential toxicity for humans and domesticated animals. Mycotoxins are toxic chemicals produced by many fungi and they vary in their degree of toxicity. They are not confined to *Fusarium* species that infect cereals. Many other fungal species such as *Aspergillus, Alternaria* and *Pennicilium* can also produce mycotoxins. Infection can reduce yield and quality, and result in mycotoxin accumulation in the grain (Kommendahl *et al.*, 1981; Marasas *et al.*, 1984). In South Africa, many of these fungi predominate in grain crops. These fungi together with the most important mycotoxins that each can produce, are given below for maize (Viljoen, 2003):

Table 2.2 Infecting fungi and their associated mycotoxins produced in maize

Fungal species	Main mycotoxins produced	Reference
Fusarium verticillioides (Previously known as *F. moniliforme*)	Fumonisins (FBs)	Gelderblom *et al* (1988); Thiel *et al* (1991); Marasas (2001); JECFA (2002)
Fusarium subglutinans	Moniliformin (MON)	Kriek *et al* (1977); Marasas (2001)
Fusarium graminearum	Deoxynivalenol (DON), or nivalenol (NIV), zearalenone (ZEA)	Marasas *et al* (1984); Marasas (2001)
Aspergillus flavus	Aflatoxins	IARC (1993); JECFA (1998)
Penicillium spp	ochratoxin A (OA), Citrinin (CIT), Patulin (PAT)	Scott (1994)
Stenocarpella maydis (known as *Diplodia maydis*)	Unidentified,causing diplodiosis in cattle and sheep	Rabie *et al* (1985a); Kellerman *et al* (1985)
Stenocarpella macrospora	Diplosporin	Gorst-Allman *et al* (1983)
Alternaria alternata	Alternariol monomethyl ether (AME)	Visconti & Sibilia (1994)

Wheat, grain sorghum and sorghum malt are among other grains that are infected by these fungi. Toxins produced by the *Fusarium* species include the trichothecenes, moniliformin, zearalenone and fumonisins. All these mycotoxins have different toxicological properties and may be produced singly or in combination depending on the strain and growth conditions. The most significant of these toxins produced by *F. verticillioides* are the fumonisins (Desjardins *et al.*, 1995; Nelson *et al.*, 1993). Since fumonisins can be detected in symptomatic and asymptomatic kernels, control of fumonisin contamination has become a priority area in food safety research (Brown *et al.*, 2001).

2.2.3 Existing limits for fumonisms

Many countries have set regulatory maximum tolerable levels (MTLs) for selected mycotoxins in grain crops and their products intended for human and animal consumption. So far, three countries have formulated MTLs for fumonisins: Switzerland (being the only country that adopted a legislative regulatory limit), has an MTL of 1 µg/g for maize products (Zoller et al., 1994). Additionally advisory limits (for USA), were published by the Food and Drug Administration (FDA, reviewed in Viljoen, 2003). In South Africa where the daily intake of maize products in rural and urban areas, are relatively high, a recommended MTL of 122 ng/g for rural people and 202ng/g for urban people were set (Gelderblom et al., 1996; Marasas, 1997).

2.2.4 Toxicity of Mycotoxins

Fumonisin mycotoxins are highly toxic to horses, donkeys and mules causing eucoencephalomalacia (necrosis of the brain). They also cause experimental liver cancer in rats and lung diseases in swine (Marasas, 1995). Furthermore, some studies have implicated fumonisins in the high occurrence of human oesophageal cancer in certain regions of South Africa and China (Rheeder et al., 1992; Shephard, et al., 1996). The International Agency for Research on Cancer of the World Health Organisation (IARC), has categorised toxins derived from *F. verticillioides* (in particular Fumonisin B1) as a Group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2002; JEFCA, 2002). To date, the only maize mycotoxin directly linked to human diseases, are aflatoxins. Additionally, mycotoxins (strong immuno-suppressive agent) exposed to pregnant mothers have been shown to pose a threat to foetal development (Dutton et al., 2001).

The effect of mycotoxin exposure to HIV positive individuals is of further concern. In African rural areas, where per capita maize consumption is the highest: individuals will be ingesting fumonisins at an average rate of between 124 and 253 µg/70kg person/day (Viljoens, 2003). On a daily and on-going basis, accumulation of these mycotoxins could pose a serious health hazard. These mycotoxins are heat-stable: ordinary cooking and heat processing procedures do not substantially reduce toxin levels (reviewed in Doyle, 1997). It is therefore important to prevent the growth of *Fusarium* species in foods.

2.2.5 Preventing fungal infection

Theoretically, preventing fungal infection of the growing plant or the stored commodity could prevent mycotoxin contamination of food. In practice, however, mycotoxins in food are unavoidable because fungi are ubiquitous and there is no cost-effective way available to prevent fungal infection of crops in the field.

Fungicide treatments do reduce incidence of *Fusarium* infections in wheat, however no fungicides are approved for use at the flowering stage (reviewed in Doyle, 1997). Research into the potential for using microbes antagonistic to *Fusarium* species was being conducted with rice plants susceptible to *F. verticillioides* (Rosales *et al.* 1997). Furthermore, a biological control system using an endophytic bacterium, *Bacillus subtilis*, has been developed showing great promise for reducing mycotoxin accumulation. This bacterium occupies the identical ecological niche within the plant and is therefore considered as an ecological homologue to *F. verticillioides*. The inhibitory mechanism is thought to operate solely on the competitive exclusion principle (Bacon *et al.*, 2001). The polishing of rice, removal of small particles from corn, treatment of corn flour with calcium hydroxide, or wet milling of corn during processing methods, can substantially reduce toxin levels (reviewed in Doyle, 1997). These efforts basically control toxins levels, or reduce infection but do not prevent it. The only real prospect of achieving resistance is through conventional plant breeding or through genetic modification.

Traditional breeding for resistance to *Fusarium* infection was performed with several wheat varieties developed in China showing some degree of resistance (Mesterhazy, 1997). Breeding programs in Canada also produced varieties of corn with resistance to infection by fungal growth through the corn silk (reviewed in Doyle, 1997).
Additionally, genetic engineering techniques have also been utilized to transfer antifungal genes from plants or microbes into wheat and potatoes demonstrating enhanced resistance to *Fusarium* species (Mesterhazy, 1997).

Despite intensive research, prevention or elimination of *Fusarium* fungal infection and the presence of its mycotoxins in food, have not met with a great deal of success. Thus, this study aims towards terminating the fungal infection at an early stage of infection in both ear- and stalk-rot, by expressing plant-derived antifungal transgenes.

2.3 PLANT TRANSFORMATION

Since the advent of recombinant DNA technology in the 1970's, the last few years have shown remarkable advances in plant genetics and the rise of plant transformation. These new advances, both biological and mechanical have given researchers the ability to express foreign genes in desired plant hosts, essentially breaking the plant barrier species. Plant transformation has been defined as the stable incorporation and expression of foreign genes into plants. In 1983, Robert Fraley and colleagues reported the successful incorporation of a kanamycin resistance gene into the tobacco genome. Within a year other researchers reported similar accomplishments (De Block *et al.*, 1984; Horch *et al.*, 1984; Paszkowski *et al.*, 1984), marking the beginning of plant transformation.

Since then, successful transformations have been described for over 120 species in 35 different families (Birch, 1997) and the number is increasing every year. With respect to cereals, efficient transformation systems (both biolistic and *Agrobacterium*- mediated) have been established for maize (Armstrong & 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 & Lemaux, 1994).

The Hi-II and elite white maize genotypes used in this study, each have extremely diverse tissue culture and transformability characteristics, which is why bombardment of these genotypes are reported in two separate chapters. IZEs of elite white maize genotypes, when cultured, produce type-I callus tissues. IZEs of the laboratory strain, Hi-II, on the other hand produces a type-II callus tissue when cultured (explained further in Section 2.4.1).

The process of plant transformation is currently a long complex multi stage procedure involving three general phases: Phase 1 involves the selection and application of a delivery system that incorporates the DNA of interest into a viable host cell. Phase 2 involves selection of the transformed tissues, indicating proper integration and expression of the gene product. Finally, Phase 3 involves the recovery of a viable transgenic plant. In many cases, recovery involves tissue culture and regeneration, which can be a rate-limiting step. There are three main classes of delivery systems: biological, physical and alternative, which can be use in transformation protocols.

2.3.1 Biological Delivery System

Agrobacterium-mediated transformation occurs via a soilborne bacterium (*Agrobacterium tumefaciens*) that has been called "nature's own genetic engineer" because it naturally transfers its own DNA into the plant it infects (Yusibov *et al.*, 1994; Hansen *et al.*, 1996, Hansen *et al.* 1997). Attenuated strains of this bacterium have been created to carry the transgene of interest, but will not induce the tumors typically associated with wild type infection. The new transgene is incorporated into the plant DNA through the *A. tumefaciens* border sequences, which facilitates its transfer and stable integration, and in most cases does not transfer unwanted bacterial DNA. *Agrobacterium*-based DNA transfer system offers many advantages in plant transformation: (1) the simplicity of *Agrobacterium* gene transfer makes it a "poor man's" vector; (2) a precise transfer and integration of DNA sequences with defined ends; (3) a linked transfer of genes of interest along with the transformation marker; (4) the higher frequency of stable transformation with many single copy insertions; (5) reasonably low incidence of transgene silencing, and (6) the ability to transfer long stretches of T-DNA (>150 kb) (reviewed in Veluthambi *et al.*, 2003).

The inability of *Agrobacterium* to transfer DNA into monocotyledonous plants was considered a major limitation. However, with effective modifications in Ti plasmids and finer modifications of transformation conditions, many monocotyledonous plants including rice (Hiei *et al.*, 1994; Rashid *et al.*, 1996; Chen *et al.*, 1998), wheat (Cheng *et al.*, 1997), maize (Ishida *et al.*, 1996), sugarcane (Arencibia *et al.*, 1998) and barley (Tingay *et al.*, 1997), were transformed. *Agrobacterium* T-DNA transfer is now viewed as "universal" due to successful transformation of yeast (Bundock, *et al.*, 1995), *Aspergillus* (de Groot *et al.*, 1998) and human cells (Kunik *et al.*, 2001).

2.3.2 Physical Delivery System

Microprojectile-mediated transformation (discovered in 1987) can drive foreign DNA past biological barriers and has the unique ability to target all genomes within a cell, including choloroplasts (Ye *et al.*, 1990; Daniell, 1993) and mitochondrial genomes. This method was first used to deliver DNA and RNA into epidermal cells of *Allium cepa* (Klein *et al.*, 1987). Since then this technique was used to transform yeast and filamentous fungi (Armaleo *et al.*, 1990), algae (Zumbrunn *et al.*, 1989), cereals (Tyagi *et al.*, 1999) and pulses (Gupta *et al.*, 1999). The first successfully transformed monocot was the Black Mexican Sweet corn (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). This new ability to transform and regenerate monocot plants marked a significant advance in plant transformation. As more species (including many monocots) were being added to the transformation successes, researchers were concurrently studying and developing particle bombardment methods, maximizing techniques for both monocots and dicots (Russel *et al.*, 1992).

In the early 1990's, DuPont Biolistics Device PDS-1000 (Russel-Kikkert, 1993) was introduced which used high-pressure helium or nitrogen gas (approximately 150kg per cm^3) to accelerate particles. This system achieved a velocity of approximately 440 ms^{-1} needed for penetration. A cheaper alternative (used in this study) was the Particle Inflow Gun (PIG), which uses a solenoid to direct a helium stream accelerating particles into an evacuated chamber (Finer *et al.*, 1992). Metal particles were used for microprojectile transformation because a minimum density (19 g cm^{-3}) was needed to generate enough force to penetrate plant cells. Tungsten or gold particles are commonly used, as they are sufficiently dense and cost effective. Gold particles, have an advantage over tungsten particles as it will not oxidize or clump together (Hinchee *et al.*, 1994).

The advantages of particle bombardment are: (1) plants that are not infected by *Agrobacterium* can be transformed; (2) DNA may be transferred without using specialized vectors; (3) introduction of multiple DNA fragments/plasmids can be accomplished by co-bombardment, thus eliminating the necessity of constructing a single large plasmid containing multiple transforming sequences; (4) false positives resulting from only reporter gene expression in *Agrobacterium*, are avoided; (5) transformation protocols are applicable to plants lacking good regeneration systems, and (6) organelle transformation is achieved only by particle bombardment (reviewed in Veluthambi *et al.*, 2003). Certain drawbacks are also observed: low yield of stable transformants, high copy number and rearrangements of transgene(s) occur thus causing gene silencing. Many recent improvements were made to overcome gene silencing (Johnston *et al.*, 1988) and genetic integrity (Qu *et al.*, 1996).

Additionally, the overall transformation using biolistics was improved by osmotic conditioning of cells (Arencibia *et al.*, 1998). The first commercially released transgenic maize variety was developed using the gene gun (Gordon-Kamm *et al.*, 1990). Three years later, Koziel and co-workers (1993) published a paper on field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringienses*, using biolisitcs. Subsequently, stable transformation of elite maize by microprojectile bombardment of immature embryos, were performed by a few laboratories (Armstrong & Songstad, 1992; Hill *et al.*, 1995). Recent studies have also reported on biolistic transformation of Hi-II (laboratory maize strain) with the *uid*A reporter gene and *bar* selectable marker gene (O'Kennedy *et al.*, 1998). Elite white maize genotypes were also transformed with the *bar*, *uidA* and/or *pgip-1* genes (O'Kennedy *et al.*, 2001). With respects to *Agrobacterium*-mediated transformation of maize: high efficiency transformation was achieved for the laboratory strain of maize A188 (Ishida *et al.*, 1996) but not for elite maize genotypes.

2.3.3 Alternative Delivery Systems

Most alternative approaches attempted to develop genotype-independent, cost effective procedures for introduction of foreign DNA into cereals. Prominent among these methods are PEG-mediated transformation, liposome-mediated transformation (Gad *et al.*, 1990), electroporation (Fromm *et al.*, 1987), silicon carbide fibre-mediated transformation (Kaeppler *et al.*, 1990), microinjection (Crossway *et al.* 1986), electrophoresis (Ahokas, 1989), laser microbeam transformation (Weber *et al.*, 1989) and dessication (Topfer *et al.*, 1989). Although these methods have advantages, transformation using *Agrobacterium* and microprojectile bombardment are currently the most extensively deployed methods for plant transformation.

The reduced ability of *Agrobacterium* to infect monocots has become a fundamental limitation in transformation. Even though attempts to extend the transformation range of *Agrobacterium* by using different strains or altering the physiology and co-cultivation techniques of both the bacteria and the host, have been successful, this technique continues to have problems and limitations and the genotype dependency demonstrated is a disadvantage. It is for this reason that microprojectile (particle) bombardment using the Particle Inflow Gun (PIG), was chosen for use in this project.

2.4 PLANT TISSUE CULTURE

The entire transformation process is dependant on the ability of the species and tissues to produce totipotent cells that can be induced to regenerate into a complete viable plant. This regenerative ability is a necessity for all the different transformation systems. *In vitro* callus culture is the most commonly used totipotent tissue from which whole plants can be regenerated. Callus is defined as an amorphous mass of loosely arranged, thin-walled parenchyma cells arising from the proliferating cells of the parent tissue (Dodds & Roberts, 1985). Depending on the species, callus can be induced from a variety of tissues, including immature embryos, leaves, shoots and roots. Callus is initiated by culturing the excised tissue in a growth medium containing growth hormone (auxin) such as 2,4-dichlorophenoxyacetic acid (2,4-D).

A transformation example would include: excised immature zygotic embryos placed in a nutrient rich medium, with an appropriate delivery system (such as microprojectiles) being applied to the tissue. After treatment, the tissue would be transferred to new nutrient rich medium containing 2,4-D and the appropriate selectable or reporter gene substrate. After sufficient selection of transformed callus tissues, it is then transferred to new medium containing regeneration hormones, a balanced ratio of auxins and cytokine. Once both shoots and roots have formed, plants are transferred from the culture medium and potted in soil, where they are grown to maturity. The whole procedure, involving regeneration can take approximately 10-16 weeks (Birch, 1997). Thus plant transformation is a long and involved procedure. However, its time commitment is completely defined by the tissue culture and regeneration techniques. Efficient plant regeneration systems are primarily determined by two main factors: the explant and its regeneration capacity and the composition of the nutrient media chosen.

2.4.1 Explants

Young, meristematic tissues were shown to be more suitable for embryogenic culture initiation than older, more differentiated tissues, i.e. immature developing embryos and tissue segments obtained from young inflorescences and bases of young leaves were used to initiate cultures (Dale, 1980; Vasil & Vasil, 1980, 1981a; Wernicke & Brettell, 1980; Haydu & Vasil, 1981; Lu & Vasil, 1981a). Such explants were largely made up of meristematic and undifferentiated cells, which were not yet committed to any specific developmental pathways. The cultures derived from such cells were highly regenerable.

Since the introduction of immature embryos as donor tissues for establishing callus cultures of maize (Green & Phillips, 1975), plant regeneration from such cultures has become a common practice. Four factors were found to influence the development of embryogenic calli: (i) the size of immature embryos, (ii) the genotype, (iii) the callus media and (iv) the environment where the initial plants were grown (reviewed by Bohorova et al., 1995). The size of the explant and nutrient media, which can be easily optimised, will be explained in this review.

Maize embryos of 1.0 to 1.5 mm were found to be at the ideal physiological developmental stage for culture (Kamo et al., 1985) as older embryos, often germinated in culture and did not produce embryogenic callus. Two types of embryogenic callus have been observed for maize: Type I callus: the nodular, white, compact and organized embryogenic callus, was found by many researchers (Lu et al., 1982, 1983; Tomes & Smith, 1985). The embryogenic capacity of this type of callus however, is short-lived and the callus is difficult to maintain (Lu et al., 1982). The second type of callus (Type II) is soft, friable, yellowish and fast growing (Green, 1982; Vasil et al., 1984; Lowe et al., 1985; Vasil et al., 1985). This callus is suitable for the establishment of embryogenic suspension cultures, as it is dispersed easily and rapidly and maintains its embryogenic competence for a long period (Lu & Vasil, 1981; Vasil & Vasil, 1981a, 1982a; Ho & Vasil, 1983b). Type-II callus is often only initiated from laboratory strains of maize.

Somatic embryos either form directly from the partially organised structures at the coleorhizal end of the scutellum, or they give rise to embryogenic callus from which embryoids are later formed (Lu & Vasil, 1985). These tissues then form leafy structures when placed on suitable media, where enlargement and greening of the scutella of the somatic embryos occurs (Ozias-Akin & Vasil, 1982; Ho & Vasil, 1983a; Vasil et al., 1983a). This is then followed by shoot production and regeneration into plantlets on suitable tissue culture media.

2.4.2 Composition and quality of Nutrient Media

The quality and composition of tissue culture media used for plant cell culture and regeneration, is very important. No other factor has received as much attention and, as a result, numerous formulations have been published to date. Formulations designed by Murashige and Skoog (1962) (MS media), Gamborg et al. (1968) (B5 media), Chu et al. (1975) (N6$_{10}$ media) and Schenk & Hildebrandt (1972) (SH media), can be regarded as standard. Today, the MS-based medium is the most preferred tissue culture medium.

In this project, the N6$_{10}$ based medium developed by Armstrong and Songstad (1993) was mostly used. Other media used for regeneration studies were the G2+Pro medium (G2-based (Gless et al., 1998) supplemented with L-proline), and the 2 MS+Pro medium (MS-based (Murashige and Skoog, 1962) supplemented with L-proline). Immature embryos call for a far more critical medium composition than that required for mature embryos and this explains the different media compositions for callus initiation, maturation and regeneration outlined in Table 2.2. Both mature and immature embryos however, require macro-elements, micro-elements and sugar. Usually a solid medium with pH 5.0 to 6.0 is used (reviewed by Pierik, 1987).

Table 2.3 Macro- and Micro-nutrients included in the respective tissue culture media

	G2	MS	N6
Macroelements (mg l^{-1})			
NH_4NO_3	200	1650	0
KNO_3	1750	1900	2830
$CaCl_2 \times 2H_2O$	450	440	166
$MgSO_4 \times H_2O$	350	370	185
KH_2PO_4	200	170	400
NaEDTA	37	0	0
$FeSO_4 \times 7H_2O$	28	0	0
$(NH4)_2SO_4$	0	0	463
Microelements (mg l^{-1})			
H_3BO_3	5.00	6.3	1.6
$MnSO_4 \times 4H_2O$	25.00	22.3	3.3
$ZnSO_4 \times 4H_2O$	7.50	8.6	1.5
KI	0.75	0.83	0.8
$Na_2MoO_4 \times 2H_2O$	0.25	0.25	0.25
$CuSO_4 \times 5H_2O$	0.025	0.025	0.025
$CoCl_2 \times 6H_2O$	0.025	0.025	
Vitamin (mg l^{-1})			
Thiamine-HCl	10	0.1	1
Pyridoxine-HCl	1	0.5	0.5
Nicotinic Acid	1	0.5	0.5
Myo-inisitol	100	100	100
Glycine	0	2	2
L-glutamine	420	0	0

Table 2.4   Tissue Culture Media Composition:

Nutrient	MS based media[c]			N6₁₀ based media[d]			G2 based media[e]		
	2MS+Pro (Initiation)	0MS (Regeneration)	½ MS (Germination)	N6₁₀ (Initiation)	OT6S (Maturation)	OT2S (Regeneration)	G2+Pro (Initiation)	G2 Mat. (Maturation)	G2 Reg. (Regeneration)
MS macro-, micro-nutrients[a]	+	+	½+		+	+			
MS Fe-source[b]	+	+	½+		+	+	+	+	+
N6 macro-, micro-[a] and Fe-source[b]				+					
L3 macro-, micro-nutrients[a]							+	+	+
MS vitamins[a]	+	+							
B5 vitamins[a]					+	+			
N6 vitamins[a]				+					
HL2 vitamins							+	+	+
Sucrose	30g/l	30g/l	15g/l	20g/l	60g/l	20g/l			
Maltose							30g/l	60g/l	30g/l
PH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Bacto agar						4g/l			
Gelrite						2g/l	4g/l	4g/l	4g/l
Agarose					6g/l	6g/l			
Agar	8g/l	8g/l	8g/l						
AgNO₃				10mg/l					
2,4-D	2mg/l			1mg/l			2.5mg/l		
L-Proline	20mM			25mM			20mM		
Casein hydrolysate				100mg/l					

+ = Indicates inclusion of the particular nutrients in that specific media
a = Constituents outlined in Table 2.4
b = The source of iron (Fe) used in these media are outlined in the respective media references below
c = MS-based media developed by Murashige and Skoog (1962)
d = N6₁₀ based media developed by Armstrong and Songstad (1993)
e = G2-based media developed by Gless *et al.* (1998)

Sugar is primarily important as an energy source, although it also has the role of lowering (negative) the osmotic potential of the nutrient media, especially with young embryos (Rijven, 1952). Sucrose is generally used as sugar source, although glucose and fructose are sometimes suitable. Mature embryos are usually grown on 2-3% sucrose, while immature embryos thrive better on higher sugar concentrations (8-12%); the sugar demand decreases with the embryo size (Monnier, 1980). Rapela (1985) indicated that a lower sucrose concentration (less than 2-3%) in the initiation medium, resulted in organogenesis rather than embryogenesis. In the media used in this study, a 2-3% sugar content was used in the callus initiation media. This content was doubled in maturation media to promote embryogenesis and somatic embryoid formation (Close & Ludeman, 1987). It was further lowered in regeneration media to promote formation of leafy structures (Vasil *et al.*, 1983a).

The majority of explants are maintained on solid media, solidification being achieved by media supplements of agar, agarose or gelrite. An agar concentration of 0.6 - 0.8% is usually chosen, as higher concentrations result in the inhibition of growth (Stolz, 1971). The higher purity of agarose compared to agar may make for better response of an explant to *in vitro* culture (reviewed by Vasil & Thorpe, 1994).

2.4.3 Plant Growth substances

Plant hormones (known as Plant Growth Substances (PGS) or phytohormones) added to tissue culture media are of great importance. They have the ability to regulate many aspects of plant growth and development from seed germination through to senescence and death of the plant. There are five classical hormones identified in table 2.5: auxin, gibberellin, cytokinin, abscissic acid and ethylene. These are small molecules synthesized by the plant, and are active at 10^{-6} to 10^{-8} M. Auxins are distributed within tissues from cell to cell, while cytokinins are distributed via vascular bundles, and ethylene via intercellular spaces.

Auxin was the first plant hormone to be discovered (Darwin, 1880). Today, known as indole-3-acetic acid (IAA), it is produced in apical and root meristems, young leaves and seeds in developing fruits. It induces cell elongation in stems; stimulates cell division in combination with cytokinins in tissue culture, and also stimulates root initiation on stem cuttings. Many synthetic auxins have been developed, e.g. indole-3-butyric acid (root initiation), napthaleneacetic acid (apple fruit thinning) and 2,4-dichlorophenoxyacetic acid (2,4-D) for growth. 2.4-D together with sucrose in media has an influence on callus quality. Embryogenesis was promoted by higher 2,4-D level when a high sucrose concentration was used, and lower 2,4-D levels when a low sucrose concentration was used (Woodward & Furze, 1989).

Table 2.5 Plant Hormones and their functions

PGS	Where Found in Plant	Major Functions
Auxin	Embryo of seed, young leaves, meristems of apical buds	Stimulates cell elongation; involved in phototropism, gravitropism, apical dominance, and vascular differentiation; inhibits abscission prior to formation of abscission layer; stimulates ethylene synthesis; stimulates fruit development; induces adventitious roots on cuttings
Cytokinin	Synthesized in roots and transported to other organs	Stimulates cell division, reverse apical dominance, involved in shoot growth, delay leaf sequence
Ethylene	Tissues of ripening fruits, nodes of stems, senescent leaves and flowers	Stimulates fruit ripening, leaf and flower senescence, and abscission
Abscissic Acid	Leaves, stems, green fruit	Stimulates stomatal closure
Gibberellin	Meristems of apical buds and roots, young leaves, embryo	Stimulates shoot elongation, stimulates bolting and flowering in biennials, regulates production of hydrolytic enzymes in grains

Adapted from http://www.sidwell.edu/sidwell.resources/bio/VirtualLB/plant/hormone.html

Cytokinins (named because of the ability to produce cytokinesis (cell division)), stimulates cell division (shoot initiation/bud formation in tissue culture) and promotes some stages of root development. Cytokinins are produced in root meristems, young leaves, young fruits and seeds. The most common form of naturally occurring cytokinin in plants is zeatin, isolated from *Zea mays*. Kinetin was the first cytokinin discovered - it is referred to as a plant growth regulator as it is not made in plants. Auxins and cytokinins are not generally used with embryo cultures, as they often induce callus formation (Monnier, 1976). Their relative amounts determine whether calli differentiates into roots or shoots. Root formation generally occurs in a medium with relatively high auxin and low cytokinin concentration, whereas shoot formation requires low auxin and high cytokinin concentration (reviewed by Pierik, 1987).

Gibberellins (unlike auxins) are classified on the basis of their structure as well as function. Gibberellins are diterpenes synthesized from acetyl coA via the mevalonic acid pathway. Gibberellins, also known as gibberellic acid (GA), were first isolated from *Gibberella fujikuroi* now known as *F. moniliforme*. They have also been isolated from *Phaseolus vulgaris*, lower plants such as mosses and algae, and some bacterial species. Some of the functions of gibberellins include stimulating stem elongation (dwarf plants do not make enough GA), controlling flowering in biennial plants and breaking seed dormancy in some plants (Raghavan, 1980).

Other additional substances gaining recognition as hormones include: polyamines, jasmonates, salicylic acid and brassinosteroid.

2.4.4 Macro- and Micro-elements

The composition of macro- and microelements in media (Table 2.3) may have an effect on embryogenic callus formation and plant regeneration. The concentration of nitrogen in the media has a crucial role in callus formation and regeneration. Basic N6 medium (containing a lower nitrogen concentration than MS medium) proved to be excellent for embryogenic callus initiation and maintenance with most maize lines tested (Bohorova et al., 1995). The high salt concentrations are also implicated in inducing regeneration: raising the ammonium concentration can promote organogenesis, while lowering the concentration results in formation of adventitious shoots on callus tissue (Pierik & Steegmans, 1976). Other substances added to the tissue culture media include casein hydrolysate, silver nitrate and amino acids such as glutamine, proline and asparagines. It is assumed that these amino acids are important nitrogen sources, with glutamine being necessary for growth of immature embryos (Raghavan, 1980). However, glutamine cannot be used as the sole nitrogen source since it inhibits embryogenesis but does not interfere with cell division and growth (Mordhorst & Lorz, 1993).

The addition of L-proline to N6 culture medium resulted in the routine initiation of friable, embryogenic (Type II) callus from immature maize A188 embryos (Armstrong & Green, 1985). Its physiological role however, was not clear. Proline was initially suggested to serve as a source of nitrogen in plant metabolism (Britikov et al., 1970), however this suspicion was proven wrong by results obtained by Armstrong & Green (1985). They found a significant linear relationship between L-proline (up to 25mM) and embryoid formation in maize cultures. Proline was also thought to serve as a readily available source of energy and reducing power (Stewart et al., 1966). More recently, Nanjo and co-workers (1999) showed a positive correlation between L-proline accumulation and stress tolerance. They were thus being able to conclude that the addition of L-proline in tissue culture media might benefit cultured tissues.

Silver nitrate addition to the regeneration medium was reported to increase plant regeneration (Duncan & Widholm, 1987b) by reducing the ethylene in the gaseous atmosphere of the culture vessel in comparison to controls. Ethylene accumulation occurs during the growth of tissue and this accumulation in an enclosed surrounding (tissue culture conditions) was identified as one of the factors responsible for poor regeneration. The 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). Thus, Pius and co-workers (1993) concluded that inhibitors of ethylene, such as $AgNO_3$, make it possible to sustain regeneration in cultures that lose their ability to regenerate.

2.5. CONSTRUCTS FOR PLANT TRANSFORMATION

2.5.1 Promoters

Genes in all organisms have switches (promoters) which direct its expression at defined times in defined cells. Expression of a transgene may depend on the strength of the promoter (Vain et al., 1996) and may also depend on the copy number, position in the plant genome and the recombination ability of the transgene sequences (Kohli et al., 1997). A way to predict level of expression of a transgene is to use a promoter sequence that has been shown to impart high levels of transgene expression in that plant species (reviewed by Wilmink & Dons, 1993).

The cauliflower mosaic virus (CAMV) 35S is one such promoter. It was used in the transformation of rice (Christou et al., 1991), wheat (Vasil et al., 1992), tobacco (McElroy et al., 1991) and maize (Gordon-Kamm et al., 1990), however it is inactive in pollen (Bruce et al., 1989; Christensen et al., 1992; McElroy & Brettel, 1994). The constitutive CAMV35S promoter has been selected to drive expression of both the maize b32 and bean pgip2 genes in this project. Once inside the host genome, this promoter will maintain a high and constant non-regulated level of transcription.

The maize alcohol dehydrogenase (Adh1) promoter has also been used in monocot transformation (Fromm et al., 1990), but its activity was restricted to root and shoot meristems, endosperm, and pollen (Kyozuka et al., 1991). The rice actin (Act-1) (McElroy et al., 1990) and maize ubiquitin (Ubi-1) (Christensen et al., 1992) were investigated as potentially useful alternatives to CAMV35S and Adh1 sequences, because of their expected involvement in fundamental processes in all cell types. Both these monocot promoters were shown to be significantly more active than the CAMV35S promoter in monocot cells (Bruce et al., 1989; Christensen et al., 1992; Cornejo et al., 1993; McElroy & Brettel, 1994) with Ubi-1 promoter being somewhat stronger than Act-1 promoter when compared directly (Cornejo et al., 1993; Gallo-Meagher & Irvine, 1993; Schledzewski & Mendel, 1994; Wilmink et al., 1995).

The high activity of the maize Ubi-1 promoter was demonstrated by transient and stable transformation in a number of monocot systems including rice (Bruce et al., 1989; Toki et al., 1992; Cornejo et al., 1993; Takimoto et al., 1994), wheat (Taylor et al., 1993; Weeks et al., 1993), barley (Wan & Lemaux, 1994), sugarcane (Gallo-Meagher et al., 1993; Taylor et al., 1993), maize (Christensen et al., 1992; Gallo-Meagher et al., 1993), Pennisetum (Taylor et al., 1993) and Lemna (Rolfe & Tobin, 1991).

Furthermore, Cornejo and co-workers (1993) reported that the stress-inducible maize *Ubi-1* promoter fused to *gus*, displayed high-level gene expression of this marker in rice, facilitating efficient transformation of monocots. Thus, the *Ubi-1* promoter was used in this study to drive expression of the *bar* selectable marker gene as well as the strawberry *inors* and *tnors* terpene biosynthesis genes. The original intron present in the 5' untranslated region of the *Ubi-1* gene (Christensen *et al.*, 1992) was retained as numerous previous studies have shown that introns frequently strongly enhance transgene expression in cereals (Callis *et al.*, 1987; Bruce & Quail, 1990; McElroy *et al.* 1990; Vasil *et al.*, 1993). As more plant genes are identified and sequenced, the specificity of promoters will be expanded.

2.5.2 Terminators

Significant regulation of DNA transcription, and hence of gene expression, occurs after the initiation of RNA synthesis during elongation of the RNA chain. A principal means by which elongation is regulated, is through the action of transcription terminators. These terminators are DNA sequences that trigger the release of the RNA transcript and DNA template from RNA polymerase (RNAP) (von Hippel *et al.*, 1992; McDowell *et al.*, 1994; Roberts, 1996; von Hippel, 1998). Transcription termination is carried out by two mechanisms (Richardson & Greenblatt, 1996). In intrinsic termination, an mRNA stem loop structure is thought to induce RNAP to pause, promoting the release of both the polymerase and the RNA transcript from the template DNA. The second mechanism is protein-mediated and relies on an enzyme found throughout bacteria known as the Rho factor (Brown *et al.*, 1981; Opperman & Richardson, 1994). The rho factor is an essential protein that acts as an ancillary termination factor, which recognises RNA and acts at sites where RNAP has paused. In general, only a fraction of the RNAP molecules that reach a terminator, stop transcription there; the remainder read through the terminator to transcribe downstream DNA sequences. This fraction called termination efficiency is affected by sequences in terminator and non-terminator segments of the DNA, and by accessory proteins that bind to the transcription complex (reviewed in Yin *et al.*, 1999).

Additionally, the 3' end of most nuclear mRNA is also modified by the addition of multiple adenylate residues through the action of the enzyme poly(A) polymerase. A conserved sequence AAUAAA that is about 30 nucleotides upstream from the poly(A) site is required to generate the proper 3' end of the RNA with the co-operation of other sequence elements that are downstream from the adenylation site. The presence of this 25 adenylate residues at the 3' end of the mRNA has been shown to enhance the half-life of a transcript by about 3-fold (Gallie *et al.*, 1989), and it has also been implicated in stimulating mRNA translation (reviewed in Lam, 1997).

Translational termination occurs via stop codons. Of the 64 possible combinations of triplet codons using the four different bases, three so-called 'stop codons' serve as signals for the termination of translation. Various terminators are in widespread use, e.g. one derived from CAMV and one derived from the nopaline synthase gene, to name but a few. The nopaline synthethase (nos) terminator, isolated from *Agrobacterium tumefaciens*, contains 26 stop codons and was used in the plasmid vectors bombardment within this project.

2.5.3 Introns

Most plant genes contain intervening sequences (introns) that are transcribed into pre-mRNA and later removed by splicing. The observation that some introns stimulate gene expression was first made in animal systems (Hamer *et al.*, 1979; Gasser *et al.*, 1982), and extended to plants when Callis *et al.* (1987) demonstrated that maize (*Zea mays*) *Adh1* first intron increased the expression of several genes. Other maize introns that have increased expression include the *Bz1* intron (Callis *et al.*, 1987), *Hsp82* first intron (Silva *et al.*, 1988), *Sh1* first intron (Vasil *et al.*, 1989), *Adh1* introns 2 and 6 (Mascarenhas *et al.*, 1990), actin third intron (Luehrsen and Walbot, 1991), *GapA1* first intron (Donath *et al.*, 1995), *Ubi1* first intron (Vain *et al.*, 1996), and the *RpoT* fourth intron (Bourdon *et al.*, 2001).

Plant introns that stimulate expression have been documented in petunia (*Petunia hybrida*; Dean *et al.*, 1989; Vain *et al.*, 1996), oat (*Avena sativa*; Bruce and Quail, 1990), rice (*Oryza sativa*; McElroy *et al.*, 1990; Snowden *et al.*, 1996; Rethmeier *et al.*, 1997), castor bean (*Ricinus communis*; Tanaka *et al.*, 1990), potato (*Solanum tuberosum*; Leon *et al.*, 1991; Fu *et al.*, 1995a, 1995b), Arabidopsis (Rose & Last, 1997; Chaubet-Gigot *et al.*, 2001), soybean (*Glycine max*; Kato *et al.*, 1998), and tobacco (*Nicotiana tabacum*; Plesse *et al.*, 2001). The 2- to 10-fold range of intron-mediated enhancement usually seen in dicots is much less than increases observed in monocots, which can be more than 100-fold (reviewed in Simpson & Filipowicz, 1996). However not all plant introns enhance gene expression. Three dicot introns do not enhance gene expression in transgenic plants (Chee *et al.*, 1986; Kuhlemeier *et al.*, 1988; Vancanneyt *et al.*, 1990) and two maize introns are non-enhancing in transient assays: *Adh1* intron 9 (Mascarenhas *et al.*, 1990) and *Hsp81* intron 1 (Sinibaldi *et al.*, 1992).

Potential mechanisms for intron-mediated enhancement include increased transcription, splicing facilitated transcript maturation, stabilization, export and targeting of spliced transcripts for protein synthesis. Most observations to date in plant systems indicate that intron-mediated enhancement occurs by a co-transcriptional or post-transcriptional mechanism (reviewed in Clancy & Hannah, 2002). Introns were used in this study due to the above collective observations that introns generally enhance gene expression in monocots.

2.5.4 Selectable marker genes

Selectable markers are usually necessary for efficient production of transgenic cells and plants. After gene transfer, transformed cells are greatly outnumbered by non-transformed cells. A selectable marker gene allows the preferential growth of transformed cells in the presence of the corresponding selective agent. To date, most plant transformation systems employ resistance selection, using either herbicide or antibiotic resistance marker, although visual reporter genes (the *gus* gene, Jefferson 1987) are currently available.

2.5.4.1 Antibiotic Resistance

Antibiotics inhibit protein synthesis in prokaryotic cells (reviewed Nap *et al.*, 1992). Mitochondrial and chloroplast ribosomes of higher plants are related to bacterial ribosomes, and are therefore susceptible to the same antibiotics (reviewed by Wilmink & Dons, 1993). These aminoglycoside antibiotics bind to the 30S ribosomal subunit and thus, inhibit initiation of translation and consequently, protein synthesis.

The most widely used selectable marker is an *E.coli* bacterial gene (*aphA2*) which codes the enzyme aminoglycoside-3'-phosphotransferase II (APH 3'-II), also known as neomycin phosphotransferase II (NPT II). This enzyme inactivates a number of related antibiotics including kanamycin, neomycin and puromycin (Bevan et al 1983). Hygromycin, on the other hand, has been used for selection of transformed maize (Walters *et al.*, 1992) and rice (Shimamoto *et al.*, 1989). Hygromycin is inactivated by the enzyme hygromycin phosphotransferase (HPT), and is encoded by the *hpt* or *aphIV* gene (van Elzen *et al.*, 1985), which was isolated from *E.coli.*

Other antibiotics used for selection include gentamicin, streptomycin and spectinomycin. A possible concern about the use of antibiotic resistance as a selectable marker is the potential to compromise the use of antibiotics used in human and animal therapy. The possible transfer of resistance to gut microorganisms and the presence of gene products in food or feed is of concern.

2.5.4.2 Herbicide Resistance

Herbicide resistance marker genes have provided a more effective system for plant transformation in many cases (Chandler, 1995). They may provide considerable advantage over antibiotic resistance genes in cases where either high levels of the antibiotic may interfere in the plant regeneration process, or where plant tissues may exhibit a high level of intrinsic resistance. In addition, cell death in the presence of herbicide is generally more rapid and complete, thus providing more efficient selection.

Efficient s election and r egeneration w as displayed i n transformation examples o f l egumes such as peas, luoomo and lupine (Schroeder *et al.*, 1993).

Genes conferring resistance to a number of herbicide groups including the triazines, the sulfonylureas, bromoxynil, glyphosate and phosphinothricin are readily available. Of these, the *bar* gene isolated from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987), has been widely used as an effective selectable marker in the presence of the herbicide L-phosphinothricin (PPT), also known as glufosinate.

PPT is an analogue of glutamate and acts as a competitive inhibitor of glutamine synthetase (GS) (Kishore & Shah, 1988). The assimilation of ammonia is catalysed by GS and plays a key role in the regulation of nitrogen metabolism (De Block *et al.*, 1987). Inhibition of the enzyme causes accumulation of ammonia to toxic levels in the cell. This accumulation, rather than the lack of glutamine, causes the death of plant cells (Tachibana *et al.*, 1986). Resistance to the herbicide is conferred by phosphinothricin-N-acetyltransferase (PAT). This enzyme inactivates PPT by acetylation using acetyl coenzyme A as a cofactor and it is encoded by two similar genes: *bar* from *Streptomyces hygroscopicus* (Murakami *et al.*, 1986) and *pat* from *S. viridochromogenes* (Wohlleben *et al.*, 1988). This enzyme detoxifies the herbicide Basta® (active ingredient is PPT) which is sprayed onto putative transformants, or b ialaphos (L-phosphinothricyl-L-alanyl-L-alanine, a t ripeptide f orm of this h erbicide) (De Block *et al.* 1987; Gordon-Kamm *et al.*, 1990). Bialaphos was added to the tissue culture media as it was shown to be more effective in selection of transformed calli, than that obtained with Basta® addition (Dennehey *et al.*, 1994). PPT or bialaphos selection has thus become the agent of choice for regenerating transgenic maize (Gordon-Kamm *et al.*, 1990) and was therefore chosen for use in this study. This selection has also been successfully used in the transformation of potato, tobacco, *Brassica* species (De Block *et al.*, 1987; 1989), maize (Spencer *et al.*, 1990) and rice (Cao *et al.* 1992). Other herbicides that have been used occasionally as selective agents are glyphosate (Comai *et al.*, 1985, 1989), chlorsulfuron (Haughn *et al.*, 1988) and bromoxynil (Stalker *et al.*, 1988).

2.5.4.3. Marker-free transgenic plants
Antibiotic resistance markers (e.g. *npt*ll or *hpt*) or herbicide resistance markers e.g. *bar*) are essential for selectively propagating transformed c ells and t issues. However, s ubsequent maintenance of markers in transgenic plants is unnecessary. Elimination of markers is advocated since the antibiotic resistance genes may be transferred to pathogenic bacteria, or the herbicide resistance genes may be transferred to weeds. With the exception of the above concerns, removal of marker gene offers the following research advantages.

It enables (i) successive rounds of transformation to occur allowing useful transgenes to be stacked without crossing, and (ii) it prevents retention of promoters along with selectable markers (multiple copies of a promoter), thereby preventing transcriptional gene silencing.

In principle, there are four ways to either avoid, or eliminate 'problematic' selectable marker genes before transgenic plants are brought out into the field: (i) Avoiding selectable marker genes at all (reported recently for potato by Romano *et al.*, 2003); (ii) Use of marker genes that possibly has no 'harmful' biological activities; (iii) co-transformation with two transgenes, one carrying the desired trait and the other the selection marker, followed by the segregation of the two; and (iv) excision of selectable marker gene from the integrated DNA fragment (after successful selection) by using site specific recombination, transposition or homologous recombination (reviewed by Puchta, 2003).

In combination with marker elimination, a new set of markers has been developed (positive selection). The rational behind this system is that non-transformed cells are not killed (as in procedures using antibiotic or herbicide resistance genes) but transformed cells experience a metabolic or developmental advantage. This might even increase the efficiency of regeneration of transformed plants as reported by the use of the phosphomannose isomerase (*pmi*) genes (Joersbo *et al.* 1998, Negrotto *et al.* 2000). This selection system uses the *pmi* gene, which encodes for the phosphomannose-isomerase (PMI) enzyme that converts mannose-6-phosphate to fructose-6-phosphate. Only transformed cells are capable of utilizing mannose as a carbon source. Cells genetically transformed to express PMI acquire a growth advantage (positive selection) on mannose-containing media, which makes mannose a useful selection agent for the future generation of transgenic plants.

2.5.5 Linear Minimal DNA Transgene Cassettes

A potential problem still exists with antibiotic resistance genes present in recombinant vector backbone sequences, even with the use of a positive selection marker gene. This resistance gene can also be integrated into the genome with the transgenes. Integrated vector backbone DNA has been detected in transgenic plants generated by *Agrobacterium*-mediated transformation (Fu *et al.*, 2000) and direct delivery procedures such as particle bombardment (Kohli *et al.*, 1999). Vector backbone sequences promote transgene rearrangements and result in high copy number integration patterns, which inhibit transgene expression and contribute to transgene silencing (Matzke *et al.*, 1996). Only 20-30% simple integration events occur.

However, with bombardment of linear minimal transgene expression cassettes (promoter, open reading frame and terminator – no antibiotic resistance gene), predominantly 'simple' integration events (80%) resulted in rice, producing low-copy-number transgenic plants (77%

single transgene copy) with a low frequency rearrangement (Fu *et al.*, 2000). Additionally, no silencing in transgenic plants was reported. It is therefore believed that use of particle bombardment to deliver minimal gene cassettes into regenerable plant tissues, will become the method of choice for generating transgenic plants expressing genes of agronomic and industrial interest. Thus, bombardment experiments with minimal transgene cassettes of the maize *b32* and bean *pgip2* were initiated in the study.

2.6 ANTIFUNGAL TRANSGENES FOR RESISTANCE TO *F. VERTICILLIOIDES*

Plants respond with a wide spectrum of defense reactions producing pathogenesis-related proteins as a result of pathogen attack, which include antifungal proteins and enzymes involved in plant cell wall reinforcement or breakdown of pathogen infection structures. Most of these proteins show antifungal activity against specific pathogens while few possess enzymatic activity (chitinases, glucanases, peroxidases and ribonucleases) (reviewed by Caruso *et al.*, 2000).

In cereal crops, current research focuses on introduction of genes with antifungal potential into plants. The maize ribosome inhibiting protein (RIP), b32, was introduced into tobacco plants conferring resistance against the tobacco fungal pathogen *Rhizoctonia solani* (Maddaloni *et al.*, 1997). This *b32* gene was also introduced in selected Italian rice cultivars, to exploit its action against rice blast (*Pyricularia oryzae*) (Balconi *et al.*, 2000). Expression of the b32 protein in wheat leaf tissues prevented disease development caused by rust (*Puccinia recondite* f.sp.*tritici*) and powdery mildew (*Blumeria graminis* f.sp.*tritici*) (Lupotto *et al.*, 2002). Further research showed that the ectopic expression of barley and a pokeweed RIP, led to increased tolerance to fungal and viral infection (Logemann *et al.*, 1992; Lodge *et al.*, 1993). In addition, tomato and *Arabidopsis* plants overexpressing a polygalacturonase inhibiting protein (PGIP), showed a significant reduction of symptoms caused by the growth of fungal pathogen *Botrytis cinerea* (Powell *et al.*, 2000; Ferarri *et al.*, 2003). The most recent results indicate that PvPGIP2 (from bean) introduced into wheat plants, could possibly show resistance to *F. graminearum* and *F. culmorum* (Janni *et al.*, 2003).

The development of engineered maize plants with defense genes acting against fungal pathogens is a major objective in modern breeding strategies. Pathogen denfense genes may provide direct protection of plants in the field, contributing to alleviate damages such as ear- and stalk-rot produced by *F. verticillioides,* and subsequent development of storage molds associated with mycotoxin production (*Fusarium, Penicillium, Aspergillus*). Engineering and correct expression of antifungal genes are therefore the first requirements for such goals.

Three genes (of different origins shown to be implicated in the process of fungal infection) were chosen as a potential source of resistance for use in this study, because of their different characteristics and modality of action.

2.6.1 MAIZE *B32* GENE

One of the antifungal genes selected for introducing resistance to *F. verticillioides*, is the maize *b32* gene. This gene encodes for a cytosolic albumin protein expressed in the endosperm, which is synthesized in a temporal and quantitative coordination with the deposition of storage proteins (Soave *et al.*, 1981). The sequence of *b32* showed homologies with Ribosome Inhibiting Proteins (RIPs) and in 1991, *b32* was shown to be a functional RIP (Maddaloni *et al.*, 1991).

2.6.1.1 Ribosome Inhibiting Proteins (RIPs)

RIPs are identified as a group of plant proteins that catalytically damage eukaryotic ribosomes: this irreversible modification prevents the ribosome from binding to the elongation factor (EF2). This interaction is essential for the elongation step of protein synthesis (Olsnes & Pihl, 1982; Roberts & Selitrennikoff, 1986; Stirpe & Barbieri, 1986). The RIPs most frequently occur as single polypeptide chains (type 1 RIPs) and, in some cases, the RIP is linked by hydrophobic bonds and a disulphide bridge to a galactose-binding lectin domain (type 2 RIPs), for example ricin from *Ricinus communis*. Type 2 RIPs can enter cells through the interaction of their lectin moiety with the cell membrane, and are among the most potent natural toxins (reviewed by Stirpe *et al.*, 1992). Additionally, two type-2 RIPs that are not cytotoxic (even though they have the RIP/lectin structure) have been reported (Girbes *et al.*, 1993). Type 3 RIPs are synthesized as inactive precursors (proRIPs) that require proteolytic processing events to occur between amino acids involved in formation of the active site (Mundy *et al.*, 1994).

The maize b32 RIP is a type 1 RIP (Walsh *et al.*, 1991). Type 1 RIPs (no lectin domain) do not bind easily to cells and consequently have a relatively low native cytotoxicity activity. Most type 1 RIPs are produced in an inactive form with a C-terminal signal-peptide that directs the export of newly synthesized molecules to the cell wall matrix. Post-translational processes cleave the signal peptide to yield a mature, toxic RIP. This compartmentalisation protects the undamaged plant cell from the poisonous effects of endogenous RIP (reviewed by Stirpe *et al.*, 1992). Alternative mechanisms are possible: for example, maize RIP (*b32*) accumulates in the seed as an inactive precursor and is converted to an active form by removal of 16 amino acids from the N-terminus, several amino acids from the C-terminus and 25 amino acids from the centre of the polypeptide.

The activated form is composed of two tightly associated polypeptide chains, which are not covalently linked. An immunoblot survey of several related species showed that pro-RIP (or *b32*) homologues are not unique to maize – the most distant relative of maize that contained pro- and mature *b32* homologue was sorghum (Walsh *et al.*, 1991). A second type 1 RIP from maize was found and its gene cloned by Bass *et al* (1995). The deduced amino acid sequence of this RIP revealed little similarity to the *b32* internal and C-terminal regions that undergo proteolytic cleavage. In contrast, sequences of this protein and the active form of *b32* were highly conserved. The existence of multiple RIPs and in some cases organ specific isoforms, have been observed in other plant species (Stirpe *et al.*, 1992).

RIPs are classified as N-glycosidases which cleave the N-glycosidic bond of a specific adenine in the conserved sequence 5'-AGUACGaGAGGA-3' of animal and yeast ribosomal RNA (Endo & Tsurugi, 1988; Endo *et al.*, 1988; Stirpe *et al.*, 1988). To summarise, RIPs inhibit protein synthesis by enzymatic modification of the 28S rRNA. It is important to note that wheat and maize ribosomes, but not fungal ribosomes are resistant to the b32 protein. The maize b32 protein synthesis is modulated by the regulatory genes *Opaque-2* and *Opaque-6*, and it was demonstrated that the O2 protein product is a strong transcriptional activator of the b32 promoter (Lohmer *et al.*, 1991). Like other RIPs present in cereal seeds, b32 may play a double role of storage and a defense protein during seed germination.

2.6.1.2 Transgenic expression of *b32*

Ectopic expression of *b32* in maize might result in a wider defense action for other tissues. The introduction of maize *b32* into tobacco resulted in enhanced tolerance to the fungal pathogen *Rhizoctonia solani* AG4 (Maddaloni *et al.*, 1997). Further work by Lupotto *et al.* (2002) showed the maize *b32* protein being constitutively expressed in wheat (*Triticum aestivum* L.), and in rice (*Oryza sativa* L.) by the CAMV35S and Ubi-1 promoters (Reali *et al.*, 2001). The *b32* protein was expressed during all vegetative and reproductive stages of the plant life, with an initial quantitative estimate of wheat leaf-expressed *b32* comprising between 0.5 and 2% of the total soluble protein (Lupotto *et al.*, 2002). Most recently, A188 x B73 F_2 maize IZEs were transformed with maize *b32* under the constitutive control of the CAMV35S promoter (Lanzanova *et al.*, 2002). Evidence of expression of the engineered b32 protein in all parts of the T_0 plants was reported. This maize work was also developed within the framework of the EU-funded project SAFEMAIZE (ICA4-CT2000-30033) (http://www.up.ac.za/academic/botany/safemaiz.html). The expression vector (carrying the cassette CAMV35S-*b32* and ubi-*bar* as a selectable marker), used for the maize transformation by the Italian group, was provided for transformation experiments in South Africa, reported in this study.

2.6.2 BEAN *Pgip2* GENE

The next antifungal gene chosen for engineering resistance towards *F. verticillioides* in this study, was the *pgip2* gene (isolated from *Phaseolus vulgaris* L.) which encodes a polygalacturonase inhibiting protein (PGIP). No PGIP proteins have been identified to exist in maize to date. However bean *pgip2* was recently introduced into wheat and results from agarose diffusion assays displayed inhibitory activity against PGs from *F. verticillioides* (Janni *et al.*, 2003). PGIPs are located in the plant cell wall and limit fungal invasion of the plant, by interacting with polygalacturonases (PGs), which are secreted by phytopathogenic fungi (De Lorenzo *et al.*, 2001).

Plant cell walls are a key factor in plant-pathogen interactions as they come into direct contact with the invading organism. Plant cell defense relies on the capability of each cell to recognise the presence of pathogens and subsequently, activate defense responses. The majority of organisms need to breach this cell wall barrier to gain access to the plant tissue and produce enzymes that degrade the wall polymers (De Lorenzo *et al.*, 2002). Pectin is the main component of the middle lamella, which holds neighbouring plant cells together. Much research on cell-wall degrading enzymes produced by fungal pathogens has thus, concentrated on pectinases.

2.6.2.2 Poygalacturonases

Polygalacturonases (PGs) (EC 3.2.1.15) are pectinases secreted by fungal pathogens, that cleave the polygalacturonan backbone of the pectin molecule internally, thereby weakening the cell wall, exposing other cell wall components to degradative enzymes, and providing a carbon source for the pathogen (De Lorenzo *et al.*, 2001). Fungi secrete PG enzymes that not only macerate the plant cell wall so that the fungi can enter, but PGs are also thought in some cases to elicit a defense response from the plant. The current hypothesis is that the protective response of plants can be triggered by fragments of its own cell wall, called oligogalacturonides (OGs), that are released when PG interacts with PGIPs (common in plant cell walls). PGIP recognizes fungal PGs and modulates its enzymatic activity so that OGs accumulate, thus 'sounding the alarm' for activation of the plant defense response (Cervone *et al.*, 1989).

2.6.2.3 PGIPs from different plants

PGIP (like most plant resistance genes) belong to the superfamily of leucine-rich repeat (LRR) proteins: a class of proteins specialized for protein-protein interactions (Kobe and Kajava, 2001). Plants use the LRR fold for their 'immune' functions and recognition of non-self molecules (Jones, 2001).

The occurrence of PGIPs has been reported in a variety of dicotyledonous plants and in the pectin-rich monocotyledonous plants, onion and leek (Favaron et al., 1993). Some of the PGIPs identified and characterized include those from apple (Brown, 1984), bean (Cervone et al., 1987), pear (Stotz et al., 1993), raspberry (Johnston et al., 1993), tomato (Stotz et al., 1994), soybean (Favaron et al., 1994), pea (Hoffman et al., 1984), sweet-potato (Uritani et al., 1961). These PGIPs are effective against some fungal PGs (Cook et al., 1999; Desiderio et al., 1997; Sharrock et al., 1994; Stotz et al., 1994) but ineffective against bacterial or plant PGs (Cervone et al., 1990).

PGIPs from different plant sources differ in their inhibitory activities. PGIPs from a single plant source however, also inhibit PGs from different fungi or different PGs from the same fungus with different strengths (reviewed in De Lorenzo et al., 2001). For example, crude preparations of bean PGIP are effective against PGs from *Aspergillus niger, Botrytis cinerea*, and *Fusarium verticillioides* (Cook et al., 1999; Pressey, 1996). Interestingly, bean PGIP is significantly more effective against PG of *Colletotrichum lindemuthianum* than against PG of a related non-pathogenic *Colletotrichum,* suggesting that compatability provides a selection pressure for more efficient PGIPs that can better counteract fungal infection (Lafitte et al., 1984). PG from *F. verticillioides* is not inhibited by PGIPs from onion (Favaron et al., 1993) or petunia.

2.6.2.4 Cloning of P*gip* genes and characterization of PGIP activity

The first gene to encode a PGIP was *pgip1*, cloned from *Phaseolus vulgaris* (Toubart et al., 1992). This gene, which was expressed in tomato plants (via *Agrobacterium*-mediated transformation) as well as in *Nicotiana benthamiana* plants (with potato virus X as a vector) (Desiderio et al., 1997), inhibited crude PG preparations from *F. oxysporum* f. sp. *Lycopersici, B. cinerea* and *A. solani,* but was unable to interact with a homogenous PG from *F. verticillioides.* The p*gip2* gene (encoding PGIP-2), from *P. vulgaris* on the other hand was shown to inhibit PGs from *F. verticillioides* as well as from *A. niger* (Leckie et al., 1999). This bean p*gip2* gene was thus chosen for use in this project to engineer resistance in maize towards *F. verticillioides.*

2.6.3 TERPENOID (TERPENE) GENES

2.6.3.1 Terpenoids

The largest class of plant secondary metabolites is the terpenoids (or terpenes). Over 36,000 individual structures of this class have been reported (Hill, 2002). The structure of terpenes is extremely variable, exhibiting hundreds of different carbon skeletons. However, this wide structural diversity has a common feature of biosynthesis: all terpenes are derived from the simple process of assembly of 5-carbon atom isoprene units. The categories of terpene compounds are those made up with one (C5 hemiterpenes), two (C10 monoterpenes), three (C15 sesquiterpenes), four (C20 diterpenes), six (C30 triterpenes), eight (C40 tetraterpenes) or even more (>C40 polyterpenes) isoprene units (Chappell, 1995; McGarvey & Croteau, 1995). These categories and their respective biosynthesis within the plant cell are displayed in Figure 2.4.

A few terpenoid-derived hormones such as gibberellins (Hedden & Kamiya, 1997) and abscissic acid (Schwartz et al., 1997) have well-established roles in plant development processes. Terpenes also play important roles in flavour and fragrance agents added to foods, beverages, perfumes, soaps, toothpaste, tobacco and other products (Verlet, 1993). Many terpenes find use in industry as raw materials in the manufacture of adhesives, coatings, emulsifiers and specialty chemicals, whilst others (such as limonene) are of commercial importance as insecticides, because of their low toxicity to mammals and lack of persistence in the environment (Way & van Emden, 2000). Many dietary monoterpenes, including limonene and its active serum-oxygenated metabolite derivatives, perillic acid and dihydroperillic acid (Crowell et al., 1992), have been shown to suppress cancer through their chemopreventive activity during the promotion phase of mammary and liver carcinogenesis (Bardon et al., 1998; Crowell, 1999). Artemisinin (a sesquiterpene endoperoxide isolated from *Artemisia annua*) is proving to be a valuable antimalarial compound (Bouwmeester et al., 1999b).

Few terpene synthase genes have been identified to date in monocotyledons. In maize specifically, a diterpene synthase gene (*an1*) involved in the synthesis of gibberellins, was identified (Shah & Cha, 2000). Shen and co-workers (2001) described a second maize terpene synthase gene (*stc1*), involved in the biosynthesis of a naphthalene-type sesquiterpene. Recently, the maize terpene synthase 1 gene (*tps 1*) was isolated and characterised (Schnee *et al.*, 2002) – this gene encodes an enzyme catalysing the formation of three acyclic sesquiterpenes: (*E*)-β-farnesene, (3*R*)-(*E*)-nerolidol and (*E,E*)-farnesol, emitted by herbivore-damaged plants.

2.6.3.2 Biosynthesis of terpenes

The biosynthesis of terpenoids (Figure 2.4), can be divided into four major processes: (i) conversion of acetyl-coenzyme A (CoA) to the active isoprene unit, isopentyl pyrophosphate (IPP); (ii) various phenyltransferases generated from IPP, the higher order terpenoid building blocks: geranyl pyrophosphate (GPP; C10), farnesyl pyrophosphate (FPP, C15), and geranylgeranyl pyrophosphate (GGPP; C20); (iii) these branch point intermediates may then undergo internal addition (cyclization) to create the basic parent skeletons of various terpenoid families; and (iv) oxidation, reduction, isomerization, conjugation, or other secondary transformations that finally produce the representative terpenoid (Gershenzon & Croteau, 1993).

The initial step of the pathway involves the production of IPP (basic C5 building block) via the mevalonic acid pathway. IPP itself is insufficiently reactive to undergo ionisation to initiate the condensation to higher terpenoids. Therefore, it is first isomerised to the allylic ester (DMAPP) by IPP isomerase (reviewed by McGarvey & Croteau, 1995). Isoprene (the simplest of the terpenoids) is synthesized directly from DMAPP by diphosphate elimination. GPP synthase forms GPP from DMAPP and IPP. Monoterpenes (e.g. linalool) are then synthesized from GPP by a class of enzymes termed monoterpene synthases. FPP synthase similarly forms FPP from DMAPP and two units of IPP, to then produce sesquiterpenes (e.g. nerolidol) via sesquiterpene synthases (outlined in Figure 2.4). It is important to note that monoterpenes, diterpenes and tetraterpenes are exclusively synthesized in plastids. Sesquiterpenes and triterpenes, on the other hand, are synthesied in the cytosol and the cytosol/endoplasmic reticulum boundary (as reviewed in McGarvey & Croteau, 1995).
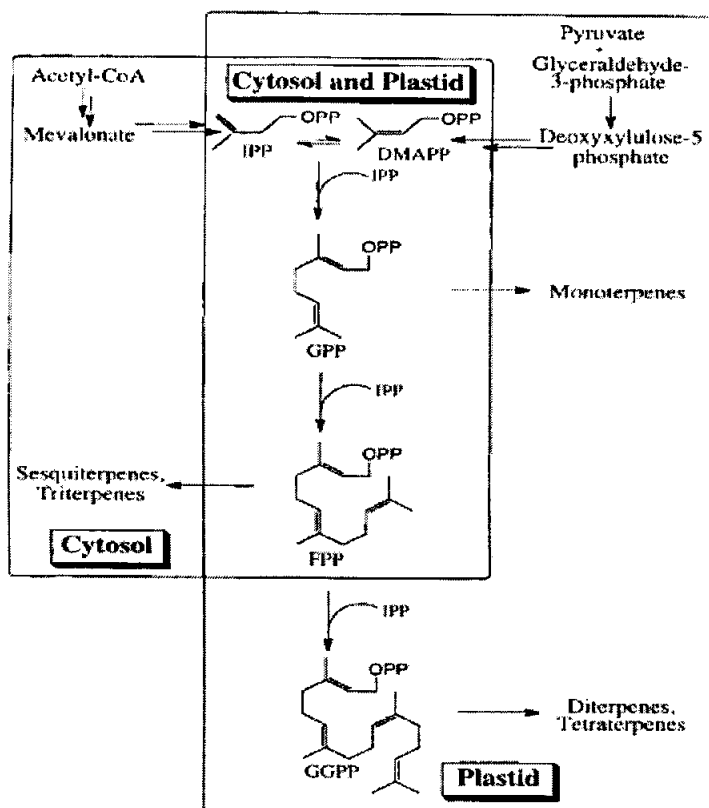
Fig 2.4 Overview of terpenoid biosynthesis in plants. The intracellular compartmentalization of the mevalonate and mevalonate-independent pathways for the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and of the derived terpenoids, is illustrated. The cytosolic pool of IPP, which serves as a precursor of farnesyl diphosphate (FPP) and, ultimately, the sesquiterpenes and triterpenes, is derived from mevalonic acid (left). The plastidial pool of IPP is derived from the glycolytic intermediates pyruvate and glyceraldehyde-3-phosphate and provides the precursor of geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) and, ultimately, the monoterpenes, diterpenes, and tetraterpenes (right). Reactions common to both pathways are enclosed by both boxes. (Adapted from Trapp & Croteau, 2001)

2.6.3.2.1 Monoterpene synthase genes

A large number of genes encoding enzymes catalysing the biosynthesis of monoterpenes in plants have been cloned to date. For example, the linalool synthase (LIS) enzyme, initially isolated from the evening primrose flower (*Clarkia* breweri) by Duradeva and co-workers (1996), was introduced into petunia plants (Lucker *et al.*, 2001) and tomato fruits (Lewinsohn *et al.*, 2001). Other enzymes cloned include (-)-limonene synthase (Colby et al., 1993; Bohlmann et al., 1997), myrcene synthase (Bohlmann et al., 2000; Fishbach et al., 2001), R-ocimene synthase (Bohlmann et al., 2000) and (+)-limonene synthase (Maruyama et al., 2001; Maruyama et al., 2002). Enzymes leading to production of primary monoterpene skeletons all appear to be active in plastids: all genes of this pathway that were cloned to date, have plastid targeting signals (Haudenschild & Croteau, 1998) and have been located in chloroplasts of parenchyma cells (Bouvier et al.,2000) and in leucoplasts of secretory cells (Turner et al., 1999).

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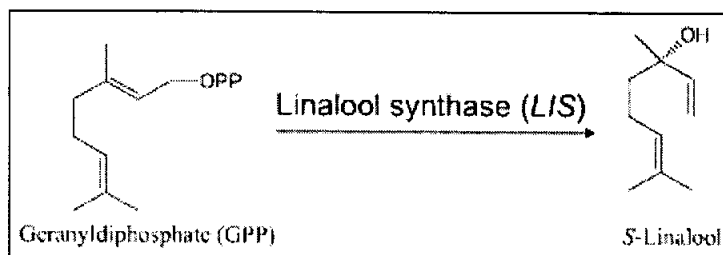
Fig 2.5 Linalool synthase (LIS) enzyme is responsible for the acyclic monoterpene S-linalool. This enzyme was first isolated from the flower *Clarkia breweri* (Duradeva *et al.*, 1996)

Linalool has been detected in maize only after herbivore damage (Schnee *et al.*, 2002). In this study, linalool expression is aimed to be constitutive and not induced.

2.6.3.2.2 Sesquiterpene synthase genes

Sesquiterpene s ynthases a ccept t he l inear C_{15} precursor farnesyl d iphosphate (FPP), a nd generate carbocation by releasing t he n egatively charged diphosphate g roup. Cyclization into a wide variety of cyclic products then occurs, through an unusual carbocation mechanism initiating the various hydrocarbon condensation and cyclization reactions (reviewed in van der Hoeven *et al.*, 2000).

Nerolidol, the sesquiterpene analog of linalool (monoterpene) that is produced in the cytosol, is a component of many essential oils and flower headspaces (Bauer *et al.*, 1990; Knudsen *et al.*, 1993). Nerolidol has been isolated from maize cvs B73 plants after herbivore damage, however it w as r apidly c onverted to (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Donath & Boland, 1994). The maize terpene synthase gene 1 (*tps 1*) has been isolated and identified in catalyzing the formation of this sesquiterpene (Schnee *et al.*, 2002).
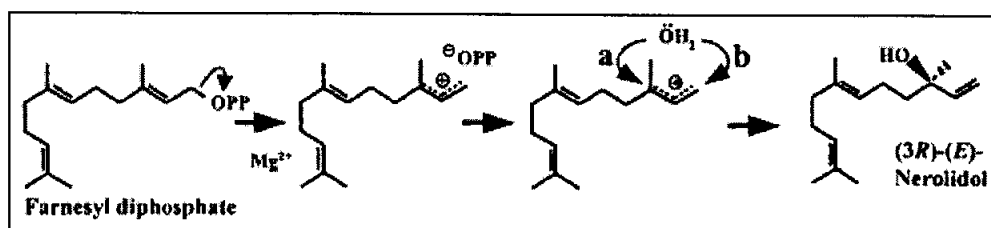
Fig 2.6 Proposed reaction mechanism for the formation of one of the sesquiterpenes, (*E*)-nerolidol, by the *tps 1* gene, in maize (Adadped from Schnee *et al.*, 2002)

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2.6.3.4 Engineering of terpene metabolism in plants

Metabolic engineering of the monoterpene biosynthesis pathway in plants has been already achieved (Mahmoud & Croteau, 2001; Lücker et al., 2001). Flower scent has seldom been a target trait in commercial flower breeding programs, but rather color, longevity and form (Zuker et al., 1998). Some groups have tried to produce fragrance compounds in transgenic plants, in an attempt to improve floral scent (Vainstein et al., 2001). For example, in old varieties of carnation, eugenol used to contribute up to 85% of total amount of headspace volatiles, but in modern varieties it is below human perception threshold detection levels, and therefore these varieties lack the characteristic original fragrance (Clery et al., 1999). However, the introduction of novel genes (encoding enzymes involved in formation of a specific fragrance-related product), has proven to be insufficient by itself. In one case, the glycosylated form of the final product, which also occurs naturally (Watanabe et al., 1993), did not allow subsequent product volatilization and emission (Lücker et al., 2001). In other cases, the level of precursor had been either limiting to allow product formation (Dudareva & Pichersky, 2000), or the product was emitted at too low levels for human pannelists to detect any olfactory alteration in floral scent (Lavy et al., 2002).

Of particular relevance to this study are the linalool/nerolidol synthase genes. These genes result in the production of linalool (a monoterpene) or nerolidol (a sesquiterpene), depending on targeting and substrate availability: with chloroplast targeting, this synthase enzyme enters the chloroplast where it finds GPP (the universal precursor for all monoterpenes) as the substrate, thus producing linalool. Without targeting, the enzyme occurs in the cytoplasm and converts FPP (the universal precursor for sesquiterpenes) into nerolidol.

Linalool is an acyclic monoterpene alcohol having a peculiar creamy, floral, sweet taste (Arctander, 1969). In *Clarkia Breweri* (daffodil flower), linalool among other compounds, is responsible for attracting pollinating moths (Raguso & Pichersky, 1995) and it acts as a repellent against aphids (Hori, 1998). Furthermore, it is one of the volatile compounds released as a semiochemical after herbivore attack in some plant species (Pare & Tumlinson, 1997; Rose *et al.*, 1996; Weissbecker *et al.*, 1997), and as such may attract predators of the herbivores. The corresponding cDNA to the enzyme (linalool synthase) has recently been isolated (Dudareva *et al.*, 1996).

Recent, unpublished results indicate that linalool or nerolidol show inhibition of mycelium growth of several isolates from *Fusarium*, *Botrytis* and *Phytopthora* (Scholten, Plant Research International, unpublished). It is for this reason that the linalool/nerolidol synthase genes (from strawberry) have been included in this study, to possibly introduce resistance to *Fusarium verticillioides*. The linalool synthase gene (chloroplast targeted) is denoted *tnors* and the nerolidol synthase gene (no organelle targeting) is denoted *inors*, in this study. These genes were recently constitutively expressed in *Arabidopsis* plants (Aharoni *et al.*, 2003) and are currently being investigated for resistance against fungal infection.

GPP and FPP are the central precursors for monoterpenes and sesquiterpenes respectively. Terpenes dominate the volatile compound blend released in maize upon herbivore attack (Turlings *et al.*, 1991), thus GPP and FPP are present in maize. The maize terpene synthase gene (*tps1*) has been isolated and expressed into a bacterial system (*E.coli*) (Schnee *et al.*, 2002), however no research to date has successfully reported the transformation of a linalool/nerolidol synthase gene into maize genotypes.

CHAPTER THREE:

REGENERATION AND TRANSFORMATION STUDIES OF SELECTED ELITE WHITE MAIZE GENOTYPES

3.1   ABSTRACT

This study reports on the regeneration and transformation studies of selected elite white maize genotypes. Pre-cultured immature zygotic embryos (IZEs) were used as the explant in this study. Initial regeneration studies examined the callus induction and regeneration potential of six elite white maize genotypes (A483-1, A483-4, A483-10, A483-12, Z206-131 and M37W) on three different callus induction media (N6$_{10}$, 2MS+Pro and G2+Pro). The number of embryogenic, white compact calli (Type-I) produced per immature zygotic embryo ranged from 53-83% for A483-1, 73-92% for M37W and 29-50% for A483-4 on N6$_{10}$ callus induction media. A483-1 and A483-4 (CIMMYT elite white maize genotypes) and M37W (South African elite white maize genotype), were found to produce the highest number of regenerants per explants (3.6, 0.8 and 2.8 respectively) on the N6$_{10}$ callus induction media. These three highly regenerable selected genotypes were bombarded with the antifungal genes (*b32* and *pgip2*) and the selectable m arker gene (*bar*). Bombarded IZEs were selected on N6$_{10}$ callus induction media containing the selective agent, bialaphos. Positive transformants were screened using PCR and Southern blotting techniques. Although a low transformation efficiency of 0.17% was obtained, stable integration of the antifungal *b32* gene (1-2 copies) in two events, BBE 13.2.3 (A483-1) and BBE 16.1.1 (A483-4), was obtained by Southern blot analysis of the T$_1$ progeny. Event 16.1.1A (T$_2$ plant) also displayed a low copy integration of the *b32* gene and T$_3$ seeds of this event, was produced. None of the events showed integration of the *pgip2* gene.

3.2 INTRODUCTION

Transformation is a process dependant on the ability of the species and tissue to produce totipotent cells that can be induced to regenerate into a complete viable plant (as reviewed in Friedberg, 1998). 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 is generally limited to selected genotypes (Lambè *et al.*, 1999). Therefore, it is essential to establish an effective regeneration system for elite maize genotypes before transformation, since improved regeneration frequency often leads to improved transformation efficiency, as demonstrated for oat plants (Gless *et al.*, 1998a, b).

Maize transformation was first achieved by Gordon and co-workers in 1990, where a laboratory strain of maize (A188) was transformed using biolistics. Subsequently, biolistic-mediated transformation of elite maize with a truncated version of the insecticidal *Bacillus thuringiensis* (*Bt*) gene was achieved (Koziel *et al.*, 1993). Since then, stable transformation of elite maize using the Particle Inflow Gun (PIG) was performed by a many laboratories (Armstrong & Songstad,1993; Hill *et al.*,1995, O'Kennedy *et al.*, 2001).

Immature zygotic embryos (IZEs) were proposed as donor tissues for establishing callus cultures of maize (Green & Phillips, 1975) and therefore, plant regeneration from such cultures has become a common practice. In this study, six elite white maize genotypes were screened *in vitro* for tissue culture amenability, on three different callus induction and regeneration media using IZEs as explants. Media used were 2MS+Pro, $N6_{10}$ and G2+Pro (refer to Section 2.4.2 for media composition). Therefore, the aims of this study were (i) to test the regeneration potential of each of the six elite white maize genotypes on the different callus induction media, identifying the genotype and callus induction media combination producing the highest number of regenerants per explant and (ii) to demonstrate stable transformation of the selected antifungal genes into these highly regenerable genotypes using the PIG. The CIMMYT elite maize genotypes used within this study were chosen because of their susceptibility to *F. verticillioides* infection, and also due to their good adaptability to African conditions.

The antifungal genes co-transformed into the elite maize genotypes were the maize *b32* gene (present in only the endosperm and expressing a ribosome inhibiting protein), and the bean *pgip2* gene (expressing a polygalacturonase-inhibiting protein). The *bar* selectable marker gene was bombarded, which conferred resistance to bialaphos (tripeptide form of the herbicide Basta®). Bialaphos was added to the tissue culture media for selection.

3.3   MATERIAL AND METHODS

3.3.1   Excision of IZEs

Five African elite white maize genotypes obtained from CIMMYT (A483-1, A483-4, A483-10, A483-12 and Z206-131) and one South African genotype (M37W), were cultured on three different callus induction media: G2+Pro; 2MS+Pro, and $N6_{10}$ (Table 3.1). Immature maize kernels (10 – 14 days post-pollination) obtained from greenhouse grown maize, were soaked in 70% (v/v) ethanol for one minute and sterilised for 20 minutes in a 2.5% (v/v) sodium hypochlorite solution, containing 0.1% (v/v) of the surfactant Tween 20 (Merck Chemicals), before being rinsed thoroughly with sterile distilled water. IZEs (0.8mm – 1.2mm long) were aseptically excised from the immature kernels and plated onto each callus induction media, with the axes in contact with the medium and the scutellum exposed. For bombardment experiments, IZEs (obtained from field grown maize at the University of Pretoria Experimental farm, South Africa) were pre-cultured for 4-6 days on $N6_{10}$ callus induction media before bombardment.

3.3.2   Tissue culturing of IZEs

IZE cultures were sub-cultured every 2 weeks to fresh media, with incubation under low light conditions (1.8 μmol m^{-2}s^{-1}). After 4 weeks, calli induced on 2MS+Pro were subsequently cultured onto regeneration media (0MS). Calli induced on G2+Pro and $N6_{10}$ however, had a two-week maturation phase on G2 maturation and OT6S media respectively, before being regenerated on G2 regeneration and OT2S media for a further 2 weeks. Regeneration of all plantlets was performed under a 16/8h (day/night) photoperiod with light supplied by fluorescent tubes (70-80 μmolm^{-2}s^{-1} intensity) at 25°C. Regenerated plantlets that developed shoots greater than 3 cm and roots greater than 1 cm were counted. Selected regenerants were hardened off in a mist bed, as described by O'Kennedy et al. (1998), before being transferred into pots to the greenhouse to assess growth and fertility. Regenerants were cross-pollinated within the same experiment and maize genotype. Three replicates (24 embryos per replicate) were conducted for each experiment (Table 3.1).

3.3.3 Plasmid Preparation

The pSC1b32 plasmid (obtained from Dr. Lupotto, Bergamo, Italy) contains the *b32* gene driven by the CAMV35S promoter, and the selectable marker gene (*bar*) driven by the maize ubiquitin promoter (Figure 3.1A). The pGEMpgip2 plasmid (obtained from Prof. Cervone, Rome, Italy) harbours the *pgip2* gene under the control of the CAMV35S promoter (Figure 3.1B). These plasmid DNAs were multiplied and extracted from overnight *E.coli* JM109 cell cultures using the Qiagen (Southern Cross Biotechnologies) maxiprep kit according to the supplier's protocol.
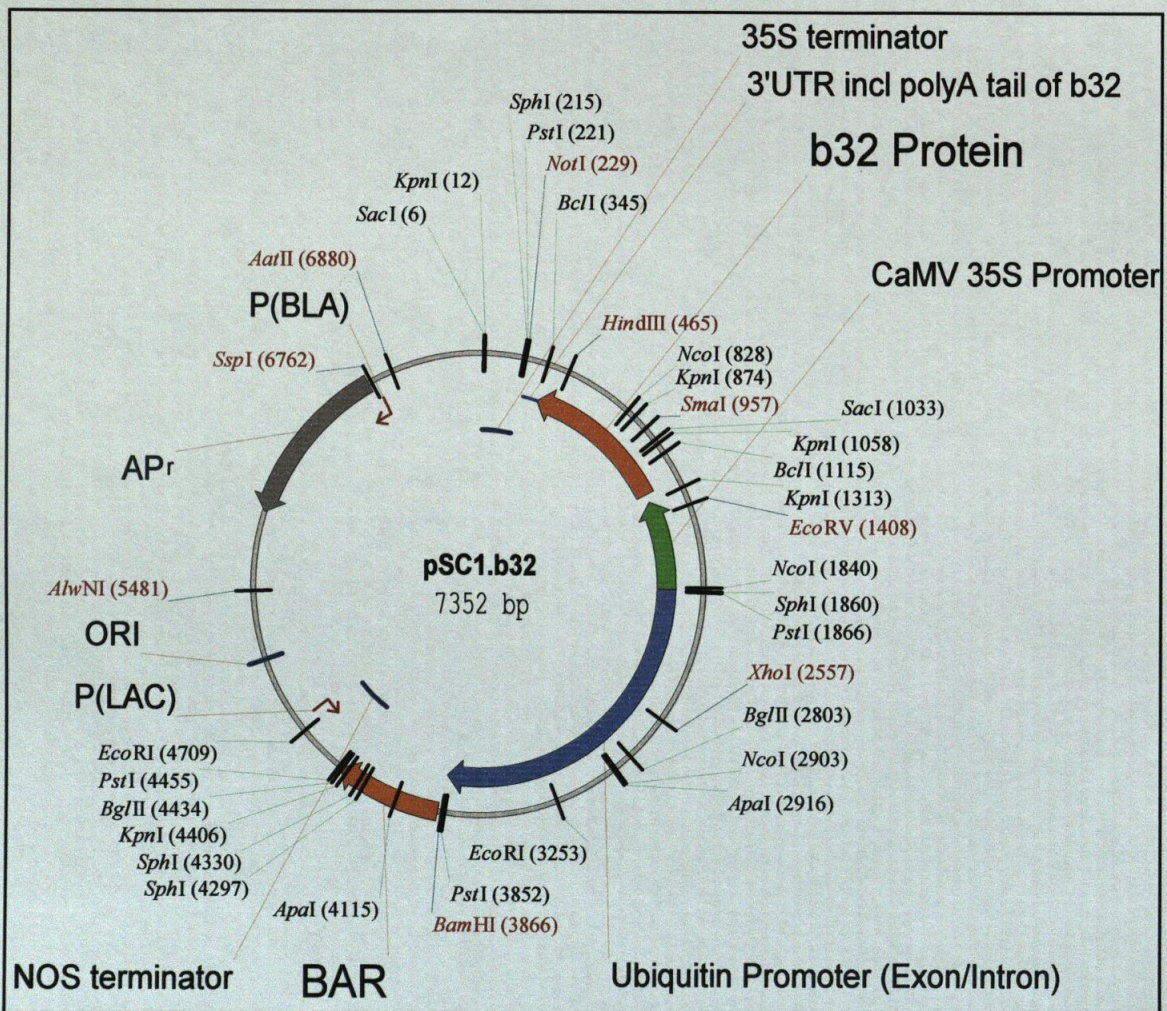
Fig 3.1A Plasmid vector pSC1b32 with selected restriction sites indicated. pSC1b32 contains the maize *b32* gene driven by the constitutive cauliflower mosaic virus (CAMV) promoter. The selectable marker gene *bar* also occurs within this vector and is under the control of the maize ubiquitin promoter. This vector is 7352 bp in size.
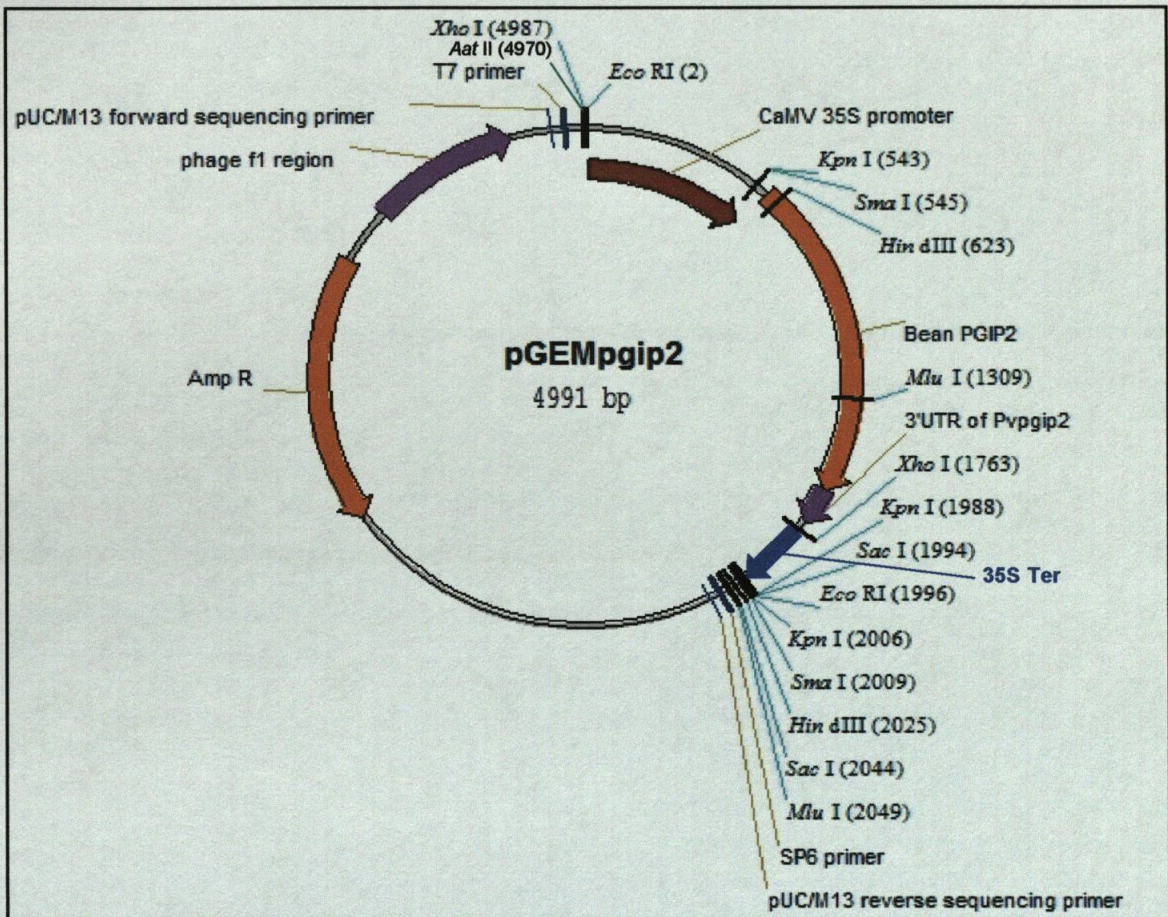
Fig 3.1B Plasmid vector pGEMpgip2 with selected restriction sites indicated. pGEMpgip2 contains the bean *pgip2* gene driven by the constitutive cauliflower mosaic virus (CAMV) promoter. This vector is 4991 bp in size.

3.3.4 Microprojectile Bombardment

Pre-cultured IZEs were placed in the middle (0 – 1 cm radius) of a 9 cm Petri dish. Media used were composed of $N6_{10}$ callus induction medium supplemented with 25% increased solidifier (i.e. 8 gl^{-1} agarose), 0.2M sorbitol and 0.2M mannitol as osmoticum, as described by Vain and co-workers (1993). The bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles as described by O'Kennedy *et al.* (1998). DNA delivery parameters were: 800-1100 KPa helium pressure; 0.16μg DNA per shot; 15.5 cm distance from syringe filter to target tissue; and a 500μm nylon mesh baffle placed 9 cm above the target tissue. Two microlitres of the bombardment mix was bombarded under a vacuum of – 87 KPa. Plasmids were co-bombarded (0.8μg per plasmid DNA per bombardment mix), producing a 1.6μg total plasmid DNA content per mix. All experiments were conducted with the PIG under sterile conditions at room temperature.

3.3.5 Selection and regeneration of transgenics

The bombarded IZEs were transferred to osmoticum-free $N6_{10}$ selection media ($N6_{10}$ callus induction media supplemented with 3 mg l^{-1} bialaphos) after 2-4 days. The herbicidal formulation Herbiace® (Meiji Seika Kaisha Ltd., Japan) containing bialaphos, was added to the media. Bialaphos content was increased from 3 mg l^{-1} to 5 mg l^{-1} after 4 weeks, with each transfer to fresh selection media occurring every 2 weeks. After 4-6 weeks on selection media, cultured IZEs that produced white, compact, type-I calli (presumably embryogenic) were transferred to maturation (OT6S) media containing 3 mg l^{-1} bialaphos for 2 weeks. Callus induction and maturation occurred under low light conditions (1.8 μmol $m^{-2}s^{-1}$). OT2S medium containing 1 mg l^{-1} bialaphos was used for regeneration of putative transformants under a 16/8h (day/night) photoperiod with a 70-80 μmol $m^{-2}s^{-1}$ light intensity. Developing plantlets were subcultured at 2-3 week intervals, until they developed shoots of greater than 3 cm and roots greater than 1 cm, before being hardened off (as described by O'Kennedy *et al.*, 1998). All experiments were performed at 25°C.

3.3.6 Chlorophenol Red (CPR) Assay

$N6_{10}$ callus induction medium supplemented with chlorophenol red (Sigma) and bialaphos was used: each of the 24 wells on the cell culture plate contained 1 ml of autoclaved media. Sterile chlorophenol red aqueous solution was added to each well to a final concentration of 1 mg ml^{-1}. For selection "Kill Curve" studies, a range of bialaphos concentrations (0-3 mg l^{-1}) was added to the wells. For screening of putative transgenics, a 3 mg l^{-1} bialaphos concentration was used. Non-transgenic tissue plated in wells without herbicide represented positive controls, while those in wells containing bialaphos served as negative controls.

Assay of Tissues: Leaf fragments (1cm x 0.5cm long), aseptically cut from putative transgenic plantlets, were individually placed in the wells with the abaxial side in contact with the medium. Plates were sealed and incubated at 25°C under low light conditions (1.8 μmol $m^{-2}s^{-1}$) for 1 - 2 weeks. Each well (containing maize tissue) was scored according to medium colour change: orange-to-yellow colour change noted as a positive, while red-to-purple colour change as negative (Wright *et al.*, 1996).

3.3.7 Herbicide Resistance (Basta® painting)

A 2 % (v/v) Basta® (200 g l^{-1} of the active ingredient, glufosinate ammonium, obtained from Hoecht AG, Germany) and 0.01% (v/v) Tween 20 (Merck Chemicals) solution, was applied to the darker, dorsal surface of selected leaves of transgenic maize plants (3-4 leaf stage), as previously described by O'Kennedy *et al.* (1998).

Glufosinate (commercial formulation Basta®), and the tripeptide bialaphos (commercial formulation Herbiace®), are both phosphinothricin (PPT)-based selective agents. Tissue necrosis on the painted leaf surface was observed within 5 days (or more) after painting and the painted leaves were clearly labeled as being herbicide resistant (R) or herbicide sensitive (S).

3.3.8 DNA Extraction

Genomic DNA was extracted from putative transgenic maize leaf material using the method outlined by Dellaporta *et al.*, 1983.

3.3.9 Polymerase Chain Reaction (PCR) Analysis

The PCR reaction mixture contained 1x reaction buffer (160mM $(NH_4)_2SO_4$, 670mM Tris-HCl, pH 8.8, 0.1% Tween-20), 0.8mM of each dNTP, 1.5mM $MgCl_2$, 0.8µM of each primer, 0.5U of Taq DNA polymerase, 1µl of the respective genomic or plasmid DNA, and sterile distilled water to make up the final volume of 25µl. All reagents used were from Bioline. For the positive controls, 1ng of plasmid DNA was used, whereas for putative transgenics, 50ng of genomic DNA was used. PCR was conducted using a Gene Amp 9700 PCR thermocycler (PE Applied Biosystems). Pgip2 – (5'- GGC TTC CAA CCA CCG ACT G –3') and (5'- GCA GAA ACT TTA GCT GCG –3'), b32 – (5'- CGC ACA ATC CCA CTA TCC TTC GC –3') and (5'- TGT CCC ACT TCT GCA CCT GCT TCC –3'), and bar (5'- CAT CGA GAC AAG CAC GGT CAA CTT C –3') and (5'- CTC TTG AAG CCC TGT GCC TCC AG –3') specific primers were used to amplify 0.73kb, 0.87kb and 0.28kb internal fragments respectively, from genomic DNA preparations of putative transgenic maize plantlets. NAD primers (Mannerlof & Tenning, 1997) were 5'-TAG CCC GAC CGT AGT GAT GTT AA -3' and 5'- ATC ACC GAA CCT GCA CTC AGG AA -3' (the *nad* gene is a conserved multicopy gene of the mitochondria and thus occurs in all plants). PCR samples were denatured at 94 °C for 2 min, and then subjected to 35 cycles of a denaturing step at 94°C for 45 sec, an annealing step (at 55°C for pgip2, 61°C for b32 and 64°C for bar) for 30 sec and an elongation step at 72°C for 45 sec. The final step at 72°C is for 2 minutes. PCR products were separated on a 1.2% (w/v) agarose gel and visualized by ethidium bromide staining under UV light.

3.3.10 Preparation of Probes and Southern Blotting

The *pgip2, b32* and *bar* genes (from their respective plasmids in figure 3.1) were labelled with digoxigenin (DIG) dNTPs, by the PCR-DIG probe synthesis kit as described by the supplier (Roche Biochemicals). The primers for the *pgip2* and *bar* genes are outlined in Section 3.3.9. The primer for the *b32* DIG probe, however, was 5'- TCAGGCCTCGTCATCGTCGTTG -3' and 5'- ATGGCCGAGACAAATCCAGAGTTGAG -3', which amplified the entire CAMV35S promoter (571 bp) and approximately 300 bp of the b32 gene. Genomic DNA (5µg) was digested with the *Eco*RI restriction enzyme and separated on a 0.8% (w/v) agarose gel and transferred onto positively charged nylon membranes (Roche Diagnostics). Prehybridisation (3 hours) and hybridisation (16 hours) with the respective DIG-labelled probes, washing and chemiluminescent detection of signals (using autoradiography) were performed as outlined in the Southern blot protocol (O'Kennedy et al., 1998).

3.4 RESULTS

3.4.1 REGENERATION STUDIES OF SELECTED ELITE WHITE MAIZE GENOTYPES

Embryogenic type-I c alli w ere i nitiated when I ZEs w ere cultured on the r espective callus induction media. This white, compact, cup-shaped calli is displayed in Figure 3.2A. The percentage of Type-I calli formed for each elite maize genotype varied on the different callus induction media (Table 3.1). For example, on the $N6_{10}$ medium, A483-1 had a 38-52% type-I calli formation while A483-12 had 19-35% type-I calli being formed. For the G2+Pro media, a low percentage of type-I calli formation occurred, with the highest percentage (26%) achieved with M37W, and the lowest (0%) occurring for at least two replicates of all the remaining genotypes. This percentage (listed per replicate for all the genotypes in Table 3.1) is representative of the number of IZEs out of 24 (per replicate) that produced type-I calli. It is important to note that although these type-I calli were regarded as embryogenic, not all calli matured to form shoots (displayed in Figure 3.2B) and roots (regenerating plantlets).

Table 3.1 The percentage of callus formation and number of regenerants obtained per IZE explant, for each of the six elite white maize genotypes, when tested on different callus induction media. Three replicates (24 embryos per replicate)

Elite White Maize Genotype	Experiment Replicate #[a]	% Callusing embryos			# Regenerants per Explant (IZE) [b]		
		2MS + Pro	$N6_{10}$	G2 + Pro	2MS + Pro	$N6_{10}$	G2 + Pro
A483-1	1	41	62	6	0.8	5.8	0.0
	2	38	69	0	1.3	2.7	0.0
	3	52	64	0	1.3	2.3	0.0
A483-4	1	39	33	0	0.04	1.2	0.0
	2	49	44	0	0.1	0.5	0.0
	3	25	23	1	0.2	0.8	0.04
A483-10	1	17	48	0	0.0	0.0	0.0
	2	19	51	0	0.0	0.0	0.0
	3	20	46	0	0.0	0.0	0.0
A483-12	1	19	36	0	0.0	0.0	0.0
	2	40	38	0	0.7	0.6	0.0
	3	35	25	0	0.2	0.04	0.0
Z206-131	1	0	1	0	0.0	0.0	0.0
	2	20	9	0	0.04	0.4	0.0
	3	17	17	0	0.8	0.3	0.0
M37W	1	23	92	26	0.5	1.8	0.5
	2	54	89	5	1.3	2.1	0.0
	3	92	74	5	1.6	4.5	0.1

were performed per experiment.

a = Three replicate experiments performed for each genotype on each callus induction media
b = Number of regenerants per explant listed for each replicate, is the average number of regenerants (rooted and shooted plantlets) produced per replicate experiment

Three individual replicate experiments (24 embryos per replicate) were done for each genotype on each callus induction media: the number of regenerated plantlets produced per explant (IZE) for each of the genotypes on the respective callus induction media, are listed in Table 3.3. A483-1 produced the highest number of regenerants per explant (5.8), with M37W closely following with 4.5, both on the $N6_{10}$ callus induction media. The G2+Pro callus induction media did not result in many regenerants being produced for most of the genotypes tested, with the highest value of 0.5 regenerants per IZE being reported for the M37W genotype.

The average of the number of regenerants per explant from all three replicates, were plotted for each genotype and callus induction media combination in the histogram in Figure 3.3. The three genotypes producing the highest number of regenerants per explant were: A483-1, M37W and A483-4 (all on $N6_{10}$ callus induction medium), producing 3.6, 2.8 and 0.8 regenerants per explant respectively.

All of the T_0 regenerating plantlets, after successful hardening-off in the greenhouse, formed abnormal phenotypic characteristics: dwarfism, sterile flowering parts and underdeveloped tassels and tassel ears. However, when the respective T_1 seeds were germinated *in vitro* and transferred directly into pots under greenhouse conditions, fertile plants exhibiting normal development and growth occurred (Figure 3.2C).
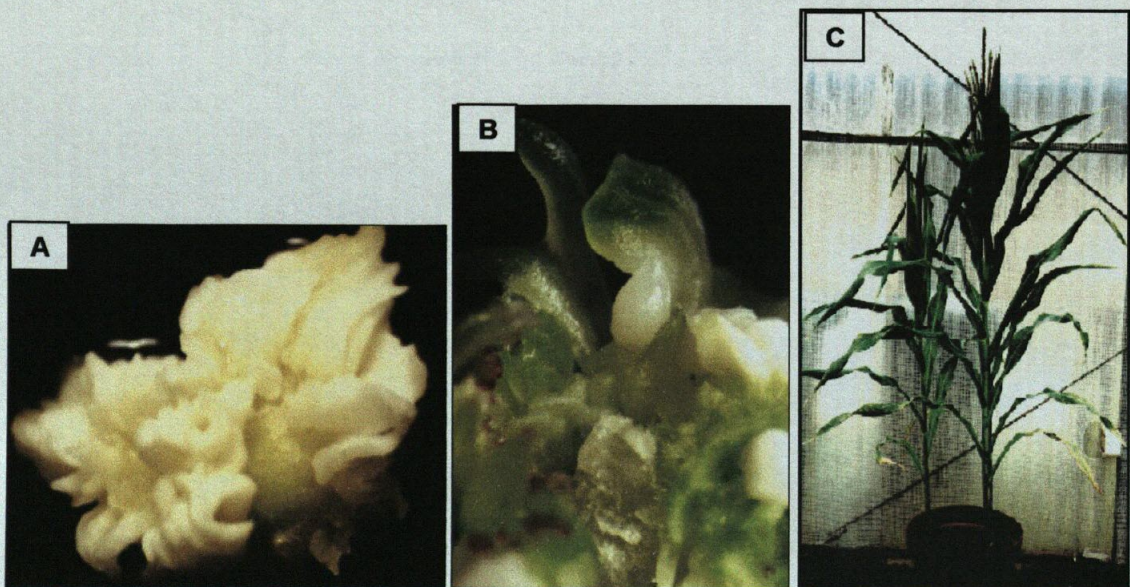
Fig 3.2 A483-1 displaying **A)** type-I calli formation after 2 weeks on $N6_{10}$ callus induction medium, **B)** shoot formation after 2 weeks on OT6S maturation medium, and **C)** T_1 progeny plants showing healthy, fertile phenotypic growth in greenhouse.

**Average Number of Regenerants per Explant
obtained per Callus induction Media**
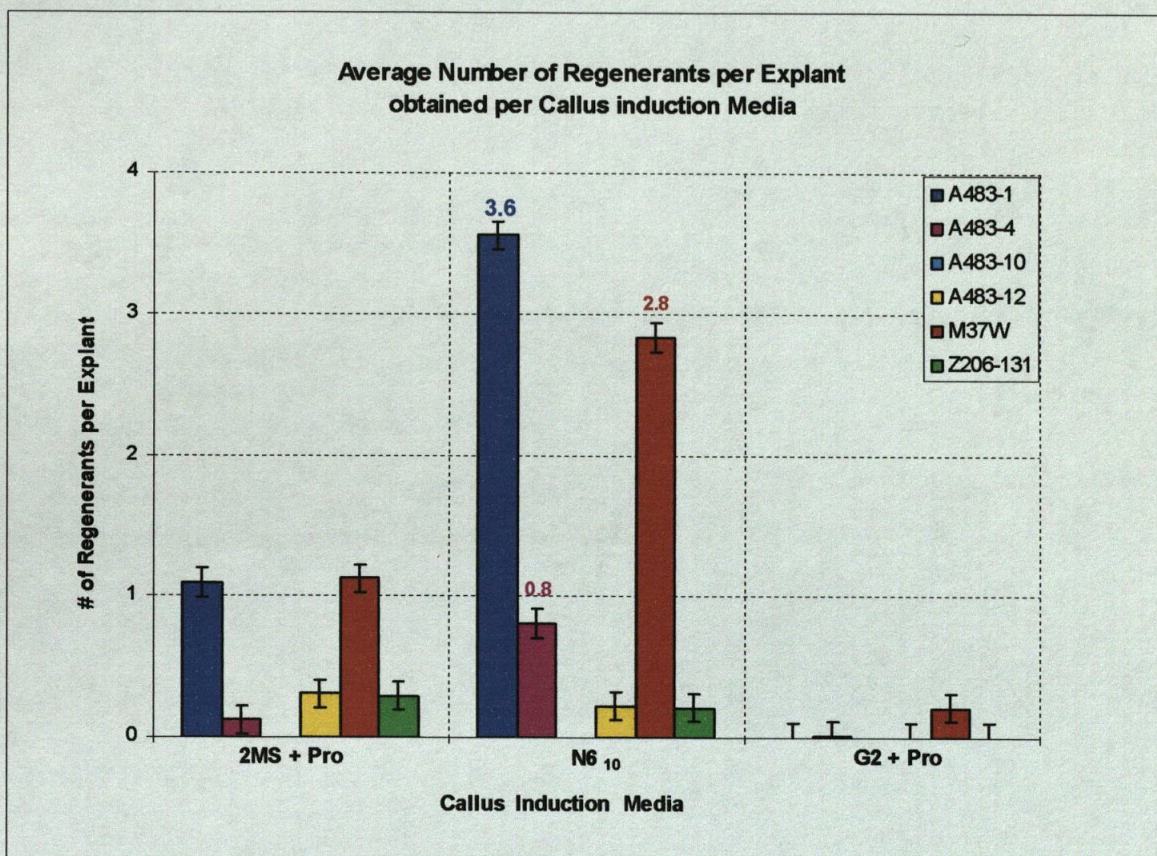
Fig 3.3 Histogram displaying the average number of regenerants obtained per explant for each elite white maize genotype per callus induction media

3.4.2 TRANSFORMATION OF SELECTED ELITE WHITE MAIZE GENOTYPES

3.4.2.1 Selection of transgenic tissues and regeneration of T_0 plants

Twenty-eight independent bombardment experiments were performed (Table 3.2). The number of experiments for each of the elite white maize genotypes was dependant on the number of IZEs available at the time of bombardment. Putatively transformed IZEs were identified by their vigorous growth to produce Type-I calli on bialaphos-containing medium, while non-transformed IZEs turned brown and watery on selection media (Figure 3.4A). Bialaphos-resistant type-I calli or calli clumps, when subcultured onto OT6S maturation media (3 mg l^{-1} bialaphos), matured to form somatic embryos. These somatic embryos produced shoots and roots (Figure 3.4B and C) when transferred to OT2S regeneration medium (with a lower 1 mg l^{-1} bialaphos concentration).

3.4.2.2 Chlorophenol Red Assay

A "kill curve" study was carried out to determine the bialaphos concentration at which untransformed Hi-II calli would be eliminated (transgenic tissue is expected to survive at this chosen herbicide concentration). Figure 4.2 (in Section 4.4) displays minimal growth for untransformed Hi-II calli at bialaphos concentrations greater than 1 mg l^{-1}, and calli turned brown and watery at concentrations above 2 mg l^{-1}. Therefore, a bialaphos concentration of 3mg l^{-1} was chosen for putative transgenics tested, to eliminate escape calli. In figure 3.5A, untransformed M37W leaf segments display a yellow colour change on the $N6_{10}$ medium (without bialaphos herbicide) indicating survival of the tissue (positive control). However, when these untransformed leaf tissues were exposed to 3 mg l^{-1} bialaphos, a red medium colour occurred as these tissues do not contain the *bar* gene as expected in figure 3.5A. This confirmed that the CPR assay could be used for rapid identification of elite maize transformed tissues containing the selectable marker gene. All of the putative elite transgenics were tested on CPR plates (Figure 3.5B). Many positives (indicated by yellow coloured wells showing presence and functionality of the *bar* gene), as well as possible negative escape plants (red coloured wells) were observed. All CPR results were confirmed by PCR positive testing for the *bar* gene presence. One-hundred-and-eight independent maize transformation events (rooted and shooted plantlets) in total were obtained from 28 independent experiments (Table 3.2). These putative transgenic plantlets were successfully hardened off.

3.4.2.3 Analysis of T_0 putative transgenic plants

Only 21 (of the 108 putative transgenic plants hardened off) grew to maturity in pots under greenhouse conditions. Basta® painting and PCR screening results of these T_0 putative transgenics are listed in Table 3.3. All 21 events tested CPR positive and Basta® paint resistant (R) (Table 3.3). This result correlated with the PCR results for the *bar* gene in all events (Table 3.3). Furthermore, four independent events were PCR positive for all three genes (*b32*, *pgip2* and *bar*), with 6 events testing positive for the *b32* and *bar* genes only, and an additional 2 events positive for the *pgip2* and *bar* genes. None of the events tested PCR positive for either of the antifungal genes (*b32* or *pgip2*) without the selectable marker (*bar*) gene (Table 3.3).

Many T_0 putative transgenics displayed stunted growth and abnormal ear production and only a few grew into tall plants (Figure 3.6A). Only two of the 21 surviving putative transgenics were fertile and produced T_1 seed when pollinated (events BBE 13.2.3 and BBE 16.1.1).

Table 3.2   Conditions for bombardment of IZEs of selected elite maize genotypes

Maize Line	Exp #[a]	# IZEs BB	Pre-culture (days)[b]	Helium Pressure (KPa)	Post-culture (days)[c]	% IZEs surviving[d]	# of events identified[e]
A483-4	BBE3	15	4	900	3	0	0
A483-1	BBE4.1	24	6	1000	4	25	1
	BBE 4.2	29	6	1000	4	56	0
	BBE 4.3	23	6	1000	4	44	0
M37W	BBE 5.1	29	4	800	2	81	1
	BBE 5.2	25	4	800	2	80	0
M37W	BBE 6.1	28	4	800	3	75	3
	BBE 6.2	17	4	900	3	65	1
M37W	BBE 7.1	37	3	900	3	81	4
	BBE 7.2	32	3	1000	3	100	3
M37W	BBE 8.1	46	5	1000	4	39	0
	BBE 8.2	44	5	1100	4	45	5
M37W	BBE 9.1	42	4	1000	4	29	0
	BBE 9.2	28	4	1100	4	61	3
M37W	BBE 10	7	6	900	5	43	0
A483-1	BBE 11.1	21	5	800	4	47	8
	BBE 11.2	20	5	900	3	60	2
	BBE 11.3	19	5	1000	2	58	3
M37W	BBE 12.1	27	5	900	4	89	6
	BBE 12.2	22	5	1000	3	91	6
A483-1	BBE13.1	22	4	1000	3	41	1
	BBE 13.2	28	4	1100	2	57	14
	BBE 13.3	17	4	1100	3	53	0
M37W	BBE14	24	6	1000	3	92	7
A483-4	BBE15.1	9	6	1000	3	44	0
	BBE 15.2	9	6	1100	4	67	0
A483-4	BBE 16.1	26	5	800	3	31	1
	BBE 16.2	41	5	900	4	27	0
	BBE 16.3	15	5	1000	3	53	0
	BBE 16.4	17	5	1000	4	65	0
A483-4	BBE 17.1	28	6	800	3	18	0
	BBE 17.2	23	6	900	2	30	0
A483-4	BBE 18	20	5	900	4	55	0
M37W	BBE19	22	5	900	4	86	4
A483-4	BBE 20.1	27	6	800	4	59	1
	BBE 20.2	18	6	800	6	50	0
	BBE 20.3	32	6	900	4	50	6
	BBE 20.4	30	6	900	6	47	4
	BBE 20.5	30	6	1000	4	57	9
	BBE 20.6	26	6	1000	6	58	0
	BBE 20.7	10	6	1100	4	70	0
A483-4	BBE21	26	5	1100	5	38	0
M37W	BBE22	18	5	1100	5	50	2
A483-4	BBE 23	10	5	1000	4	35	0
A483-1	BBE 24	24	5	900	1	44	7
A483-4	BBE 25	30	5	900	1	20	3
A483-4	BBE 26	19	5	1000	1	10	1
A483-1	BBE 27.1	18	5	800	6	45	1
	BBE 27.2	20	5	800	6	51	2
	BBE 27.3	14	5	800	6	47	0
M37W	BBE 28	23	5	800	6	48	0

a   =   Experiment number of bombarded IZE cultures on N6$_{10}$ media

b   =   Number of culture days of IZEs on N6$_{10}$ media before bombardment

c   =   Number of culture days of bombarded IZEs on osmoticum media before transfer to N6$_{10}$ selection media (3 mg l^{-1} bialaphos)

d   =   Percentage of bombarded IZEs proliferating to form Type-I calli after 4 weeks on N6$_{10}$ selection media (3 mg l^{-1} bialaphos)

e   =   Number of rooted and shooted plantlets produced on OT2S regeneration media with 1 mg l^{-1} bialaphos selection
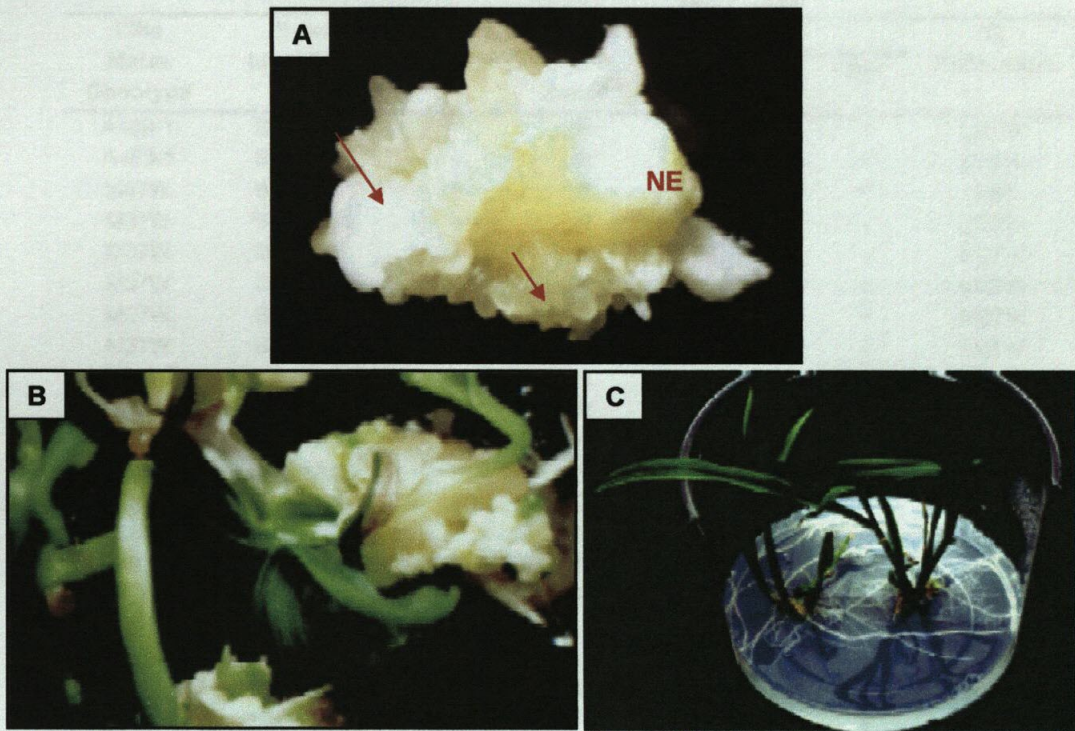
Fig 3.4 A) Putative transformants are identified as proliferating sectors of callus tissue (red arrows) among a background of non-embryogenic (NE) calli on $N6_{10}$ callus induction media containing 3 mg l^{-1} bialaphos herbicide; **B)** Putative transgenic somatic embryos developing shoots on OT6S maturation medium supplemented with 3 mg l^{-1} bialaphos herbicide; and **C)** Putative transgenic maize plantlets displaying extensive root development on OT2S regeneration medium, supplemented with 1 mg l^{-1} bialaphos.
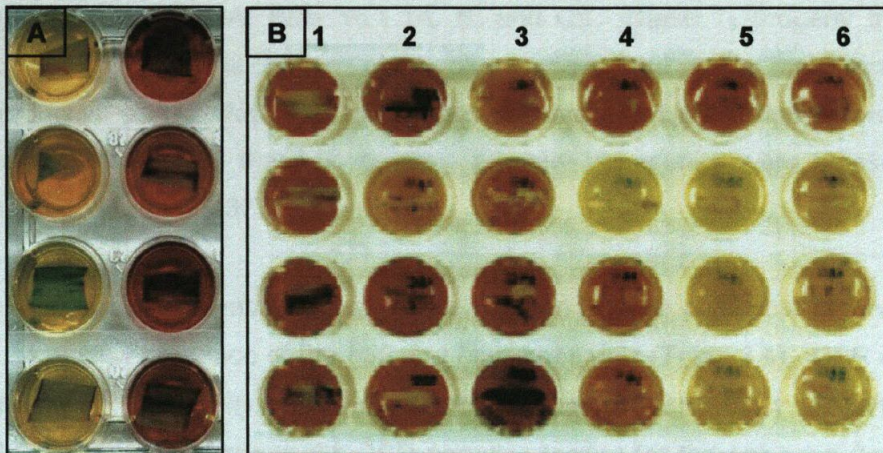
Fig 3.5 Chlorophenol red assay using $N6_{10}$ callus induction medium supplemented with different bialaphos concentrations, after two weeks in culture. **A)** Leaf pieces of untransformed elite maize M37W plantlets cultured with 0 mg l^{-1} (left column) and 3 mg l^{-1} (right column) bialaphos and **B)** Leaf pieces of elite maize putative transgenic plantlets (selection 3 mg l^{-1} bialaphos). Wells of column 1 contains leaf pieces from non-transformed M37W (control) plantlets. Each well of columns 2-6 contains leaf pieces from different events of putatively transgenic T_0 plantlets.

Table 3.3 Summary of results obtained for the T_0 putative transgenics

Elite Maize Genotype	Event identified [a]	CPR Result	Basta® Paint Result [b]	PCR			T_0 Pollination
				b32	*pgip2*	*bar*	
A483-1	BBE 4.2.1	+	R	-	-	+	M37W
A483-1	BBE 4.3.1	+	R	-	+	+	M37W
M37W	BBE 6.1.1	+	R	-	-	+	Self
M37W	BBE 6.1.2	+	R	+	-	+	M37W
M37W	BBE 7.2.1	+	R	-	-	+	M37W
M37W	BBE 8.2.2	+	R	-	-	+	M37W
M37W	BBE 8.2.4	+	R	-	-	+	M37W
M37W	BBE 9.2.3	+	R	-	-	+	M37W
A483-1	BBE 11.2.1	+	R	+	-	+	M37W
A483-1	BBE 11.3.1	+	R	+	-	+	M37W
M37W	BBE 12.1.3	+	R	+	-	+	M37W
M37W	BBE 12.2.2	+	R	-	+	+	Self
A483-1	BBE 13.2.2	+	R	-	-	+	M37W
A483-1	BBE 13.2.3	+	R	+	-	+	M37W
A483-1	BBE 13.2.7	+	R	-	-	+	M37W
M37W	BBE 14.1.5	+	R	+	+	+	M37W
M37W	BBE 14.1.6	+	R	+	+	+	M37W
A483-4	BBE 16.1.1	+	R	+	+	+	M37W
M37W	BBE 19.1.3	+	R	-	-	+	M37W
M37W	BBE 22.1.3	+	R	+	+	+	M37W
M37W	BBE 22.1.5	+	R	+	-	+	M37W

a	=	T_0 putative transgenic plants that survived under greenhouse conditions
b	=	'R' represents herbicide resistance while 'S' represents herbicide sensitivity

3.4.2.4 Analysis of T_1 progeny transgenic plants

The two fertile T_0 events (BBE 13.2.3 and BBE 16.1.1) produced their respective T_1 progeny (BBE 13.2.3A, BBE 16.1.1A, B, and C). These T_1 progeny transgenics displayed normal tall maize plant phenotypes, and both male and female reproductive parts. However, the tassels had produced the pollen before the ears developed, and thus, these T_1 putative progeny transgenics were cross-pollinated with untransformed M37W pollen.

Event BBE 13.2.3A was CPR assay positive and Basta® paint resistant, while PCR results display presence of the *b32* and *bar* genes only (Table 3.4). CPR assay and Basta® paint sensitivity occurs for the T_1 progeny of BBE 16.1.1 (BBE 16.1.1A, B and C), which correlates to the PCR negative testing for the *bar* gene (lanes 6-8 in figure 3.7).

Table 3.4 PCR results for putatively transgenic elite genotype T_1 progeny

| Maize Genotype | Event | CPR Result | Basta® Paint Result [a] | PCR | | | Pollination |
				b32	Pgip2	bar	
A483-1	BBE 13.2.3 A	+	R	+	-	+	M37W
A483-4	BBE 16.1.1 A	-	S	+	-	-	M37W
	BBE 16.1.1 B	-	S	+	-	-	M37W
	BBE 16.1.1 C	-	S	-	-	-	M37W

a = 'R' represents herbicide resistance while 'S' represents herbicide sensitivity

The PCR results (Figure 3.7) show that for event BBE 13.2.3A, the correct amplified band sizes for the *b32* and *bar* genes was present. No amplified DNA band for the *pgip2* gene in lane 5 occurred. The positive controls containing plasmid DNA (lane 1 and 4) produced the expected amplified products of 875bp, 731bp and 279bp for the *b32*, *pgip2* and *bar* genes respectively. The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA), as expected, did not give any amplification products. These results were confirmed by southern blot analysis in figure 3.9. The *b32* gene presence was confirmed by the hybridisation of a doublet band in lane 3 of figure 3.8. These bands (of approximately 7400bp and 6200bp in size) are much lighter in comparison to the hybridising band representative of 2 copies of the gene (lane 2). No hybridisation of the *pgip2* probe occurs for BBE 13.2.3A as clearly indicated by the absence of a band in lane 3 of figure 3.9.
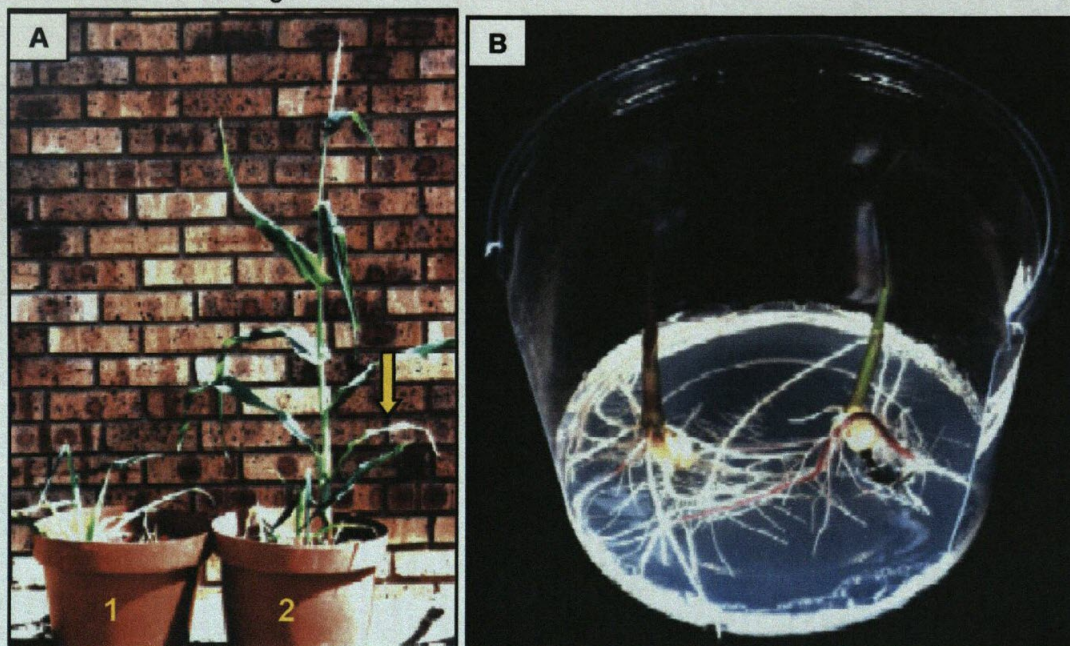
Fig 3.6 A) Putative T_0 transgenic plants (from experiment BBE 16.1) occur growing in pots under greenhouse conditions. Some transgenic plants displayed stunted growth (1), while others displayed fertile and tall phenotypes (2). Basta® painting sensitivity is also displayed by the dying of the leaf painted (arrow); and **B)** T_1 progeny (BBE 13.2.3A) germinating on ½ MS media supplemented with 1 mg l^{-1} bialaphos. Extensive root development is displayed.

The progeny of event BBE 16.1.1 did not yield any amplified fragments for the *bar* and *pgip2* genes via PCR testing (lanes 6-8 in figure 3.7). For the *b32* gene however, faint bands occurred for the progeny A and B (lane 6-7). Progeny C (lane 8) did not yield any amplified bands. The positive controls containing plasmid DNA (lane 1 and 4) produced the expected amplified products of 875bp, 731bp and 279bp for the *b32*, *pgip2* and *bar* genes respectively. The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA), as expected, did not give any amplification products. PCR of the *nad* gene (figure 3.8) for the progeny were positive, confirming that the genomic DNA was of good quality. BBE 16.1.1C (lane 8 of figure 3.7) PCR result was therefore negative for *b32*, *pgip2* and *bar* and not due to poor DNA quality.
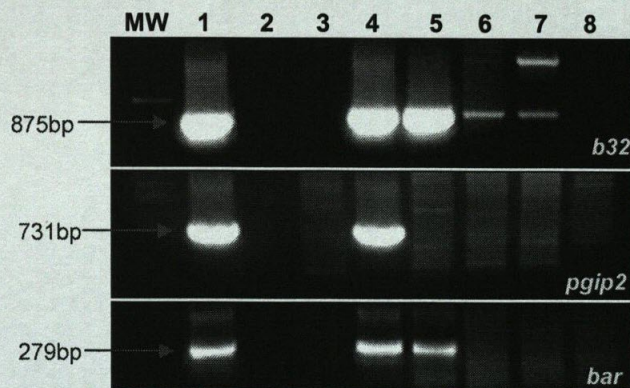
Fig 3.7 PCR of genomic DNA of putatively transgenic T$_1$ plants for *b32*, *pgip2* and *bar* genes. Lane MW: DNA molecular weight marker IV; Lane 1: positive control of the respective plasmid DNA (pSC1b32 for *b32* and *bar* and pGEMpgip2 for *pgip2* PCRs); Lane 2: PCR mix without DNA; Lane 3: untransformed M37W genomic DNA (negative control); Lane 4: untransformed M37W genomic DNA spiked with 1ng of the respective plasmid DNA; Lanes 5 to 8: genomic DNA (50ng) of the T$_1$ putative progeny transgenics, BBE 13.2.3A, BBE 16.1.1A, BBE 16.1.1B and BBE 16.1.1C, respectively.
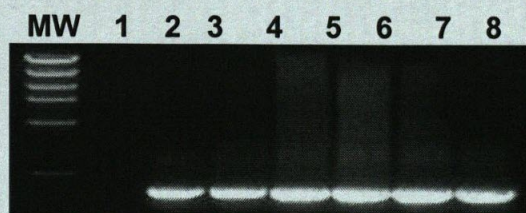
Fig 3.8 PCR of genomic DNA of putatively transgenic T$_1$ plants for *nad* gene. Lane MW: DNA molecular weight marker IV; Lane 1: PCR mix without DNA (negative control); Lane 3: untransformed M37W genomic DNA; Lane 4 to 7: genomic DNA (50ng) of the T$_1$ putative progeny transgenics, BBE 13.2.3A, BBE 16.1.1A, BBE 16.1.1B, BBE 16.1.1C respectively, and lane 8: BBE 16.1.1 A1 (T$_2$ progeny).

Southern blot analysis (Figure 3.9) show hybridising bands (of approximately 3400 bp in size) occurring with the *b32* probe, for the T_1 (lane 4-5) and T_2 (lane 6) progeny of BBE 16.1.1.   The banding pattern of the T_1 (BBE 16.1.1A and BBE 16.1.1B) and the T_2 (BBE 16.1.1A1) progeny of BBE 16.1.1 are the same, as expected. These bands are much lighter in comparison to the hybridising band representative of 2 copies of the gene (lane 2 in figure 3.9). No hybridisation of the *pgip2* probe occurs with the genomic DNA of any of the progeny of BBE 16.1.1 clearly indicated by the absence of bands in lanes 4 - 6 of figure 3.10. The negative control containing genomic DNA of untransformed Hi-II (lane 1 in both figures 3.9 and 3.10) did not produce any hybridising bands as expected.

The positive controls containing untransformed Hi-II genomic DNA spiked with 2 copies (lane 2) and 10 copies (lane 7) of the respective plasmid DNAs however, produced hybridising bands with different intensities: the 10 copy bands were comparatively darker than the 2 copy bands produced.
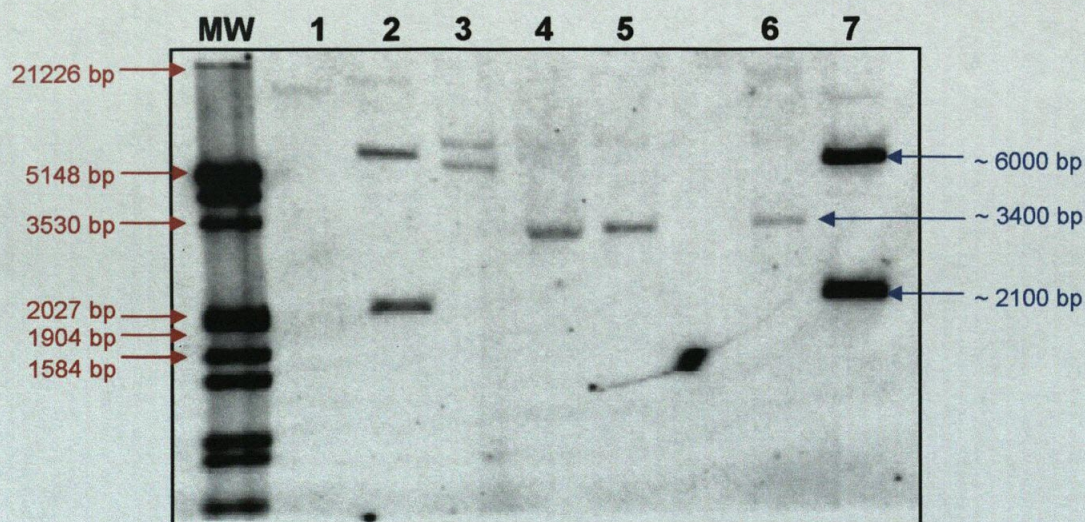
Fig 3.9 Southern blot (*b32*) analysis of T_1 and T_2 progeny of two transgenic elite maize genotypes. Five microgram of genomic DNA (purified from leaf material) for all samples, were restricted with the *EcoR*I restriction enzyme and resolved on a 0.8% agarose gel, transferred onto a nylon membrane and then probed with the *b32* probe outlined in Section 3.2.3.8. Lane MW: DIG-labelled molecular weight marker-III; Lane 1: genomic DNA of untransformed M37W; Lane 2: untransformed M37W genomic DNA spiked with 2 copies (20pg) of the pSC1b32 plasmid DNA; Lanes 3 to 6: genomic DNA of BBE 13.2.3A, BBE 16.1.1A, BBE 16.1.1B, and BBE 16.1.1A1 (T_2) respectively; and Lane 7: untransformed M37W genomic DNA spiked with 10 copies (100pg) of the pSC1b32 plasmid DNA.
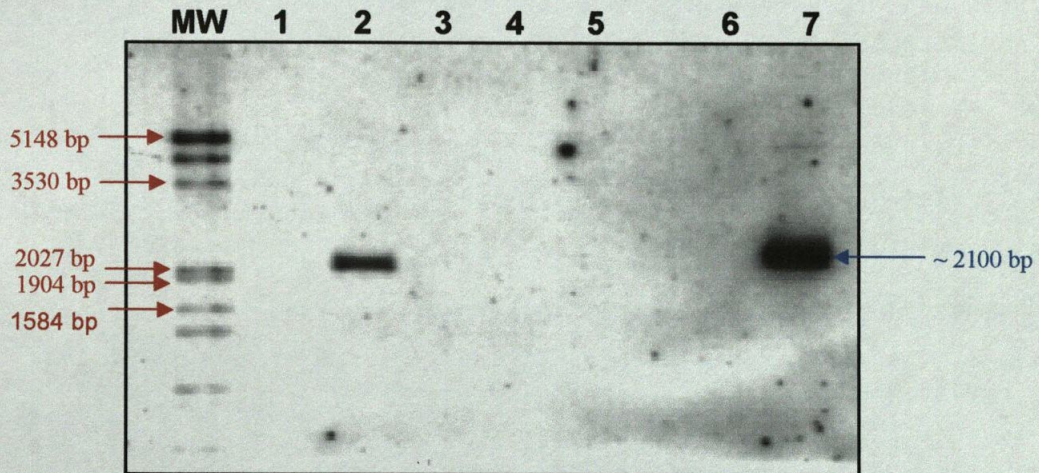
Fig 3.10 Southern blot (*pgip2*) analysis of T_1 and T_2 transgenic elite maize progeny. Five microgram of genomic DNA (purified from leaf material) for all samples were restricted with *EcoR*I, resolved on a 0.8% agarose gel and transferred onto a nylon membrane. Hybridisation with the *pgip2* probe outlined in Section 3.2.3.8 was done and exposure to hyperfilm occurred overnight. Lane M: DIG-labelled marker III; Lane 1: genomic DNA of untransformed M37W; Lane 2: untransformed M37W genomic DNA spiked with 2 copies (20pg) of the pSC1b32 plasmid DNA; Lanes 3 to 6: genomic DNA of BBE 13.2.3A, BBE 16.1.1A, BBE 16.1.1B, and BBE 16.1.1A1 (T_2) respectively; and Lane 7: untransformed M37W genomic DNA spiked with 10 copies (100pg) of pGEMpgip2 plasmid DNA.

Only one T_1 putative transgenic plant (BBE 16.1.1A) produced eight T_2 seeds. These T_2 seeds were germinated on ½ MS media (without bialaphos selection) but only one seed germinated (BBE 16.1.1A1). This T_2 plant (CPR assay negative), when hardened off and grown under greenhouse conditions, tested to be Basta® paint negative and produced T_3 seeds. When tested for the presence of the respective genes by PCR, BBE 16.1.1 A1 tested *b32* positive but pgip2 and *bar* negative (data not shown). Southern blot results additionally show integration of *b32* in BBE 16.1.1A1 (lane 6 of Figure 3.9).

3.5 DISCUSSION

3.5.1 REGENERATION STUDIES OF SELECTED ELITE WHITE MAIZE GENOTYPES

An essential requirement for the generation of transgenic plants is the availability of an efficient plant regeneration system. A highly efficient regeneration system has led to a highly efficient transformation system for oat plants (Gless et al., 1998a & b). Therefore, this section aimed at screening several elite white maize genotypes for embryogenic callus formation and plant regeneration. Genotypes producing the highest regeneration potential in vitro would be used for transformation.

In this study, the IZEs were cultured with the embryo axis in contact with the culture media, thus stimulating the proliferation of callus from scutellum cells (Armstrong & Green, 1985). Embryogenic type I calli, being generally white, nodular and compact (Lu e al., 1982, 1983; Tomes & Smith, 1985), was initiated from the IZEs. However, the frequency of type-I calli initiation varied significantly among the genotypes on the different callus induction media (Table 3.1). Although these type-I calli were regarded as embryogenic, not all of them matured to form somatic embryoids from which plantlets could be regenerated. Therefore, as suggested by O'Kennedy et al. (2001), the classification of calli as embryogenic did not necessarily imply regenerability. This variation in calli initiation among the genotypes is dependent on two main factors as outlined in Section 2.4: the explant source and its regeneration capacity; and the quality (or composition) of the nutrient media.

The composition of the nutrient media used in this study varied. The $N6_{10}$ media proved to be more efficient in the regeneration capacity for selected genotypes, with 3.6 and 2.8 regenerants per explant produced for A483-1 and M37W genotypes respectively. Therefore, the $N6_{10}$ nutrient based media was selected as the media for use in this study. The A483-4 genotype (having the third highest number of regenerants per explant (Figure 3.3)) was additionally selected for further transformation studies.

$N6_{10}$ media differed considerably from the other two media. The carbohydrate source (sucrose) is primarily important as an energy source, although it has been implicated in lowering (negative) the osmotic potential of nutrient media (Rijven, 1952). Nutrient salts contribute 20% to 50% of the osmotic potential of media, with sucrose making up the rest.

Thus, the lower sucrose concentration of 20 mg l^{-1} in the $N6_{10}$-based medium, as compared to 30 mg l^{-1} in the MS-based medium, could possibly be one of the reasons for $N6_{10}$ favouring a higher regeneration ability of the genotypes.

$N6_{10}$ additionally includes casein hydrolysate and silver nitrate. It also contains agarose for media solidification as opposed to agar used in both 2MS+Pro and G2+Pro media (Table 2.3). Silver nitrate addition has been reported to increase plant regeneration (Duncan & Wildholm, 1987b) by reducing ethylene accumulation while addition of casein hyrdolysate (a casamino acid) was said to increase callus formation and growth (Armstrong & Green, 1985). Additionally, the higher purity of agarose compared to agar possibly makes for a better response of an explant in culture (Vasil & Thorpe, 1994). Agarose is the low sulphate, neutral gelling fraction of agar, and is considerably purer than agar with respects to ionic groups. Therefore, addition of these substances to the media does reign as an advantage.

Furthermore, the $N6_{10}$ medium contains a lower nitrogen concentration than the MS-based medium (Table 2.1). Previous publications (Halperin & Wetherell, 1965; Reinert et al., 1967; Wetherell & Dougall, 1976) have demonstrated (in carrots) that the nitrogen composition of a medium plays an important role in somatic embryogenesis. The ratio of ammonium:nitrate (Grimes & Hodges, 1990) and the addition of reduced nitrogen in an organic form (amino acids), have also been reported to affect somatic embryogenesis and plant regeneration in several species (Gamborg, 1970; Stuart & Strickland, 1984; Shetty & Asano, 1991). Armstrong & Green (1985) additionally reported a 25% reduced frequency of embryoid and callus formation, when inorganic nitrogen sources in $N6_{10}$ were replaced with those normally used in MS media. Thus, this lower nitrogen concentration in $N6_{10}$ media could reign as an additional advantage over the MS medium. This better performance of the $N6_{10}$ media in comparison to the MS-based media for the genotype regeneration potential is in accordance to results reported by Bohorova et al. (1995). These scientists reported that basic $N6_{10}$ media proved to be excellent for embryogenic callus initiation and maintenance with most maize genotypes tested.

L-Proline and the synthetic auxin, 2,4-D, occurred in all three media types. These two compounds were reported to increase regeneration potentials: L-Proline was reported to have a positive function in stress tolerance of cultured tissues (Nanjo et al., 1999), while 2,4-D promotes embryogenesis (Woodward & Furze, 1989).

Another possible additional advantage is that the IZEs when cultured on $N6_{10}$ callus induction media were subjected to an extra two week period on maturation media, which is not catered for by the MS-based media. The sugar content in the maturation media was doubled (as compared to the induction media), and this has been reported to promote embryogenesis and somatic embryoid formation by Close & Ludeman (1987). The G2-based media also included a maturation period (2 weeks) however the regeneration potentials reported for the genotypes (in comparison to those obtained with the $N6_{10}$ media) were very low. Additionally, the G2-based media contained maltose as the carbohydrate source compared to sucrose in $N6_{10}$. Some genotypes could possibly show a preference to sucrose as a sugar source as compared to maltose, and this could possibly be the reason for the poor regeneration performance of most of the genotypes on the G2-based medium.

Plants that regenerated and were hardened off under greenhouse conditions displayed abnormal phenotypes (dwarfism, underdeveloped tassels and tassel ears). These phenotypic abnormalities are most likely to be the consequence of prolonged tissue culture periods. The T_1 progeny however, exposed to one week of tissue culturing (for germination), resulted in phenotypically normal maize plants.

3.5.2 TRANSFORMATION OF SELECTED ELITE WHITE MAIZE GENOTYPES

The highly regenerable genotypes (A483-1, A483-4 and M37W) selected in section 3.5.1 were used for transformation studies. IZEs were used as explant material for stable integration of the antifungal and selectable marker genes into the genome of maize. These IZEs were of 0.8 – 1.2 mm size as IZEs of this size were found to be at the ideal physiological development stage for culture (Kamo et al., 1985). Such explants were largely made up of meristematic and undifferentiated cells, which were not yet committed to any specific developmental pathways (Dale, 1980; Vasil & Vasil, 1980). Cultures derived from these cells were highly regenerable.

The IZEs were cultured with the embryo axis in contact with the nutrient media to stimulate the proliferation of scutellum cells (Armstrong & Green, 1985). IZEs were subjected to a short pre-culture period on nutrient media before bombardment, in order to attain embryogenic and transformation competence during these early stages of culture (Vasil & Vasil, 1981; 1982b).

O'Kennedy and co-workers (in 2001) reported successful transformation of M37W with a pre-culture period of 4 – 6 days. Therefore, this pre-culture period was chosen for use in this study.

Christou (1995) observed that injury to the cells by bombardment limits the recovery of the stable transformants. Several researchers have hypothesised that osmotic treatment of explants alleviates plasmolysis of cells, thereby reducing cell damage from bombardment (Vain et al., 1993). Thus in this study, explants were treated with osmoticum before (3 hours) and after (2-4 days) bombardment.

The genotypes used for the transformation process were A483-1, A483-4 and M37W. These genotypes were selected based on their high regeneration potential (figure 3.3). Scutellum cells of pre-cultured IZEs were bombarded with DNA coated tungsten particles, accelerated by PIG. Successful transformation of the scutellum cells of cultured IZEs of M37W was obtained using helium pressures of 700 to 900 KPa (O'Kennedy et al., 2001). Using these pressures as guidelines, helium pressures used in this study ranged from 800 – 1100 KPa.

Putative transformants were selected on bialaphos containing media (for details on how bar confers resistance to bialaphos refer to section 2.5.4.2). However, to determine the bialaphos concentration to be used for selection, a CPR assay (Kramer et al., 1993) was initially performed. A 1 mg l^{-1} concentration of CPR gave the tissue culture medium (pH 5.8) an orange colour. Figure 3.5A clearly shows a red medium colour change for the column containing untransformed M37W leaf pieces in the presence of 3 mg l^{-1} bialaphos, and a yellow colour change in the column without bialaphos. Chlorophenol red is a pH indicator – at pH 6.0 and higher, the solution colour is red, whereas at lower pH, the colour is yellow. Untransformed tissue (no bar gene) when cultured on media without bialaphos (left column in Figure 3.5A) was able to survive and metabolize the nutrients in the media. This metabolism produced acids that caused the medium pH to drop, and resulted in a yellow medium colour change. However, untransformed tissues when cultured in the presence of the selective agent (bialaphos) (right column in Figure 3.5A) were unable to cleave bialaphos. Therefore, necrosis occurred in these leaf tissues resulting in ammonia accumulation (increasing the pH), which in turn caused a red-medium colour change. This assay clearly indicated that untransformed tissues undergo necrosis at 3 mg l^{-1} bialaphos concentrations. Therefore, for selection of transformed tissues, a bialaphos concentration of 3 mg l^{-1} or higher was used.

Bombarded IZEs were initially selected on 3 mg l^{-1} bialaphos (4 weeks) and this was then increased to 5 mg l^{-1} to eliminate possible escapes (non-transgenic tissues that survived selective agent treatment). This selection pressure was decreased to 1mg l^{-1} during regeneration as rooting is particularly sensitive to bialaphos (Vain, 1993).

The frequency of the surviving putatively transgenic type-I calli varied significantly among the experiments (Table 3.1). This is due to the fact that not all of the embryogenic type-I calli matured to form regenerants. Putatively transgenic events were thus identified as successfully regenerating (rooting and shooting) plantlets rather than surviving calli events.

Leaf segments of all putative T_0 transgenics (rooted and shooted plantlets) were subjected to the CPR assay (Figure 3.5B) with a selection pressure of 3 mg l^{-1} bialaphos. Numerous transgenics displayed a yellow medium colour change, indicating the integration and functionality of the bar gene in its genome. However, there were also many wells that had a red medium colour change. These plants were either escape plants, or plants that were low expressors of the bar gene. All positive plants were confirmed with Basta® painting and PCR results (Table 3.3). Basta® paint positive plants demonstrated the functionality of the bar gene, (expression of PAT enzyme), by their resistance to the typical necrosis associated with localised applications of the herbicide Basta® to the maize leaves. This PAT enzyme detoxifies the herbicide as outlined in section 2.5.4.2. Untransformed plants developed necrotic lesions as expected, as they do not harbour the bar gene and thus, were susceptible to the Basta® herbicide application.

The T_0 putative transgenics displayed abnormal phenotypic characteristic, which included dwarfism and underdeveloped tassels and ears. These characteristics can be attributed to tissue culture induced stress since the T_1 and T_2 progeny were tall and fertile plants, resembling control plants that were germinated directly from seeds.

Molecular Analysis
EcoRI restriction enzyme used for digestion of genomic DNA, has two restriction sites on pSC1b32 (figure 3.1A), and thus cuts twice within this plasmid (but not within the b32 probe itself). Therefore for the 2 and 10 copy positive controls of plasmid DNA, the number of fragments hybridising to the b32 probe is expected to be two, which occurred.

The copy number estimation was determined by comparing the hybridising intensities of the resulting fragments of the putative transgenics (lanes 3 to 6 in figure 3.9), to the untransformed spiked samples with known number of copies (lanes 2 and 7).

The *b32* gene is an endosperm specific gene isolated from maize itself. However no hybridising bands occurred with the non-spiked untransformed M37W genomic DNA (lane 2 of Figure 3.9). The *b32* probe used for hybridisation is composed of the entire CAMV35S promoter sequence and 300 bp of the *b32* gene. Thus, this probe might have shown a stronger binding affinity to specific DNA fragments containing the 571bp of CAMV35S sequence (non-existent in untransformed maize for control of the *b32* gene) and 300 bp of the *b32* gene, as opposed to only 300 bp of the *b32* gene in untransformed maize. Therefore, no hybridisation of genomic DNA with the *b32* gene (1043 bp) in untransformed maize occurred in lane 1 (figure 3.9).

Eco RI digestion of the pSC1b32 plasmid is expected to yield two fragments of 1456bp and 6096 bp (calculated from the restriction sites on figure 3.1A). The 6096 bp fragment contains the CAMV35S promoter and the *b32* gene, and is therefore expected to hybridise with the *b32* probe (which contains 571 bp of CAMV35S and 300 bp of *b32* gene). Digestion with *Eco* RI (2 and 10 copy spikes) however yields two fragments instead of only one 6096 bp fragment (figure 3.9). Lanes 2 and 7 (figures 3.9 and 3.10) were both spiked with 2 and 10 copies (respectively) of each of the pSC1b32 and pGEMpgip2 plasmids. Therefore, the 2100 bp fragment produced in lanes 2 and 7 (figure 3.9) can be that of the pGEMpgip2 *Eco* RI digested fragment, as the *b32* probe recognised the 571bp of CAMV35S (also the promoter for *pgip2*) of the digested *pgip2* fragment. This is further substantiated by the fact that a similar 2100 bp fragment occurred when hybridised with the *pgip2* probe only in figure 3.10.

The *b32*, *pgip2* and *bar* gene presence were confirmed by PCR analysis before southern blot analysis was performed. BBE 13.2.3A (PCR positive for the *b32* and *bar* genes) had two bands detected via southern blotting (lane 3 of figure 3.9). The top band (approximately 7400bp) and the bottom band (approximately 6200bp) are representative of the two separate integration events. The copy number for this event can be estimated to be 2 copies.

The *b32* PCR for events BBE 16.1.1A and BBE 16.1.1B was positive (lane 6-7 of figure 3.7). These results were confirmed by southern blot analysis (figure 3.9).

Progeny BBE 16.1.1A and BBE 16.1.1B (lanes 4-5) display single bands of 3400bp in size, with very little or no undigested genomic DNA hybridisation. A low copy number integration (1-2 copies) of the *b32* gene occurs in the abovementioned elite transgenic maize events.

Southern blot analysis (figure 3.9) further demonstrated that BBE 13.2.3 and BBE 16.1.1 are two independent transformation events, due to the presence of unique banding patterns. It also demonstrates that the *b32* gene has been stably integrated into the next generation (T_2) as the integration patterns for BBE 16.1.1A1 (lane 6), is identical to that of the T_1 parent plant.

Pgip2 southern blot analysis (figure 3.10) showed no hybridising bands produced for events tested, indicating that *pgip2* was not successfully integrated into the genome of these plants. The *pgip2* gene (isolated from *P. vulgaris* (bean plants)) does not occur in untransformed maize plants, which explains why no hybridising bands occurred with untransformed M37W genomic DNA (lane 1 of figure 3.10). *Eco* RI once again has two restriction sites on the pGEMpgip2 plasmid, thus resulting in two fragments being produced: one containing the *pgip2* gene (~ 1994 bp) and the backbone fragment (~ 2997 bp). Hybridisation using the *pgip2* probe is therefore expected to bind only to fragment containing the *pgip2* gene only. This explains the presence of only one hybridising band (2100 bp) for the 2 and 10 copy controls (lane 2 and 7) in figure 3.10.

Thus, two events (BBE 13.2.3 and BBE 16.1.1) out of a total of 1211 bombarded IZEs were fertile and transgenic, resulting in a low transformation efficiency of 0.17 %. Many events were produced in the initial T_0 phase, however most of these putative transgenics did not survive and those that did, were not fertile. Therefore, one of the basic suggestions to improve this transformation efficiency would be to decrease the tissue culture selection period. This in turn is suggested to improve fertility of the putative transgenics, and could thus, improve the overall transformation efficiency. Another suggestion would be to use Agrobacterium-mediated transformation instead of biolistics, as this has been reported to have higher transformation efficiencies developed for only laboratory strains to date (Ishida *et al.*, 1996).

3.6 CONCLUSION

Three elite white maize genotypes: A483-1, A483-4 and M37W (in order of regeneration ability), were initially identified as being highly regenerable on $N6_{10}$ as the callus induction media. Transformation of these elite white maize genotypes (A483-1 and A483-4) was achieved with the antifungal *b32*, and selectable marker *bar*, genes, resulting in a relatively low transformation efficiency of 0.17%. Stable and low copy integration (1-2 copies) of the *b32* gene occurred in the two transformation events (BBE 13.2.3 and BBE 16.1.1). Event BBE 16.1.1A1 (T_2 plant) of C IMMYT l ine A483-4, was southern blot positive for only t he *b 32* gene, a nd T_3 seeds of this event was produced.

CHAPTER FOUR:

BIOLISTIC TRANSFORMATION AND MOLECULAR ANAYLSIS OF THE Hi-II MAIZE GENOTYPE WITH THE MAIZE *b32* AND BEAN *pgip2* ANTIFUNGAL GENES

4.1   ABSTRACT

This chapter reports on the stable transformation of the maize laboratory strain, Hi-II (A188 x B73) callus tissue with the *b32* and *pgip2* antifungal transgenes (*bar* used as the selectable marker gene).   Bombarded Hi-II calli tissues were selected on $N6_{10}$ callus induction media supplemented with the selective agent, bialaphos.   Forty-five to ninety percent of bombarded calli proliferated, forming embryogenic Type-II calli. Positive transformants were screened using the chlorophenol red assay (CPR), herbicide (Basta®) painting test, PCR, Southern and northern blotting techniques. Only eight events (out of 76 independent T_0 events identified) were fertile.   PCR analysis of these events showed four events (A, B, G, H) positive for the *b32*, *pgip2* and *bar* genes, while event F tested positive for the *b32* and *bar* genes only.   Three further events were positive for the *bar* gene only (C, D and E). T_1 and T_2 progeny of event B tested *b32* PCR positive only and were herbicide sensitive.   Selected T_1 and T_2 progeny of event F were *b32* and *bar* PCR positive, and herbicide resistant. Further analysis of selected T_3 progeny of event A resulted in six T_3 progeny being southern blot positive for the *pgip2* and *b32* transgenes (five of these plants were northern blot positive for both mRNA transcripts).   A further 42 transgenic T_3 progeny (among events F and H) tested PCR positive for the *b32* and *bar* genes, and southern blot analysis for the *b32* gene was confirmed for all plants.   Northern blot expression studies for these plants displayed expression of the *b32* mRNA transcript in 38 of these transgenics.   Fungal infection studies resulted in a significant (up to 50%) reduction in *Fusarium verticillioides* (MRC826) infection of the transgenic seed surface when compared to untransformed Hi-II seeds.

4.2 INTRODUCTION

Plant transformation is often performed to supplement conventional plant breeding, thereby contributing to disease or insect resistance or improved nutritional quality. The most commonly reported fungal species infecting maize is *Fusarium verticillioides* (previously known as *F. moniliforme*) (Nelson, 1992; Nelson *et al.*, 1993). Thus, the main aim of this study was to engineer resistance to this fungus by introducing the antifungal *b32* and *pgip2* transgenes into maize genotypes.

This chapter examines the particle bombardment of these antifungal transgenes into the laboratory stain of maize, Hi-II (A188 x B73). Transformation of Hi-II immature zygotic embryos with the maize *b32* gene (constitutively expressed) was recently performed (Lanzanova *et al.* 2003). Embryogenic type-II callus tissue however, was used as the explant for bombardment experiments in this study. Hi-II callus tissues have been previously successfully transformed with the *uid*A reporter gene and the *bar* selectable marker gene (O'Kennedy *et al.*, 1998). However, no recent studies on the transformation of Hi-II callus with antifungal genes have been reported.

The antifungal genes chosen were the maize *b32* and bean *pgip2* genes. The *b32* encodes for a ribosome inhibiting protein (RIP), normally expressed in the endosperm tissues (Soave *et al.*, 1981). The bean *pgip2* gene, isolated from *Phaseolus vulgaris*, codes for a polygalacturonase inhibiting protein (PGIP), which recognizes fungal polygalacturonases and activates the plant defense response (De Lorenzo *et al.*, 1994). These antifungal genes are driven by the CAMV-35S promoter and will be transformed onto callus tissue for constitutive expression in the plant. The *bar* selectable marker gene is used for selection of transformed tissues. This gene confers resistance to bialaphos (explained in section 2.5.4.2), which is added to the tissue culture media. The $N6_{10}$ based tissue culture media (composition outlined in table 2.3) is used.

Fungal infection studies (according to the method outlined by Balconi *et al.*, 2003) will be performed on the transgenic seeds. Analysis of seed germination and the seed surface area infected will be relevant in concluding resistance for *Fusarium verticillioides* infection.

4.3 MATERIALS AND METHODS

4.3.1 Excision and culturing of IZEs for Hi-II callus production

Immature zygotic embryos of Hi-II (1.2 - 1.5 mm long) were aseptically excised from immature kernels (10-14 days post pollination) as described in Section 3.3.1. Kernels were obtained from greenhouse grown maize. The excised IZEs were then placed on $N6_{10}$ callus induction medium with the axis in contact with the medium. Cultures were incubated at 25°C under low light conditions (1.8 μmol m^{-2}s^{-1}). Embryogenic Hi-II calli were selected and subcultured to fresh media every 14 days. Calli that were 2 - 4months old were used as explants for bombardment experiments.

4.3.2 Plasmid preparation

The pSC1b32 (figure 3.1A) and pGEMpgip2 (figure 3.1B) plasmids, prepared as outlined in Section 3.3.3, were used for bombardment as explained below.

4.3.3 Microprojectile Bombardment

Microprojectile bombardment was performed as outlined in Section 3.3.4, with exceptions: embryogenic type-II calli (initiated as described in 4.3.1) were used as explants instead of IZEs; and the helium pressure used was 600 KPa for all experiments. All other parameters remained as explained in section 3.3.4. The pSC1b32 and pGEMpgip2 plasmids were co-bombarded (0.8μg per plasmid DNA per bombardment mix). All experiments were conducted using the PIG under sterile conditions at room temperature.

4.3.4 Selection and regeneration of transgenics

The bombarded Hi-II calli were transferred to osmoticum-free $N6_{10}$ selection media ($N6_{10}$ callus induction media supplemented with 3 mg l^{-1} bialaphos) after 16 hours. The herbicidal formulation Herbiace® (Meiji Seika Kaisha Ltd., Japan) containing bialaphos was added to the media. Bialaphos content was increased from 3 mg l^{-1} to 5 mg l^{-1} after 4 weeks, with each transfer to fresh selection media occurring every 2 weeks. Six to ten weeks after bombardment, bialaphos-resistant Hi-II calli were transferred to maturation (OT6S) media containing 3 mg l^{-1} bialaphos for 2 weeks. Callus induction and maturation occurred under low light conditions (1.8 μmol m^{-2}s^{-1}). OT2S medium containing 1 mg l^{-1} bialaphos was used for regeneration of putative transformants under a 16/8h (day/night) photoperiod with a 70-80 μmol m^{-2}s^{-1} light intensity. Developing plantlets were subcultured at 2-3 week intervals, until they developed shoots of greater than 3 cm and roots greater than 1cm, before being hardened off (as described by O'Kennedy *et al.*, 1998).

4.3.5 Chlorophenol Red Assay (CPR)

This assay was performed as outlined in Section 3.3.6.

Table 4.1   Conditions for bombardment of Hi-II calli with the pSC1b32 and pGEMpgip2 plasmids

Exp #[a]	# plates of Hi-II calli BB	Age of Hi-II calli (months)
BBK 1	6	5 months
BBK 2	6	5 months
BBK 3	6	6 months
BBK 4	5	7 months
BBK 5	6	2 months
BBK 6	4	7 months
BBK 7	13	2½ months
BBK 8	7	3 months
BBK 9	4	8 months
BBK 10	7	3½ months
BBK 11	5	4 months
BBK 12	5	4 months
BBK 13	5	5 months
BBK 14	5	5 months
BBK 15	3	6 months
BBK 16	2	7 months
BBK 17	2	2½ months
BBK 18	2	3 months
BBK 19	3	3½ months
BBK 20	4	4 months
BBK 21	2	4½ months
BBK 22	4	5½ months

a = Experiment number of bombarded Hi-II calli on $N6_{10}$ media

4.3.6 Herbicide Resistance / Basta® painting

Herbicide resistance tests were performed as outlined in Section 3.3.7. The ends of painted leaves were cut in a small "V" shape for easier identification of the painted leaf. Distilled water (instead of 2% Basta®) was used in the application mix, for painting of selected control plants. Tissue necrosis of the leaf surface was observed within 5 days of painting, and painted leaves were clearly recorded as being herbicide resistant (R) or herbicide sensitive (S).

4.3.7 Germination and Pollination of Transgenic progeny

Transgenic seeds were germinated on ½MS media (1 mg l^{-1} bialaphos) and incubated at 25°C under fluorescent lights (70-80 μmol m^{-2}s^{-1}).

T_3 seeds were however, planted directly into soil under glasshouse conditions (Agricultural Research Council – Grain Crops Institute (ARC-GCI), Potchefstroom, South Africa). All putative transgenics were either self-pollinated within the same event, or cross-pollinated with untransformed M37W plants.

4.3.8 DNA Extraction

Genomic DNA was extracted from putative transgenic maize leaf tissue using the chloroform/isoamylalcohol (CI) method outlined below: 0.1g of tissue was crushed to a fine powder using liquid nitrogen. 800μl of extraction buffer (3% CTAB, 1.4M NaCl, 0.02M EDTA, 1M Tris-Cl pH8.0 and 1% mercaptoethanol) was added to the tissue, vortexed for 1 minute and samples were incubated at 65°C for 1 hour. 800μl of chloroform:isoamylalcohol (24:1) was then added. Samples were vortexed and centrifuged at 12500rpm for 15 minutes. The aqueous supernatant was transferred into a clean eppendorf, and 0.7 volumes of isopropanol was added and incubated overnight at 4°C. Samples were centrifuged at 12500rpm for 20 minutes. The DNA pellet was washed with 70% ethanol and centrifuged at 12500rpm for 15 minutes. The DNA pellet was air dried and dissolved in 25μl TE buffer (pH 8.0) with 20 mg l^{-1} RNAse A.

4.3.9 Polymerase Chain Reaction (PCR) Analysis

The PCR reagents, primers and PCR protocol used for the *b32*, *pgip2*, *bar* and *nad* gene amplifications are described in Section 3.3.9.

4.3.10 Preparation of Probes and Southern Blotting

The protocol for probe preparation and southern blot detection of the transgenes of interest, were performed as outlined in Section 3.3.10.

4.3.11 RNA Extraction

Total RNA was extracted from leaves from transgenic T_3 plants (grown under glasshouse conditions at the ARC-GCI, Potchefstroom) using Tri-Reagent (Sigma) according to the supplier's protocol.

4.3.12 Northern Blotting

Total RNA (10μg per lane) was size fractionated by electrophoresis on a 1% (w/v) formaldehyde agarose gel and transferred onto Hybond-N nylon membranes. These membranes were prehybridised in DIG Easy Hyb solution (Roche Molecular Biochemicals) at 42°C for 3 hours.

Membranes were then hybridised in the same solution with the addition of PCR DIG-labelled *pgip2*, or the *b32* probes (probe preparation outlined in Section 3.3.10). Washing and chemiluminescent detection of signals (using autoradiography) was performed similar to the southern blotting protocol outlined by O'Kennedy *et al.* (1998).

4.1.13 Fungal Infection Studies

Plant Material. Untransformed Hi-II maize and T_4 transgenic seeds (grown at the ARC-GCI Glasshouse trials at Potchefstroom, South Africa) were used. They were soaked in 70% (v/v) ethanol for one minute and sterilised for 20 minutes in a 2.5% (v/v) sodium hypochlorite solution, containing 0.1% (v/v) of the surfactant Tween 20 (Merck Chemicals), before being rinsed thoroughly with sterile distilled water.

Culture Media. Potato Dextrose Agar (PDA, from Sigma) was used. For positive controls, Saporin (a plant RIP from *Saponaria officinalis* seeds, Sigma S-9896) and Nystatin (a fungicidin, Mycostatin, Sigma N-3503) was added to the PDA tissue culture medium. Concentrations of 35 μg ml^{-1} saporin and 20 μg ml^{-1} nystatin were used.

Fungal Spore calculation. *Fusarium verticillioides* (MRC 826) was cultured on ½ PDA and incubated for a week at 25°C until the mycelium covered the surface of the plate. Two millilitres of distilled water was added to the fungal plate and gently shaken to form a water-spore inoculum solution. Serial dilutions were made, and the spore count per μl was determined using a haemocytometer.

In vitro infection of seeds. Conidial suspension of the fungus (125 spores in 10μl inoculum) was pipetted onto a spot on the PDA plate, with the sterile seeds plated over it. Four seeds were plated per tissue culture (PDA) plate (as explained above). All experiments were conducted in triplicate samples under sterile conditions. Plates were incubated at room temperature for 4 - 7 days. Analysis occurred according to visible fungal colonization of the seeds and a relevant infection score was allocated. Infection score was allocated according to the descriptions below:

1	=	5 – 10% infected seed surface
2	=	10 – 30% infected seed surface
3	=	30 – 50% infected seed surface
4	=	50 – 75% infected seed surface
5	=	75 – 100% infected seed surface
6	=	100% heavily infected seed surface

4.4   RESULTS

4.4.1   Selection and regeneration of transgenics

A total of 106 plates of Hi-II calli (among 22 independent bombardment experiments), were bombarded with the respective selectable marker (*bar*) and antifungal (*b32* and *pgip2*) genes (Table 4.1).   Putatively transformed Hi-II calli were identified by its vigorous proliferation on bialaphos-containing medium producing more Type-II calli, while non-transformed tissues turned brown and watery (Figure 4.1A).   Bialaphos-resistant calli, when subcultured onto OT6S maturation media (3 mg l^{-1} bialaphos), matured to form somatic embryos.   These somatic embryos produced shoots and roots (Figure 4.1B) when transferred to OT2S regeneration medium with a lower 1 mg l^{-1} bialaphos concentration.
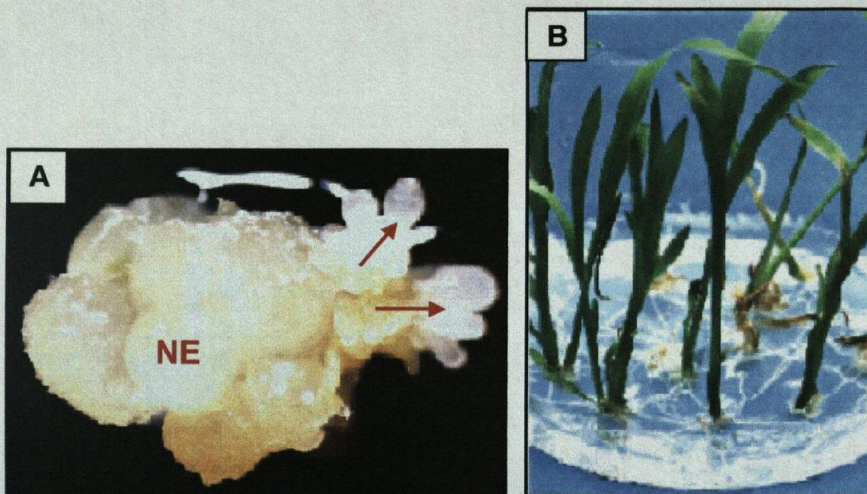
Fig 4.1 A) Putative Hi-II transformants identified as proliferating sectors of callus tissue (red arrows) among a background of non-proliferating, non-embryogenic cells (NE) on $N6_{10}$ callus induction media with 3 mg l^{-1} bialaphos selection; **B)** Putatively transgenic Hi-II plantlets displaying healthy shoot, and extensive root development on OT2S regeneration media supplemented with 1 mg l^{-1} bialaphos.

The percentage of proliferating calli on selection media ranged from 45 - 90% among the experiments (Table 4.2).   A total of 92 independent T_0 events were identified (106 plates bombarded).   Only plantlets producing shoots greater than 3 cm and roots greater than 1 cm were counted. These putative transgenics were subjected to a preliminary CPR assay before being hardened off under greenhouse conditions.

Table 4.2   Percentage of proliferating Hi-II calli and number of events identified for each of the independent bombardment experiments conducted.

Exp #[a]	% calli surviving [b]	# events identified [c]	Pollination
BBK 1	80%	15	M37W
BBK 2	80%	9	M37W
BBK 3	70%	4	M37W
BBK 4	90%	5	M37W
BBK 5	90%	12	M37W
BBK 6	85%	8	M37W
BBK 7	95%	3	M37W
BBK 8	90%	3	M37W
BBK 9	85%	0	M37W
BBK 10	80%	4	M37W
BBK 11	70%	5	M37W
BBK 12	78%	7	M37W
BBK 13	82%	3	M37W
BBK 14	90%	2	M37W
BBK 15	75%	0	M37W
BBK 16	60%	0	M37W
BBK 17	45%	2	M37W
BBK 18	48%	3	M37W
BBK 19	73%	1	M37W
BBK 20	75%	0	M37W
BBK 21	80%	2	M37W
BBK 22	71 %	4	M37W

a = Experiment number of bombarded Hi-II calli on $N6_{10}$ media
b = Percentage of bombarded Hi-II calli proliferating to form Type-II calli after 4 weeks on $N6_{10}$ selection media (3 mg l^{-1} bialaphos)
c = Number of rooted and shooted plantlets produced on OT2S-regeneration media with 1 mg l^{-1} bialaphos selection

4.4.2   Chlorophenol Red (CPR) Assay

In figure 4.2A, non-transformed Hi-II calli displayed a yellow medium colour change in wells containing 0 mg l^{-1} bialaphos, indicating survival of Hi-II calli tissue (positive control).   Non-transformed calli in wells containing bialaphos concentrations of less than 1 mg l^{-1} displayed an orange-to-red medium colour change. However, on closer examination of the callus tissue pieces, clear calli proliferation occurred.   Wells containing bialaphos at concentrations above 1 mg l^{-1} displayed an intensified red medium colour change, and closer examination of Hi-II calli showed minimal growth and tissue browning (necrosis). This indicated that non-transformed Hi-II calli did not survive at bialaphos concentrations of greater than 1 mg l^{-1}.

For selection of transformed tissues, a concentration of 3 mg l^{-1} bialaphos was therefore chosen to eliminate all potential escape tissues. Leaf pieces of putative T_0 transgenics were tested (figure 4.2B).

In lane 1 (figure 4.2B) untransformed Hi-II leaf segments (lacking *bar* gene) displayed a red medium colour change (3 mg l⁻¹ bialaphos) indicative of tissue necrosis (negative control). However, leaves of putative transgenics (lanes 2-5) displayed a distinct yellow medium colour change, indicating survival of leaf tissues at 3 mg l⁻¹ bialaphos concentration. All putative Hi-II transgenics tested were positive (yellow coloured wells). No negative escape plants (red coloured wells) were observed. All CPR results were confirmed by PCR positive testing for the presence of the *bar* gene (section 4.4.3).
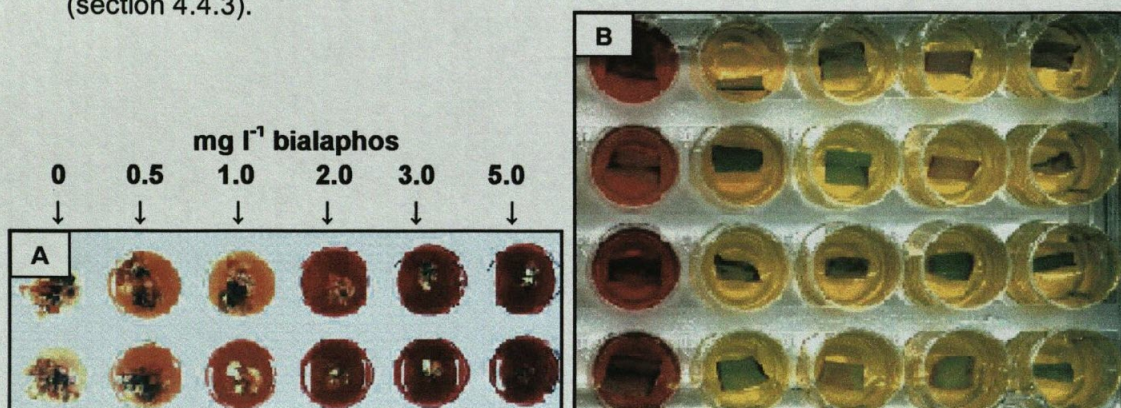
Fig 4.2A) Chlorophenol Red (CPR) assay of non-transgenic Hi-II callus using N6₁₀ callus induction medium with different concentrations of bialaphos (one month in culture). **B)** CPR assay of leaf pieces after 2 weeks in culture: non-transformed leaf pieces occur in column 1 displaying a red medium colour change, while columns 2-5 contain leaf pieces of different regenerating putatively transgenic Hi-II plantlets, displaying yellow medium colour change.

4.4.3   Analysis of the T_0 transgenic plants

Of the 92 independent T_0 putative transgenics hardened off, only 76 survived under greenhouse conditions. All surviving T_0 putative transgenics displayed stunted growth and abnormal ear production (Figure 4.3A) with all being CPR positive and Basta® paint resistant (data not shown). Only eight of these 76 surviving T_0 putative transgenic events were fertile to produce T_1 seed when pollinated (events listed in Table 4.3). For easier reference, a code allocated to the individual transformation events listed in table 4.3, will be quoted in the rest of this chapter. For example, transformation event BBK 1.3v will be referred to as event A, with its T_1 progeny named as A1 etc., T_2 progeny as A1A etc., and T_3 progeny as A1A1 etc.

All 8 events were CPR positive and Basta® paint resistant, which correlated with positive PCR testing for the *bar* gene in all events (Table 4.3). PCR results for all three genes in events A - H are listed: four independent events (A, B, G and H) were positive for all three genes (*b32*, *pgip2* and *bar*), three other events (C, D and E) were PCR positive for *bar* only, while event F tested positive for *b32* and *bar* genes.

Table 4.3   Data of T_0 fertile events producing T_1 seeds (all plants were pollinated with untransformed M37W)

Transformation Event	Code [a]	CPR Result	Basta® painting Result	T_0 PCR Results b32	pgip2	bar	# T_1 seeds produced	# T_1 seeds germinating on ½ MS
BBK 1.3v	A	+	R	+	+	+	4	4
BBK 2.4k	B	+	R	+	+	+	4	4
BBK 2.5d	C	+	R	-	-	+	8	6
BBK 2.5f	D	+	R	-	-	+	19	16
BBK 2.5o	E	+	R	-	-	+	6	6
BBK 2.5a/4.2a	F	+	R	+	-	+	7	7
BBK 2.6m	G	+	R	+	+	+	4	4
BBK 4.2a	H	+	R	+	+	+	104	12 [b]

a   =   A code was allocated to each event to ensure simplicity in future references to their progeny
b   =   Only fifteen T_1 seeds were randomly selected and germinated on ½ MS media
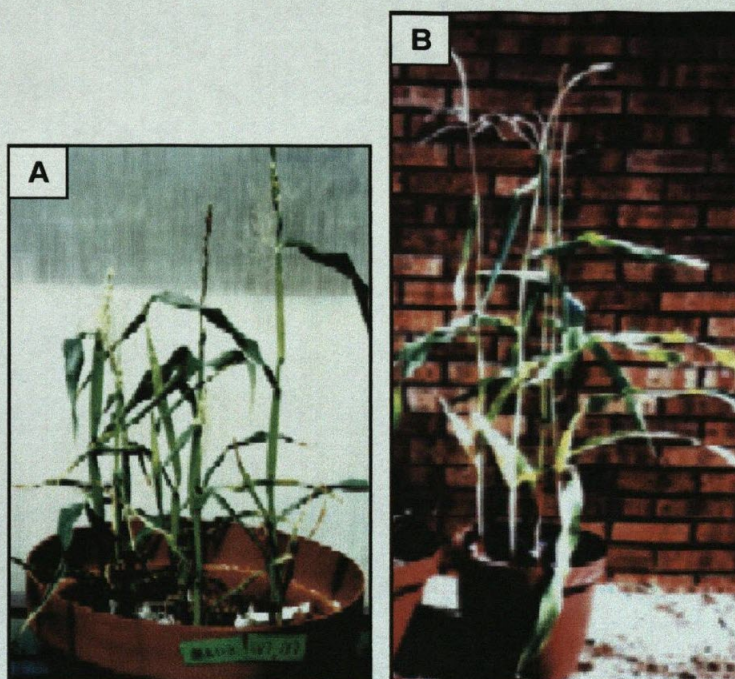
Fig 4.3 A) Putative T_0 Hi-II transgenic plants displaying dwarfism and abnormal ear and tassel formation; and **B)** T_1 putative Hi-II transgenic plants (cross pollinated with untransformed M37W), displaying normal, tall maize phenotypic growth under greenhouse conditions.

4.4.4 Analysis of the T_1 transgenic plants

T_1 seeds of the 8 events (Table 4.3) were germinated on ½ MS media containing 1 mg l^{-1} bialaphos. Extensive root (greater than 2 cm in length) and shoot (3-4cm in height) development occurred (Figure 4.4.). These T_1 plants displayed tall phenotypes with fertile male and female parts (figure 4.3B).

Fig 4.4 Putative T_1 Hi-II transgenic seeds germinating on ½ MS media supplemented with 1 mg l^{-1} bialaphos. Root development (longer than 2 cm in length) and shoot development (3-4 cm in height) were observed for all seeds that germinated.

Basta® painting of leaves was performed as outlined in Section 3.3.7. Non-transformed plant leaves were herbicide sensitive (figure 4.5B), while different putative transgenics displayed varied responses: some herbicide resistant (figure 4.5D) and others, herbicide sensitive (figure 4.5C).
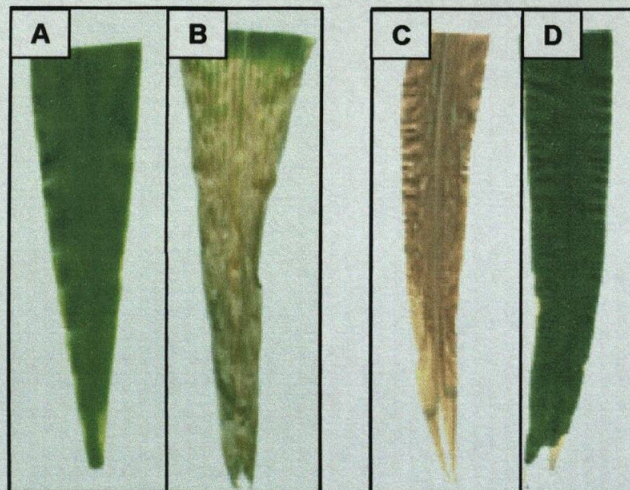
Fig 4.5 Basta® painting results of leaves obtained 7 days after painting. No necrosis observed when untransformed Hi-II (control) leaf (A) was painted with distilled water and 0.01% Tween 20. Necrotic lesions however, were evident on control leaf (B) painted with 2% Basta® herbicide and 0.01% Tween 20 (leaf eventually died after 10 days). Putative Hi-II T_1 transgenic leaves, similarly painted with 2% Basta®, display herbicide resistance (Basta® painting positive) on leaf D, whereas Basta® sensitivity occurs on leaf C (Basta® paint negative).

Basta® painting and CPR screening results of T_1 progeny (for the eight transgenic events) are listed in Table 4.4. Seventeen plants (among four events: A, F, G and H) out of a total of 46 surviving T_1 progeny, were CPR assay positive and Basta® paint resistant. However, the results of only 15 of these T_1 progeny transgenics correlated with the *bar* PCR results.

The other 2 events: event C3 (CPR assay and Basta® paint sensitive) was PCR positive for the *bar* gene, while event H2 (CPR assay and Basta® paint resistant) was contrarily negative for *bar*. PCR results for the respective genes of selected T$_1$ progeny plants are presented in figure 4.6.
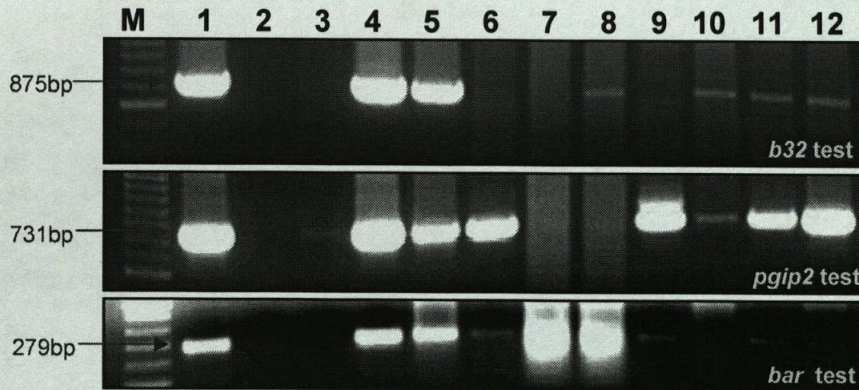
Fig 4.6 PCR of genomic DNA of putatively transgenic T$_1$ plants for *b32*, *pgip2* and *bar* genes. Lane M represents the DNA molecular weight marker (100bp ladder). DNA templates for PCR reactions were as follows: Lane 1: positive control of the respective plasmid DNA (pSC1b32 for *b32* and *bar*; and pGEMpgip2 for *pgip2* PCRs); Lane 2: PCR mix without DNA; Lane 3: 1ng untransformed Hi-II genomic DNA (negative control); Lane 4: 1ng untransformed Hi-II genomic DNA spiked with 1ng of the respective plasmid DNA; Lanes 5 to 12 represent genomic DNA (50ng) of the T$_1$ putative progeny transgenics: lane 5: A1, lane 6: B1, lane 7: C3, lane 8: E9, lane 9: G1, lane 10: H3, lane 11: H7 and lane 12: H8.

The PCR (Figure 4.6) shows the correct amplified band sizes for all three genes present for plants A1, H3, H7 and H8. Plants B1 and G1 produced amplified DNA bands for *pgip2* and *bar* genes only; E9 produced bands for *b32* and *bar*, while C3 produced a DNA band for the *bar* gene only. The positive controls containing plasmid DNA (lanes 1 and 4) produced the expected amplified products of 875 bp, 731 bp and 279 bp for the *b32*, *pgip2* and *bar* genes respectively. The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA) did not produce any amplified products. PCR results for all T$_1$ plants tested are presented in Table 4.4.

PCR of the *nad* gene (data not included) was performed for all T$_1$ putative transgenics tested in figure 4.6. The *nad* gene is a conserved multicopy gene of the mitochondria (Mannerlof & Tenning, 1997), and thus occurs in all plants. Positive amplified DNA bands for this gene was produced for all plants including that of untransformed Hi-II DNA tested. The negative control did not produce any amplified DNA bands.

Table 4.4 Summary of results obtained for the T_1 progeny of putative transgenics

Transformation Event Code	T_1 progeny [a]	CPR Result	Basta® painting Result	PCR Results			Pollination	# T_2 seeds produced
				b32	pgip2	bar		
A	1	+	R	+	+	+	Self	9
	2	+	R	+	+	+	Self	7
	3	-	S	-	-	-	Self	7
B	1	-	S	+	-	-	Self	5
	3	-	S	+	-	-	Self	92
	10	-	S	+	-	-	Self	88
C	2	-	S	-	-	-	Self	70
	3	-	S	-	-	+	Self	117
	5	-	S	-	-	-	Self	0
	6	-	S	-	-	-	Self	196
	7	-	S	-	-	-	Self	0
D	1	-	S	+	-	-	Self	0
	3	-	S	-	-	-	Self	0
	5	-	S	-	-	-	Self	55
	9	-	S	-	-	-	Self	32
	10	-	S	-	-	-	Self	46
	11	-	S	-	-	-	Self	131
	12	-	S	-	-	-	Self	150
	13	-	S	-	-	-	Self	37
	14	-	S	-	-	-	Self	24
E	5	-	S	-	-	-	Self	56
	7	-	S	-	-	-	Self	48
	9	-	S	+	-	-	Self	116
	10	-	S	-	-	-	Self	69
	11	-	S	+	-	-	Self	0
F	1	+	R	+	-	+	M37W	35
	2	-	S	+	-	-	M37W	0
	3	-	S	-	-	-	M37W	29
	4	+	R	+	-	+	M37W	40
	6	-	S	-	-	-	M37W	0
	9	-	S	+	-	-	M37W	0
G	1	+	R	+	-	+	Self	51
	4	-	S	-	-	-	Self	146
H	1	+	R	+	+	+	M37W	0
	2	+	R	+	-	-	M37W	0
	3	+	R	+	+	+	M37W	70
	4	+	R	-	-	+	M37W	5
	5	+	R	-	-	+	M37W	0
	6	+	R	-	-	+	M37W	2
	7	+	R	+	+	+	M37W	55
	8	+	R	+	+	+	M37W	75
	9	+	R	+	-	+	M37W	0
	10	+	R	-	-	+	M37W	0
	11	+	R	+	-	+	M37W	0
	12	+	R	+	+	+	M37W	31

a = T_1 progeny listed are those that survived under greenhouse conditions. Some T_1 seeds (even though they germinated successfully on ½ MS media) did not survive when hardened off and placed under greenhouse conditions. For e.g. event F had 16 T_1 seeds that germinated, however only 6 T_1 plants survived (listed in table above).

This study is part of a larger European Union funded project: SAFEMAIZE (contract number: ICA4-CT-2000-30033). Transgenic plants produced in this study were multiplied for use in future field trial analysis (T_4 generation). Thus, since sufficient plant material w as a vailable, molecular a nalyses of T_2 and T_3 p rogeny of s elected events were performed. This additionally allowed the demonstration of transgene inheritance and stability in further generations.

4.4.5 Analysis of the T_2 transgenic plants

T_2 seeds of selected events (based on Basta® painting, PCR results and the number of T_2 seeds produced per event), were germinated on ½ MS media (1 mg I^{-1} bialaphos) and transferred directly into pots under greenhouse conditions. Some PCR negative events were also chosen to represent negative controls. T_2 plants displayed tall and fertile phenotypes. PCR results for the selected plants are summarized in Table 4.5.

Table 4.5 Summary of the T_2 progeny of putative transgenic events selected to continue to the T_3 generation (glasshouse trial conditions)

T_2 progeny Code	Basta® painting Result	T_2 PCR Results			Pollination
		b32	pgip2	bar	
A1E	R	+	+	+	Self
A2D	R	+	+	+	M37W
A2E	R	+	+	+	M37W
F1A	R	+	-	+	Self
F1F	R	+	-	+	Self
F1G	S	+	-	-	Self
F4A	S	+	-	-	Self
F4F	R	+	-	+	Self
G1H	R	+	-	+	M37W
H3C	R	+	-	+	Self
H7G	R	+	-	+	Self
H7H	R	+	-	+	Self
H8C	R	+	-	+	Self
H8E	R	+	-	+	Self
H8G	R	+	-	+	Self
H12I	R	+	-	+	M37W
H12J	R	+	-	+	M37W
A1B	S	-	-	-	Self
B1C	S	-	-	-	Self
C3D	S	-	-	-	Self
D9A	S	-	-	-	M37W
E9A	S	-	-	-	Self
G4B	S	-	-	-	Self
F4J	S	-	-	-	Self
H12C	S	-	-	-	Self
Untrans.Hi-II	S	-	-	-	Self
Untrans.A188	S	-	-	-	Self

4.4.6 Analysis of the T_3 transgenic plants

Selected T_3 seeds (based on Basta® painting, PCR results and the number of T_3 seeds produced per event) were planted directly into soil under glasshouse trial conditions (ARC-GCI, Potchefstroom, South Africa). Some PCR negative events were chosen to represent negative controls. Transgenic plants are collectively listed in Appendix 1. Some plants did not germinate (absence of growth in figure 4.7A). Others that germinated, displayed normal and healthy maize phenotypes, with some plants (arrow A) producing taller and greener phenotypes when compared to the untransformed M37W control plants (arrow B) in Figure 4.7B.
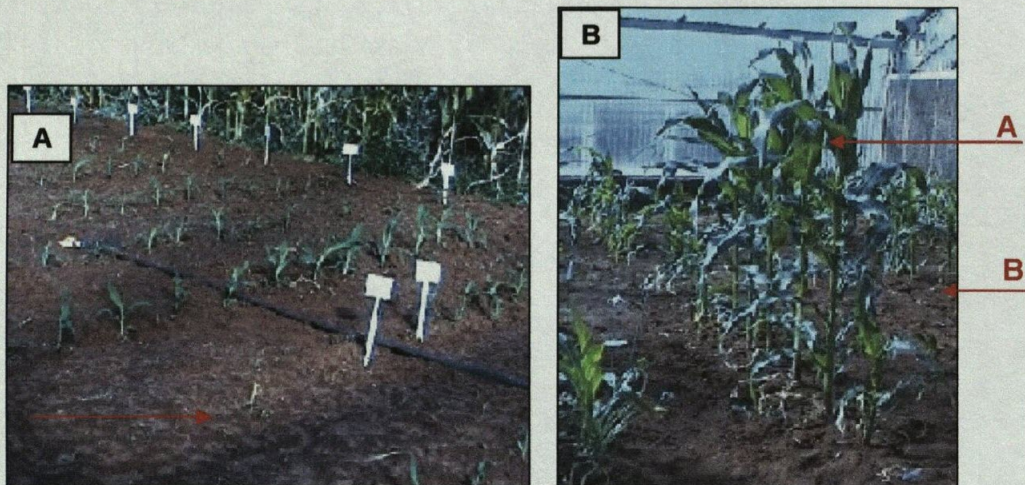
Fig 4.7 Transgenic T_3 seeds planted directly into soil and were under controlled glasshouse growth conditions at the ARC-Grain Crops Institute in Potchefstroom, South Africa. **A)** No germination and plant growth (arrow) occurred for event A2D. **B)** T_3 transgenic progeny (H7G) display healthy and tall maize phenotypes (arrow A) while untransformed M37W control plants displayed a phenotype that is shorter in height (arrow B).

Basta® painting (as outlined in Section 4.3.6) was performed on all transgenics. Only 60 plants (among 4 events) of the 181 T_3 transgenic progeny surviving under glasshouse trial conditions, tested Basta® paint positive, displaying herbicide resistance (data not included).

4.4.6.1 PCR analysis of T_3 transgenics

PCR results of selected events are shown in Figure 4.8: Five plants (A1E3, A1E5, A1E11, A2E3 and A2E5) were positive for all three genes producing amplified DNA bands of correct sizes (731 bp, 875 bp and 279 bp for the *b32*, *pgip2* and *bar* genes respectively).

Only two plants (H8E6 and H12J16) produced bands for the *b32* and *bar* genes, while two other plants (H8C1 and F1A15) produced bands for only the *bar* gene. Plant H12J9 (lane 13) did not produce any amplified bands for any of the genes. The negative controls: lane 2 (no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA) did not produce any amplification products. The positive controls (lanes 1 and 4), which contained plasmid DNA, produced the expected amplified bands. PCR results for selected T_3 progeny occur in Appendix 1: six plants (among one event, A) tested PCR positive for all three genes. A further 49 plants (among 3 independent events E, F and H) were PCR positive for the *b32* and *bar* genes, while only 2 plants (among one event, H) were only *b32* PCR positive.
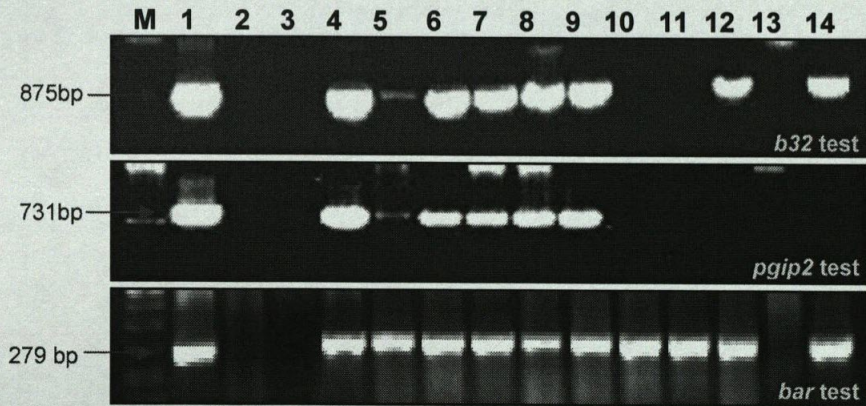
Fig 4.8 PCR of genomic DNA of putatively transgenic T_3 plants for *b32*, *pgip2* and *bar* genes. Lane M: DNA molecular weight marker (100bp ladder). DNA templates for PCR reactions were as follows: Lane 1: positive control of the respective plasmid DNA (pSC1b32 for *b32* and *bar* and pGEMpgip2 for *pgip2* PCRs); Lane 2: PCR mix without DNA; Lane 3: 1ng of untransformed Hi-II genomic DNA (negative control); Lane 4: 1 ng of untansformed Hi-II genomic DNA spiked with 1ng of the respective plasmid DNA; Lanes 5 to 14 represent genomic DNA (50ng) of T_3 putative transgenics: lane 5: A1E3, lane 6: A1E5, lane 7: A1E11, lane 8: A2E3, lane 9: A2E5, lane 10: H8C1, lane 11: F1A15, lane 12: H8E6, lane 13: H12J9, and lane 14: H12J16
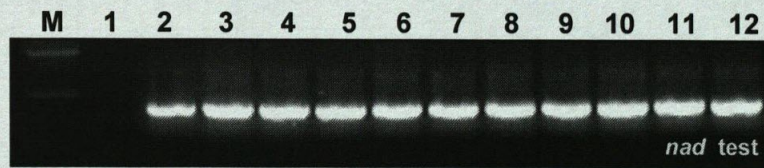
Fig 4.9 PCR of genomic DNA of putatively transgenic T_3 plants for the *nad* gene. Lane M: DNA molecular weight marker IV. DNA templates for PCR reactions were as follows: Lane 1: PCR mix without DNA (negative control); Lane 2: 1ng untransformed Hi-II genomic DNA; Lanes 3 to 12 represent genomic DNA (50ng) of T_1 putative transgenics: lane 3: A1E3, lane 4: A1E5, lane 5: A1E11, lane 6: A2E3, lane 7: A2E5, lane 8: H8C1, lane 9: F1A15, lane 10: H8E6, lane 11: H12J9 and lane 12: H12J16.

PCR amplification of the *nad* gene (figure 4.9) for the T_3 putative transgenics tested in figure 4.8, were positive for all plants tested (lanes 3 to 12) and untransformed Hi-II DNA (lane 2). The negative control (lane 1) did not produce any amplified DNA bands.

4.4.6.2 Southern Blot Analysis of T_3 transgenics

Selected PCR positive T_3 transgenics were tested by southern blot analysis for the introduced transgenes. A southern blot analysis (*Eco* RI digestion) for the *b32* gene presence is reported in figure 4.10. Genomic DNA loaded in lanes 4 and 10 were higher than the other lanes (data not shown).
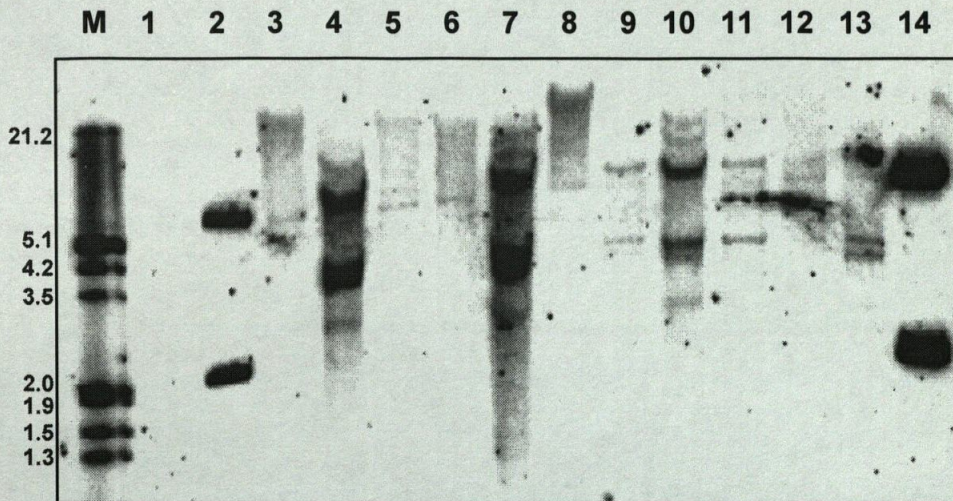
Fig 4.10 Southern blot analysis (*b32*) of T$_3$ transgenic Hi-II maize progeny. Five microgram of genomic DNA (purified from leaf tissues) for all samples, were restricted with the *Eco R*I restriction enzyme and resolved on a 0.8% agarose gel, transferred onto a nylon membrane and then probed with the *b32* probe outlined in Section 3.3.10. Lane M: DIG-labelled molecular weight marker III (lambda DNA digested with *Eco*RI and *Hind*III); Lane 1: genomic DNA of untransformed Hi-II; Lane 2: untransformed Hi-II genomic DNA spiked with 2 copies (20pg) each of the pSC1b32 and pGEMpgip2 plasmid DNAs and Lane 14: untransformed Hi-II genomic DNA spiked with 10 copies (100pg) each of the pSC1b32 and pGEMpgip2 plasmid DNAs. Lanes 3 to 13 represent genomic DNA of the following T$_3$ transgenic plants: lane 3: H3C 2, lane 4: H3C 10, lane 5: H7G 3, lane 6: H7G 5, lane 7: H8C 6, lane 8: H8C 7, lane 9: H8E 8, lane 10: H8E 9, lane 11: H12I7, lane 12: H12J2 and lane 13: H12J4. Marker sizes are indicated in kb.

B32 integration was confirmed by hybridisation of bands in lanes representing the T$_3$ transgenics (figure 4.10). Several T$_3$ transgenic plants seem to share the same banding patterns: H8C10 (lane 4), H8C6 (lane 7), H8E9 (lane 10) and H12J4 (lane 13). Other progeny also sharing similar patterns are H3C2 (lane 3), H7G3 (lane 5), H7G5 (lane 6) and H8C7 (lane 8), while H8E8 (lane 9) and H12I7 (lane 11) share the same pattern. Hybridisation of the *b32* probe was not observed in the untransformed Hi-II maize sample (lane 1). Positive controls containing untransformed Hi-II genomic DNA spiked with 2 copies (lane 2) and 10 copies (lane 14) of the pSC1b32 plasmid, produced hybridising bands (2100 and 6000 bp in sizes) with different intensities: the 10 copy bands were comparatively darker than the 2 copy bands.

Southern blot (*pgip2*) analysis (performed by A. Slaughter & L. Solms, University of Pretoria), of selected events is shown in Appendix 2. Results from this blot confirmed the successful integration of *pgip2* into the progeny of event A: hybridisation of bands with the *pgip2* probe occurred in all lanes representing the transgenic T$_3$ plants. Hybridisation of the *pgip2* probe was not observed in the untransformed Hi-II maize sample (lane 10). A list of the southern blot results for selected T$_3$ transgenics are shown in Appendix 3.

4.4.6.3 Northern Blot Analysis of T_3 transgenics

Northern blot experiments were performed by A. Slaughter and L. Solms (University of Pretoria). The *pgip2* and *b32* gene probes (outlined in Section 3.3.10) were used for hybridisation. Event A (*pgip2* and *b32* PCR and southern blot positive) was tested for mRNA transcripts (Appendix 4). Total RNA (from leaf tissues) were visualised on the formaldehyde agarose gel: fluorescing RNA bands for untransformed Hi-II and all progeny (lanes 1-7) were of similar intensities. Hybridisation analysis demonstrated a doublet band being produced (*pgip2* probed) for five of the independent T_3 progeny (lanes 2 to 6). Lane 1 (A1E3) however, showed no hybridising bands. Additionally, all transgenic progeny (lanes 1 to 6) showed a positive hybridising band for the *b32* transcript (Appendix 4B). No hybridising bands for *b32* or *pgip2* transcripts were detected in untransformed Hi-II control plant (lane 7).

T_3 progeny of event H (*b32* PCR positive) were tested via northern blot analysis for the *b32* mRNA transcripts (Appendix 5). All progenies (lanes 1 to 6) showed a positive hybridising band for the transcript. No *b32* transcripts (no hybridising bands) were detected in the untransformed Hi-II control plant (lane 6). The fluorescing RNA bands for the untransformed Hi-II and all progeny (lanes 1 – 6) were of approximately similar intensities.

Forty-eight independent T_3 transgenic plants (among events: A, F and H) were tested via northern blotting (Appendix 6). Five transgenic plants (event A) were *pgip2* positive (hybridising bands produced), while A1E3 was negative (no hybridising bands). Forty-four plants (among 3 events) were *b32* positive, and 4 plants (F1A5, F1A11, F1A14 and F1F 18) were negative. Differential banding intensities occurred among the transgenic plants: a darker band produced was labelled (in superscript) 'H' in the northern blot column (Appendix 5), while a lighter band was labelled 'L'.

4.4.7 *In vitro* Fungal Infection Studies

Untransformed Hi-II (control) and transgenic T_4 seeds (seeds of T_3 plants that were pooled) were plated on PDA with 125 spores of *Fusarium verticillioides* MRC826 inoculated per seed (figures 4.11, 4.12 and 4.13). Control experiments with saporin (a plant RIP) and nystatin (a fungicidin) were included. Figure 4.12 displayed results obtained after 4 days of incubation of infected seeds. Untransformed Hi-II seeds (figure 4.12A) displayed germination by production of shoot (of approximately 2 cm in length) and root.

High seed surface infection occurred, with fungal growth almost fully covering the surface (visible in close-up picture of untransformed Hi-II seed in figure 4.12B). Germination of transgenic seeds was evident, but shorter shoots and roots developed (figure 4.12C). Fungal mycelium growth coverage of transgenic seed surfaces were less (right plate) when compared to untransformed Hi-II seeds (left plate) in Figure 4.12D.

After 7 days of infection (figure 4.13): germination of untransformed Hi-II seeds (left plate of figure 4.13A) stopped (seedlings displayed browning and necrosis), and seeds were fully covered by fungal mycelium growth. The control experiment (containing a purified plant RIP, saporin) displayed zones of inhibition (indicated by arrows) and germination. However the seed surface was entirely covered by fungal mycelium growth (right plate of figure 4.13A). Transgenic seeds (figure 4.13B, C, D and E) continued to germinate. Growth and greening of the coleoptile (arrow 1) and root development (arrow 2) occurred in figure 4.13C.
Seed germination, visible zones of inhibition and reduced seed surface coverage by fungal mycelia, occurred in transgenic seeds.

Quantitative data (after 4 days infection)

No significant difference in fungal mycelium growth diameter occurred between untransformed and transgenic seeds (data not included). A significant difference of the infection scores however, occurred (score markings outlined in section 4.1.13). The average scores are represented in figure 4.11. A score as low as 2.25 (10-30% infected seed surface) for event F1F occurred, in comparison to a score of 6 (100% heavily infected seed surface) allocated to untransformed Hi-II seeds.
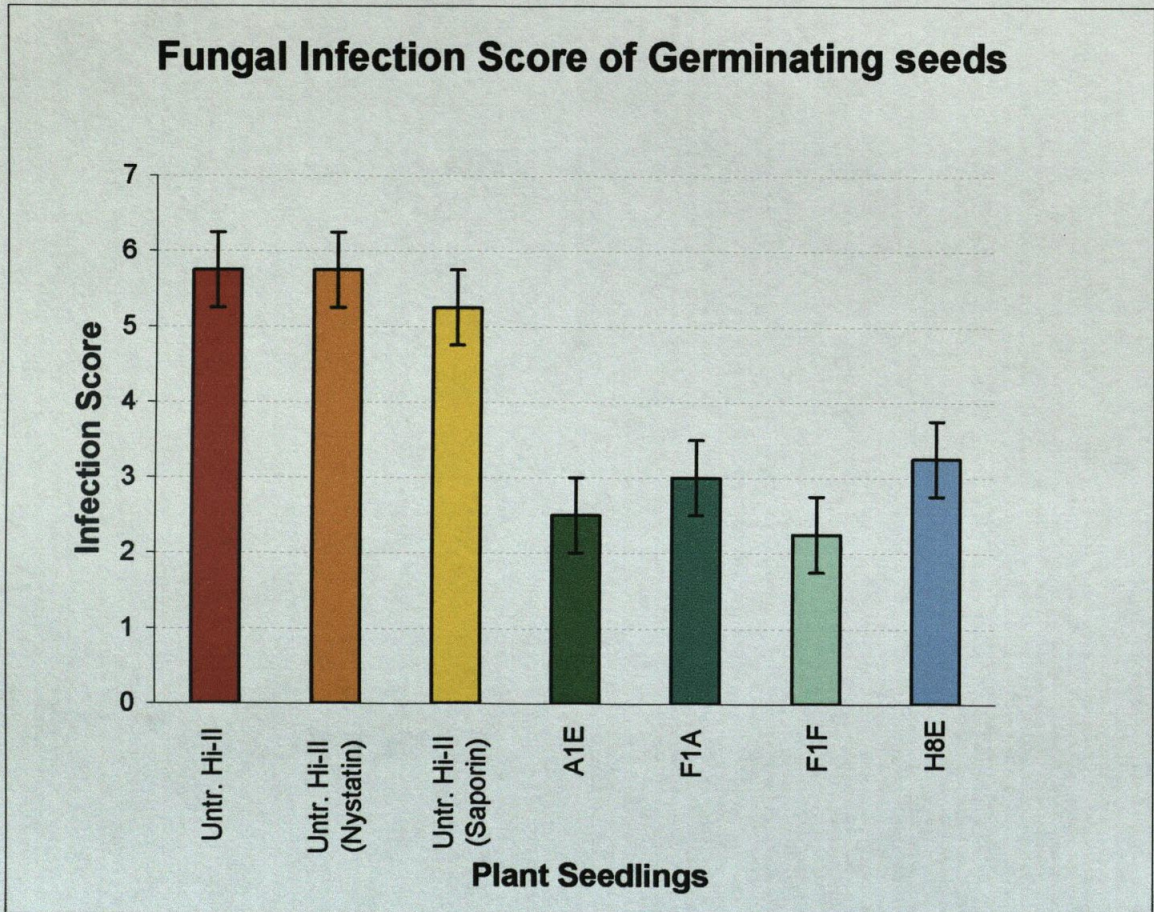
Fig 4.11 Bar histogram showing the average fungal infection score allocated to germinating seeds of untransformed Hi-II (control) and transgenic events, when infected *Fusarium verticillioides* (MRC826) spores. Results obtained after four days with 125 spores per seed infection on PDA plates. Infection scores allocated according to the infected seed surface area: 1: 5 – 10%, 2: 10 – 30%, 3: 30 – 50%, 4: 50 – 75%, 5: 75 – 100% and 6: 100% heavily infected seed surface area.
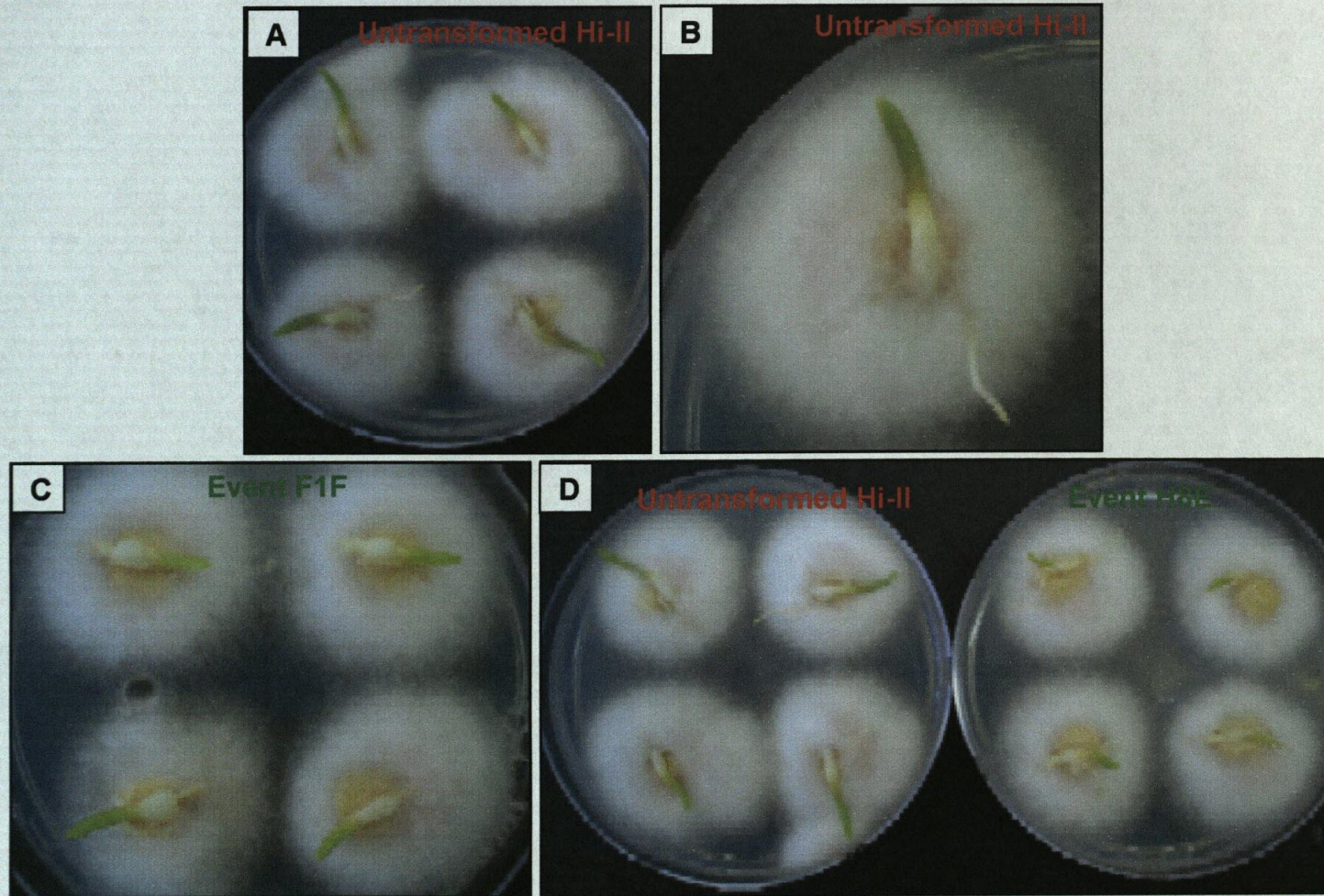
Fig 4.12 Fungal infection analysis of untransformed and transgenic seeds germinating on PDA media after 4 days of infection by *Fusarium verticillioides* (MRC826) with 125 spores infected per seed. **A)** Untransformed Hi-II seeds showing germination however in **B)** seed surface is heavily infected by fungal mycelium growth; **C)** Transgenic F1F seeds displaying seed germination and reduced seed surface infection by mycelium growth; **D)** Germination of untransformed Hi-II (left) compared to event H8E (right) seeds. Transgenic events clearly show reduced fungal infection of the seed surface area
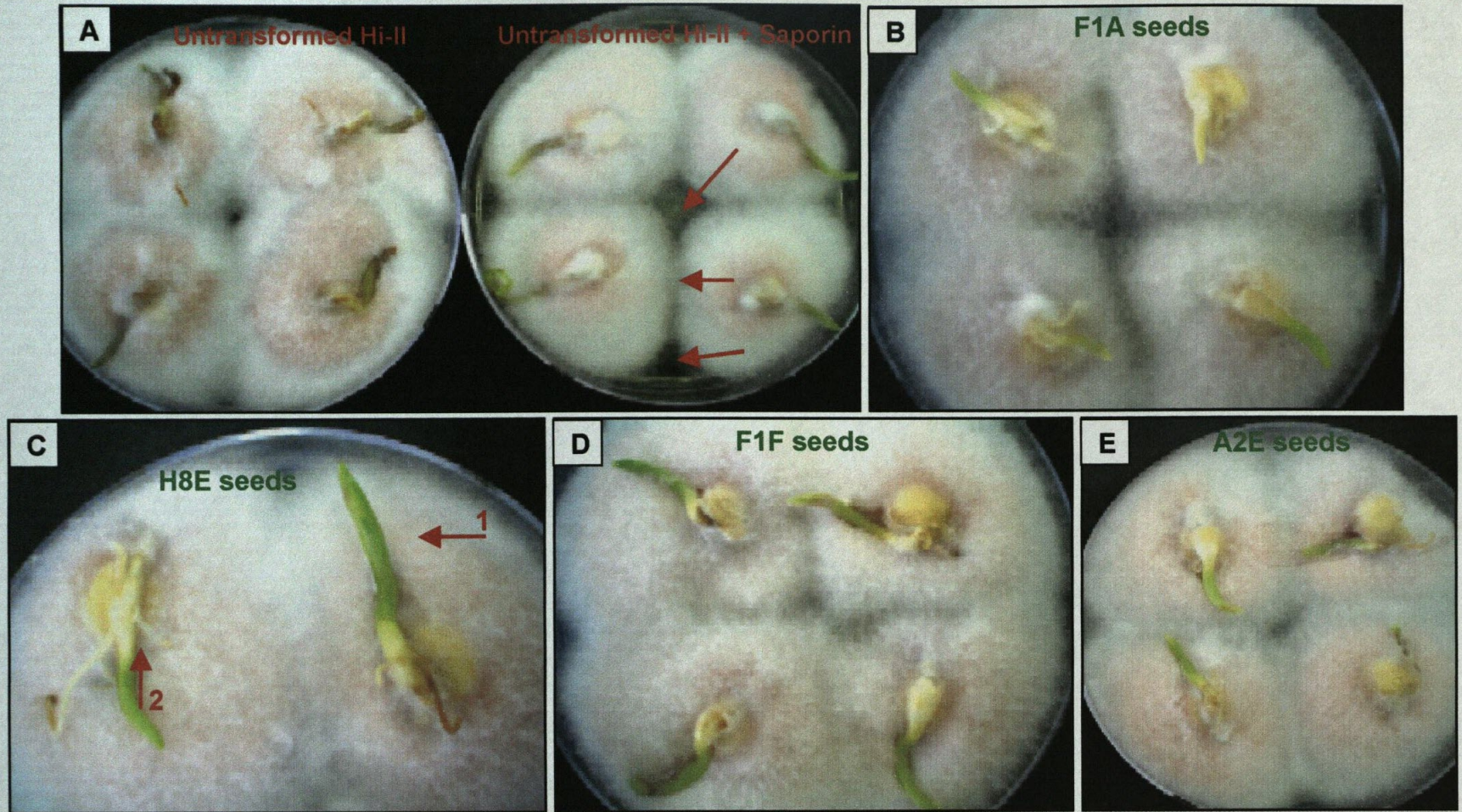
Fig 4.13 Fungal infection analysis of untransformed and transgenic seeds germinating on PDA media after 7 days of infection by *Fusarium verticillioides* (MRC826) with 125 spores infected per seed. **A)** Death of seedlings is evident in untransformed Hi-II seeds (left plate), however untransformed Hi-II seeds growing in the presence of saporin (right plate), continued germination. Fungal mycelium growth fully covered the surface of seeds in both plates, however zones of inhibition were visible in plate with saporin addition (indicated by arrows); B, C, D and E show transgenic seeds of F1A, H8E, F1F and A2E respectively. Continued germination and reduced seed surface area infection by fungal growth occurred in all seeds. Germination with production of healthy shoot development (arrow 1) and root development (arrow 2) was evident. Zones of fungal growth inhibition were also visible in plate B.

4.5 DISCUSSION

In this study, stable integration of the antifungal genes b32 and pgip2, as well as the selectable marker gene (bar) was demonstrated in Hi-II maize plants.   Hi-II is a laboratory strain of maize (A188xB73) (Armstrong et al., 1991) and embryogenic type-II callus tissue is often easily initiated from Hi-II cultured immature zygotic embryos (IZEs).   Type-II callus maintains its embryogenic competence for a long periods of time (Lu & Vasil, 1981; Vasil & Vasil, 1981a, 1982a; Ho & Vasil, 1983b), and it has been previously transformed with the uidA reporter gene and the bar selectable marker gene (O'Kennedy et al., 1998) using biolistics. It is for this reason that Hi-II callus tissue (instead of IZEs) were chosen as the explant for use in bombardment experiments.

Hi-II callus (initiated from Hi-II IZEs as explained in Section 4.3.1) were subcultured every 2 weeks (until they were 2 months old) before being used for bombardment. This time period enabled proper development of the soft, friable, embryogenic, fast growing type-II callus (Green, 1982; Vasil et al., 1984; Lowe et al., 1985; Vasil et al., 1985). Plant tissues maintained under tissue culture conditions for long periods were exposed to tissue culture stress, and often resulted in stress related abnormalities, such as dwarfism and infertility (Lowe et al., 1985).   Therefore, for bombardment experiments in this study, type-II callus tissues were constantly initiated from Hi-II IZEs: calli between 2-8 months old were only used for bombardment experiments.

A bombardment pressure of 600 KPa was used in this study. This helium pressure was reported by O'Kennedy et al. (1998) to yield the highest levels of transient expression in Hi-II callus, when compared to other bombardment helium pressures tested. These researchers also reported on the preferred use of tungsten instead of gold particles for the bombardment of Hi-II callus tissue, and thus tungsten particles were chosen for use in this study.

Pre- and post-treatment of explants with osmoticum containing tissue culture media, alleviated plasmolysis of cells (previously explained in Section 3.2.5), thereby reducing cell damage from bombardment (Vain et al., 1993). Vain and co-authors (1993) reported a 6 to 7 fold increase in the number of stable transformants obtained when osmoticum treatment was used.   Therefore, Hi-II callus tissues were pre-treated on osmoticum containing $N6_{10}$ media for 3 hours before, and 16 hours after bombardment. Putatively transformed Hi-II calli were then selected on $N6_{10}$ callus induction media supplemented with the selective agent, bialaphos.

4.5.1 Chlorophenol Red (CPR) Assay

The optimal bialaphos concentration used for selection of transgenics was determined by performing a "Kill Curve" test. This test (explained in Section 4.4) is based on the chlorophenol-Red assay developed by Wright *et al.* (1996) and tests the response of untransformed Hi-II callus tissue to various concentrations of the selective agent, bialaphos (a tripeptide form of the Basta® herbicide). Untransformed Hi-II callus tissue displayed a red medium colour change (indicating tissue necrosis) at a bialaphos concentration of 1mg l^{-1} and above. Transformed tissues (containing the *bar* gene are expected to survive above this concentration. Thus, for rapid initial screening of transformed tissues, a relatively higher concentration of 3 mg l^{-1} was used. All putative transgenics testing positive for this assay, had the *bar* gene presence confirmed by PCR analysis, indicating the reliability of this technique for initial screening of transgenics.

4.5.2 Selection, Regeneration and Germination of transgenics

Transformed callus tissues were initially selected on $N6_{10}$ callus induction media (3 mg l^{-1} bialaphos) and bialaphos concentration was later increased to 5 mg l^{-1} to eliminate the possible "escape tissues" (negative tissues that survived herbicide selection pressures). For regeneration of putative transgenics, the concentration was decreased to 1 mg l^{-1} to allow for rooting to occur, as rooting has been previously reported by Vain *et al.* (1993) to be sensitive to bialaphos. Although the frequency of putatively transgenic type-II calli surviving varied form 45 - 90% (Table 4.2), only few of these type-II calli matured to form regenerants. This could possibly be due to the tissue culture stress (preventing root or shoot development), or that the surviving calli were escapes (did not possess the *bar* gene) and were thus sensitive to regeneration in the presence of 1 mg l^{-1} bialaphos.

4.5.2.1 Phenotypic Growth Analysis

Putative T_0 transgenic plants displayed abnormal phenotypes: dwarfism and underdeveloped tassels and ears (Figure 4.3A). These characteristics can be attributed to tissue culture stress (Lowe *et al.*, 1985), thus explaining why many T_0 plants were infertile (not producing T_1 seed). Contrarily, the T_1 progeny of the eight independent events (Table 4.3) identified to have produced T_1 seeds, were tall and fertile plants (figure 4.3B). These T_1 progeny were germinated directly from seeds (figure 4.4) and therefore, were not subjected to the long tissue culture periods.

A variability in germination of T_3 transgenic seeds occurred under glasshouse conditions (Figure 4.7). Some events (for example A2D in figure 4.7A) did not germinate. This could be explained by one of two possible reasons: (i) the T_3 seeds were not healthy enough to sustain itself for germination in the soil (previous generations of transgenic seeds were initially germinated on ½MS germination media for a week before being hardened off and transferred into pots), or (ii) some gene rearrangements occurred which interfered, or silenced other genes that were imperative for the normal functioning ability of the seed to germinate (gene silencing explained further below).

On the other hand, some transgenic progeny (for example H7G indicated by arrow A in figure 4.7B) produced taller, greener phenotypes as compared to untransformed M37W control plants (arrow B in figure 4.7B). It has been previously reported that an active copy of a transgene can acquire the capacity to inactivate other copies of the gene in subsequent crosses (Meyer *et* al., 1993). This phenomenon is said to resemble "paramutation" – paramutators (inactive) alleles inhibit the expression of paramutable (active) alleles causing them to become inactive (Brink, 1956).

More recent results lead to a hypothesis that mutations introduced by transgene introduction, are capable of reactivating endogenous chromosomal information that otherwise would be transcriptionally silent (Steimer *et al.*, 2000).
Thus, the integration of the *b32* and *bar* genes in H7G (table 4.6), could have possibly occurred on the locus of one (or more) of the chromosomes, at a point where it could have (i) interfered (un-silenced) or upregulated the expression of other important growth or developmental genes, or (ii) caused inactivation (silencing) of genes that inhibited extensive phenotypic growth. Furthermore, this integration in H7G is probably different from other events (like G1H or F1A) that do not display this tall phenotype under glasshouse conditions even though they are also PCR positive for the *b32* and *bar* genes (table 4.5).

4.5.3 Basta® Painting

Basta® painting of putative transgenic leaves (figure 4.5) tested the functionality of the *bar* gene in these plants (expression of PAT enzyme and detoxification of herbicide is explained in Section 3.5.4.3). All plants (with one exception) that displayed resistance to Basta®, were confirmed PCR positive for the *bar* gene. However, plant H2 (table 4.4) tested CPR and Basta® positive but PCR (*bar*) negative.

The possibility of the genomic DNA being of poor (or degraded) quality is minimal as this plant tested PCR positive for the *nad* gene (table 4.4). Thus, extracted genomic DNA for H2 is unlikely to have secondary compounds (polyphenols and polysaccharides), which could have interfered with the Taq DNA polymerase activity (Mannerlof & Tenning, 1997). Furthermore, one transgenic plant (C3 in table 4.4) was Basta® paint and CPR negative, but tested PCR positive for the *bar* gene. This indicates that the *bar* gene is present in the genome, but it is possibly truncated or silenced within the genome, and is thus not functional. Gene silencing is further discussed below under molecular analysis.

4.5.4 Molecular Analysis of Transgenics

PCR analysis was performed for all eight T_0 transgenic events (table 4.3) and their progeny plants, to confirm the presence of the *b32*, *pgip2* and *bar* genes. However, only a selection of T_3 transgenics (based on their PCR result), were selected for Southern and northern blotting analysis.

4.5.4.1 Polymerase Chain Reaction (PCR) analysis of T_1 and T_2 progeny

PCR analysis for all putative transgenics performed correlated with the southern and northern blot results. The PCR for the *nad* gene produced positive amplified bands for all events tested, thus indicating that the genomic DNA used for PCR was of good quality. Some DNA bands produced by PCR amplification were faint: for example lane 10 in figure 4.6 produced a 731 bp DNA band for the *pgip2* gene. This faintness in the banding produced, could be attributed to this selected PCR tube reaction not being optimal for the PCR conditions, as the positive controls (lane 1 and 4) and other independent events (lanes 5, 6, 9, 11, 12) produced bright amplified DNA bands. The events producing these faint bands were regarded as PCR positive because some amplification (irrespective of how minimal) does indeed occur. Eight independent fertile events (table 4.3) were identified, out of a total of 106 plates of Hi-II calli bombarded. Thus, a transformation efficiency of 7.5% (per plate) was obtained.

Table 4.4 indicates that 26 independent T_1 progeny (among the 8 independent events) were PCR positive for at least one of the three bombarded transgenes. The T_1 progeny not listed in this table did not survive under greenhouse conditions. This could possibly be due to transgene instability or a "paramutation-like" event (explained further in section 4.5.3.1) occurring, that probably affected the growth and phenotypic development of the plant.

T_3 transgenic plants (randomly selected for analysis) listed in Appendix 1, showed only 6 plants (among event A) testing positive for all three genes. This was consistent with PCR results for the previous generations, indicating stable integration for this event. The other 49 transgenics (among 3 independent events) that were positive for the *b32* and *bar* genes, also showed correlation with previous PCR results for their respective previous generations, and thus also displayed integration stability. Integration patterns for selected T_3 progeny are explained below.

4.5.4.2 Southern Blot Analysis of transgenics

All events selected (Appendix 3) that were PCR positive for the respective antifungal genes, were confirmed by southern blot analysis.

B32 southern blot analysis

The *Aat* II restriction enzyme (having a unique restriction site on pSC1b32) was initially chosen for digestion of genomic DNA however, digestion was poor. Thus, *Eco* RI (good digestion of genomic DNA) was chosen. *Eco* RI has two restriction sites on pSC1b32 (figure 3.1A): a 6000 bp (containing the *b32* gene) and a 1456 bp fragment. Therefore, for the positive 2 and 10 copy controls of plasmid DNA (figure 4.10), the number of fragments hybridising to the *b32* probe is expected to only be one (the 6000 bp fragment). However, an additional fragment of 2100 bp occurred. Lanes 2 and 14 (figure 4.10) were both spiked with 2 and 10 copies (respectively) of each of the pSC1b32 and pGEMpgip2 plasmids. Therefore, the 2100 bp fragment produced in lanes 2 and 14 are that of the pGEMpgip2 *Eco* RI digested fragment, as the *b32* probe recognised the 571bp of CAMV35S (also the promoter for *pgip2*) of the digested *pgip2* fragment. Additionally, the copy number estimation was determined by comparing the hybridising intensities of the resulting fragments of the putative transgenics (lanes 3 to 13), to the untransformed spiked samples with known number of copies (lanes 2 and 14).

Several transgenics seem to share similar integration patterns, implying that they were clones of each other (figure 4.10). Progeny sharing similar integration patterns are in lanes 4 (H8C10); 7 (H8C6); 10 (H8E9) and 13 (H12J14). Progeny in lanes 3 (H3C2); 5 (H7G3) and 6 (H7G5) also share a similar integration pattern, as does H8E8 and H12I7, in lanes 9 and 11 respectively. These integration patterns seem to vary among the progeny tested. However, taking into consideration that these progeny originate from the same T_0 event (H), it is possible that all these plants would show similar patterns, if the blot was exposed for longer periods via chemiluminescent detection methods (allowing other lighter bands to be detected).

H8C7 (lane 8) has a broad, smeared band visible at the top of the blot, which could represent some undigested genomic DNA, while the second band (11000 bp) could represent the digested fragment (containing CAMV35S as well as the *b32* gene specifically). Multiple tandem repeats of the promoter and gene could have integrated which probably account for the high size of this band.

The copy number for events H3C10 (lane 3) and H8C6 (lane 4) is estimated to be less than 10 copies since more DNA was loaded in these lanes, thus resulting in darker bands being produced. Other events, for example H12I7 (lane 11), is estimated to be of single-copy integration. Thus for all transgenics tested in this figure, low copy number integration (2-6 copies) occurred for the *b32* gene. Similarly a low copy number integration (2-10 copies) also existed for all other transgenic events tested (blots not included).

The *b32* gene is an endosperm specific gene isolated from maize itself. However no hybridisation of bands occurred with the non-spiked untransformed Hi-II genomic DNA (lane 2 of figure 4.10). This is because the *b32* probe used for hybridisation is composed of the entire CAMV35S promoter sequence and approximately 300 bp of the *b32* gene. Thus, this probe might have shown a stronger binding affinity to specific DNA fragments containing the 571 bp of CAMV35S sequence (non-existent in untransformed maize for control of the *b32* gene) and 300 bp of the *b32* gene, as opposed to only the 300 bp of the endogenous *b32* gene in untransformed maize. Therefore, no hybridisation of genomic DNA with the *b32* gene in untransformed maize occurred in lane 1.

Pgip2 southern blot analysis

Appendix 2 presents the southern blot results for T_3 progeny of event A. The enzyme *Hind* III has two restriction sites of importance indicated in figure 4.14. Thus, *Hind* III digestion of the transgenic genomic DNA is expected to produce a single hybridising band of approximately 1402 bp, which does occur in the lanes digested with *Hind* III (Appendix 2). Southern blotting of untransformed Hi-II genomic DNA did not produce any hybridising bands as bean *pgip2* (from *P. vulgaris*) is absent in maize.

Digestion with *Eco* RI is expected to yield a single hybridising fragment of 1994bp, which does not occur in the lanes digested with *Eco* RI (Appendix 2). Lane 7 (A2E3) of Appendix 2 was digested with *Eco* RI but displayed 2 hybridising fragments of sizes 4900 and 1400 bp.

The 4900 bp band could be the band hybridising to incompletely-digested genomic DNA (*Eco*RI failing to digest at one of its restriction sites). The 1400 bp hybridising band could be representative of the fragment that was possibly sheared on bombardment, and only part of the plasmid was integrated. Thus, a smaller hybridising band occurred on *Eco* RI digestion. Other progeny, A1E5 (lane 3) and A2E6 (lane 9), also digested with *Eco* RI, show hybridising bands of approximately 4900 bp, which could be indicative of incomplete digestion of the genomic DNA (as explained above for lane 7). The patterns for all the T_3 progeny are similar, which is expected as they all are progeny from the same event.
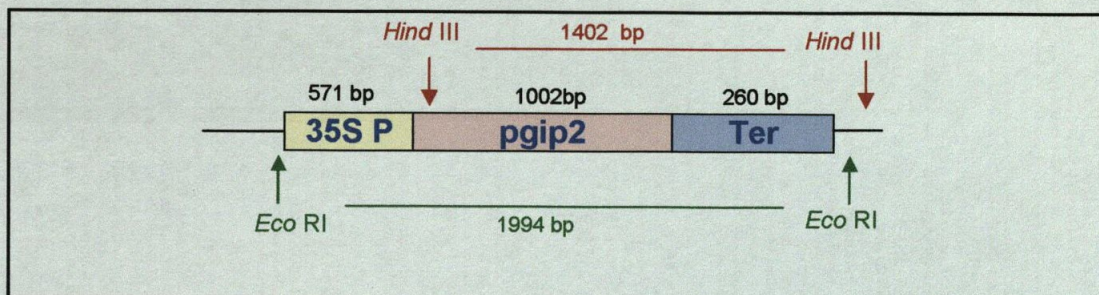
Fig 4.14 Diagrammatic representation of the CAMV-*pgip2* part of the pGEMpgip2 plasmid, indicating the *Hind* III and *Eco* RI restriction sites.

A transformation efficiency of 7.5% (per plate Hi-II calli bombarded) is reported (8 fertile T_0 events from 106 plates of Hi-II calli bombarded).

4.5.4.3 Northern Blot Analysis of transgenics

Expression of bean *pgip2* and maize *b32* mRNA transcripts in Hi-II maize plants, are presented by the northern blot results. All progeny of event A (*pgip2* and *b32* southern blot positive) except A1E3 (lane 1 of Appendix 4) produced *pgip2* transcripts. Variation in amount of transcript produced occurred: darker bands (A1E5 in lane 2) represented more transcript produced when compared to a lighter band (A1E11 in lane 4), which represents less transcript production. Thus, A1E5 (lane 2), A1E6 (lane 6) and A2E3 (lane 5) are high expressors, while A1E7 (lane 3) and A1E11 (lane 4) are low expressors of *pgip2* mRNA. A1E3 (lane 1) is a non-*pgip2*-expressor and is an example of gene silencing, where the gene has been integrated (Appendix 2), but not transcribed or expressed.

Gene silencing can occur either through repression of transcription, termed transcriptional gene silencing (TGS), or through mRNA degradation, termed post-transcriptional gene silencing (PTGS) (as reviewed by Vaucheret & Fagard, 2001). TGS is meiotically heritable (reviewed in Finnegan *et al.*, 1998), implying that if a

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plant has a gene silenced: all its progeny plants would be expected to have the gene silenced. A 1E3 shares the same integration site as the other progeny of event A (A1E5, A1E7, A1E11, A2E3 and A2E6). However, these progeny tested positive for mRNA production, thus TGS occurring for A1E3 seems unlikely.

PTGS (degradation of mRNA produced) could be the explanation as PTGS is not meiotically transmitted, and needs to be re-established in each sexual generation (reviewed in Steimer *et al.*, 2000). PTGS is not necessarily coupled with modification of the DNA template but increased amounts of DNA methylation within the protein-coding region of silenced genes have been observed (reviewed in Depicker & Van Montagu, 1997). Thus, increased methylation could have occurred in A1E3. Furthermore, reports indicate that overproduction of mRNA above a putative threshold level, can trigger the irreversible degradation of RNA (Dehio & Schell, 1994). The transgenes are constitutively expressed in A1E3 and thus, overproduction of mRNA could have occurred. Additionally, A1E3 has a low copy integration of *pgip2* therefore multiple copy gene silencing does not apply.

Further analysis of *b32* mRNA expression in event A (Appendix 4B) and other events (Appendix 5A) showed the presence of transcript in all progeny tested. The amount total RNA used for analysis of all progeny, were approximately the same (indicated in Appendix 4C and 5B). Thus, differences observed in mRNA production, is solely attributed to the transcriptional ability of the plants.

Thus, northern blot analysis confirmed the presence of *b32* and *pgip2* mRNA transcripts for 5 (out of 6) plants tested (event A). A further 38 (out of 42) plants tested (events F and H) were positive for the *b32* transcripts. Plants having no expression of the transcripts tested, were southern blot positive for the respective gene, but were subjected to gene silencing.

4.5.5   Fungal Infection Studies

Selected T_4 seeds (expressing the respective transcripts) and untransformed Hi-II seeds were tested against infection by *Fusarium verticillioides* (MRC826), while germinating on PDA media plates. Saporin (plant RIP) and nystatin (fungicidin) addition to the PDA media, served as additional positive controls, as these compounds were expected to show some inhibition of fungal infection of the seeds.

Untransformed Hi-II seeds (plates with and without saporin or nystatin) showed faster germination after 4 days, when compared to the transgenic seeds. However, on closer examination, the fungal growth coverage on the untransformed Hi-II seed surfaces was visibly higher than those of the transgenic seeds. These observations however, were not substantial enough to make any conclusions.

Germinating seedlings of untransformed Hi-II (without saporin and nystatin addition), died and germination was halted after 7 days of infection. The endogenous b32 protein (in untransformed maize) is expressed in the endosperm only. This therefore explains why germination occurred initially after 4 days, but once shoots and roots (no b32 expression in these parts) were produced, the fungi mycelia possibly infected these parts causing the plantlet to die after 7 days. Untransformed Hi-II seeds with saporin or nystatin addition (providing some source of fungal resistance), continued germination after 7 days, however the seeds surface was fully covered by fungal mycelium growth. Transgenic seeds however, displayed continued germination and reduced coverage of the seed surface by mycelium growth and germination of the seeds continued. This was expected for transgenics as the antifungal proteins are expressed constitutively, i.e. throughout the plantlet and thus, displayed some resistance to fungal infection.

Fungal infection of transgenic seeds was reduced by almost 50% when compared to infection of untransformed Hi-II seeds in figure 4.11. Standard deviations of the average for the infection scores among the experiments were either 0.5 or 1.0 (data not included). This indicated that results obtained for the individual replicates, compared closely to the average infection score and thus, a statistical comparison could be drawn. A significant difference thus exists between transgenic and untransformed seeds. Responses between the transgenics containing the *pgip2* and *b32* genes (A1E), and those with *b32* only (F1A, F1F and H8E), however showed no significant difference.

The untransformed Hi-II seeds (in the presence of nystatin), showed continued germination but no significant reduction in fungal infection of the seed. This could possibly be due to an incorrect concentration being used. A higher concentration can be examined in future experiments. Untransformed Hi-II seeds (in the presence of saporin (plant RIP)), showed some zones of inhibition forming, as well as reduced infection of the seed surface.

However, the reduced seed surface infection of transgenic seeds was comparatively lower than untransformed seeds cultured with 35 μg ml^{-1} saporin. This possibly suggests that apart from displaying increased resistance to *Fusarium verticillioides* (MRC826) infection, these transgenic seeds produced antifungal proteins at concentrations greater than 35 μg ml^{-1}.

4.6   CONCLUSION

This chapter reports on the successful transformation of maize Hi-II callus, with the antifungal *b32* and *pgip2* genes, using *bar* as the selectable marker gene. Although a low transformation efficiency of 7.5% occurred, transgenic plants displayed stable integration of the transgenes throughout several generations. Successful production of mRNA transcripts for the *b32* and *pgip2* is reported for one event, while progeny of two other events show production of the *b32* transcripts. Fungal infection studies of germinating transgenic T_4 seeds, further display significant resistance to *Fusarium verticillioides* mycelium infection of the seed surface, when compared to untransformed control seeds.

CHAPTER FIVE:

BIOLISTIC TRANSFORMATION AND MOLECULAR ANALYSIS OF Hi-II MAIZE GENOTYPE WITH ADDITIONAL TRANSGENES AND IMPROVED PLASMIDS

5.1 ABSTRACT

This c hapter r eports on t he b ombardment of Hi-II c allus tissue independently with selected transgenes. The *bar* selectable marker gene was used for all experiments (bialaphos herbicide selection). Bombardment of linear DNA minimal transgene cassettes (lacking plasmid vector backbone) resulted in 20-50% of bombarded Hi-II callus proliferating after 6 weeks of bialaphos selection. Twenty-four independent T_0 events were identified, but only 3 survived under greenhouse conditions displaying dwarfism, abnormal ear and tassel formation. One event (BBF 1.1A, *b32* and *bar* PCR positive) was fertile to produce 4 seeds. Only 1 seed germinated producing a normal, tall and fertile T_1 phenotype progeny that was PCR negative for the *b32* and *bar* genes. Co-bombardment of pSC1-mpib32 (*Fusarium* inducible promoter) and pRTL2-improved PGIP (containing an oat cell wall bound thionin signal peptide) resulted in 5-46% Hi-II calli proliferation after 4 weeks of selection, however all calli died on further selection, and no transgenics were produced. PCR analysis of bombarded calli tested negative for *b32* and *pgip2* genes. Additional bombardment of Hi-II calli with pAHCinors (containing *inors* (nerolidol synthase) gene), resulted in 10-20% Hi-II callus proliferation after 4 weeks of selection, with no calli surviving to produce transgenics on further selection. Bombardment with pAHCtnors (containing *tnors* (linalool synthase) gene with chloroplast targeting) yielded 30-50% Hi-II callus proliferating after 4 weeks of selection. Thirty-one independent T_0 events were identified – all plants displayed dwarfism, abnormal ear and tassel formation. Only 2 events survived with one event (BBT 1.1B, *b32* and *bar* PCR positive) being fertile to produce 16 seeds. Six T_1 seeds germinated on bialaphos containing media, but all progeny plants were Basta® paint negative and PCR negative for the *tnors* and *bar* genes. A sweet, floral scent was observed for selected T_0 plants and five T_1 progeny, when plants were under tissue culture conditions (in tubs). This scent was not detected when plants were placed under greenhouse conditions.

5.2 INTRODUCTION

Plant transformation with plasmid vectors results in the incorporation of unwanted vector backbone sequences (containing the antibiotic resistance gene), which promotes transgene rearrangements and high-copy number integration patterns, thus contributing to transgene silencing (Matzke *et al.*, 1996). Bombardment with linear DNA minimal transgene cassettes (promoter, open reading frame and terminator only) has resulted in low-copy number integration (77% single integration), low frequency gene rearrangements and no gene silencing in rice (Fu *et al.*, 2000). Thus bombardment of linear minimal transgene cassettes of the maize *b32*, bean *pgip2* genes and *bar* selectable marker genes were examined in this chapter.

The pSC1-mpib32 plasmid (figure 5.3A) contains the maize *b32* gene under the control of the *Fusarium* inducible, Mpi promoter (Cordero *et al.*, 1994). The pRTL2-improved PGIP plasmid (figure 5.3B) harbours the signal peptide from an oat cell-wall bound thionin (*Asthi1*) instead of the bean PGIP2 signal peptide. This leaf thionin *Asthi1* (produced in transgenic rice plants) was secreted outside cells and ionically bound to cell walls (Iwai *et al.*, 2002). Thus, this signal peptide was thought to direct the mature PGIP2 protein to the cell wall where it would exhibit its antifungal properties. These improved *b32* and *pgip2* plasmids were co-bombardment onto Hi-II calli tissue to possibly increase the number of transgenics, thereby improving the transformation efficiency.

The strawberry terpene synthase gene constructs (containing *inors* or *tnors*) encode for nerolidol and linalool synthase genes respectively. The pAHCinors plasmid harbours the *inors* gene, which lacks a plastid-targeting signal and the terpene precursor is thus active in the cytosol where it reacts with FPP to produce nerolidol. The pAHCtnors plasmid (containing *tnors*) encodes a plastid (chloroplast) targeting signal (Haudenschilde & Croteau, 1998), and this terpene precursor reacts with GPP in the chloroplast to produce linalool. Linalool and nerolidol have been recently shown to inhibit mycelium growth of several isolates of *Fusarium* among other fungi (Scholten, unpublished). These products are emitted only upon herbivory and cannot be stimulated by mechanical wounding (Turlings *et al.*, 1991). Thus, constitutive expression of these terpene synthase genes, were targeted for introduction into maize to provide resistance to *Fusarium* in this study.

5.3 MATERIALS AND METHODS

5.3.1 Excision and culturing of IZEs for Hi-II callus production

Initiation and tissue culturing of Hi-II callus was performed as outlined in Section 4.3.1.

5.3.2 Linear DNA minimal transgene cassette preparation

Minimal transgene cassettes were isolated from the source plasmids: the *bar-b32* cassette (figure 5.1A) from pSC1b32 plasmid (Figure 3.1A), and the *pgip2* cassette (figure 5.1B) from the pGEMpgip2 plasmid (Figure 3.1B) respectively.   These plasmids (prepared as outlined in Section 3.3.3), were digested with the restriction enzymes *Aat*II and *Alw*NI (for pSC1b32); and *Eco*RI (for pGEMpgip2), and incubated at 37°C for 1 hour. The digested samples were then fractionated by electrophoresis on a 0.8% agarose gel and visualised by ethidium bromide staining under UV light. DNA bands corresponding to the expected sizes of the linear DNA expression cassettes were excised from the agarose gel, and purified using the QiaQuick Gel Extraction Kit (Qiagen).   The resultant linear minimal transgene DNA cassettes are illustrated in Figure 5.1.
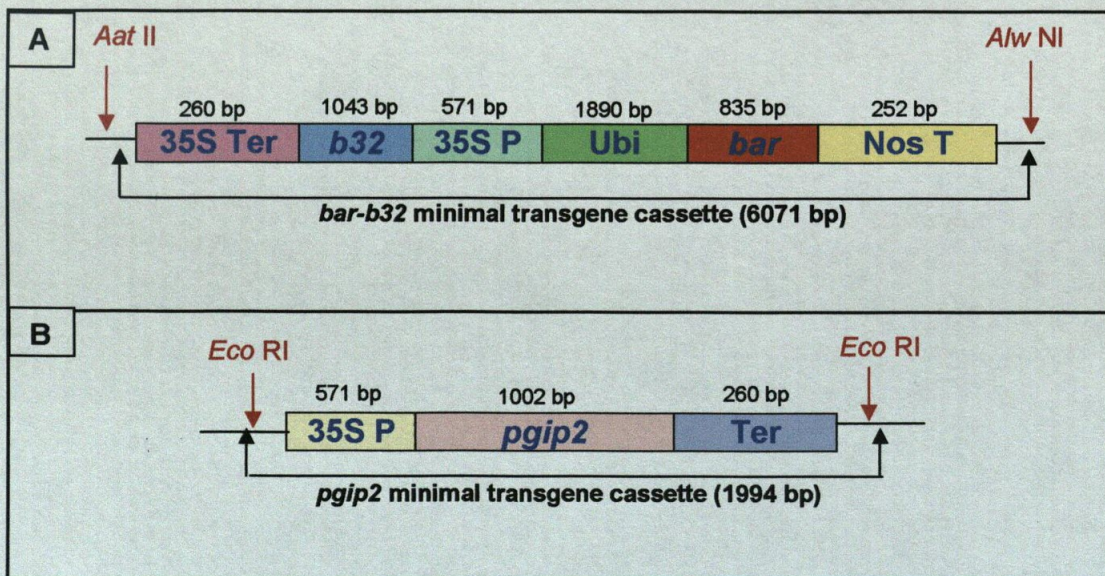
Fig 5.1 The resultant linear minimal transgene cassettes used in bombardment experiments: **A)** 6071 bp *bar-b32* minimal transgene cassette excised from pSC1b32, and **B)** 1 994 bp *pgip2* minimal transgene cassette excised from the pGEMpgip2 plasmid.

These two linear DNA minimal cassettes were co-bombarded (as explained in section 5.3.4) onto Hi-II callus tissue in different ratios (outlined in Table 5.1).
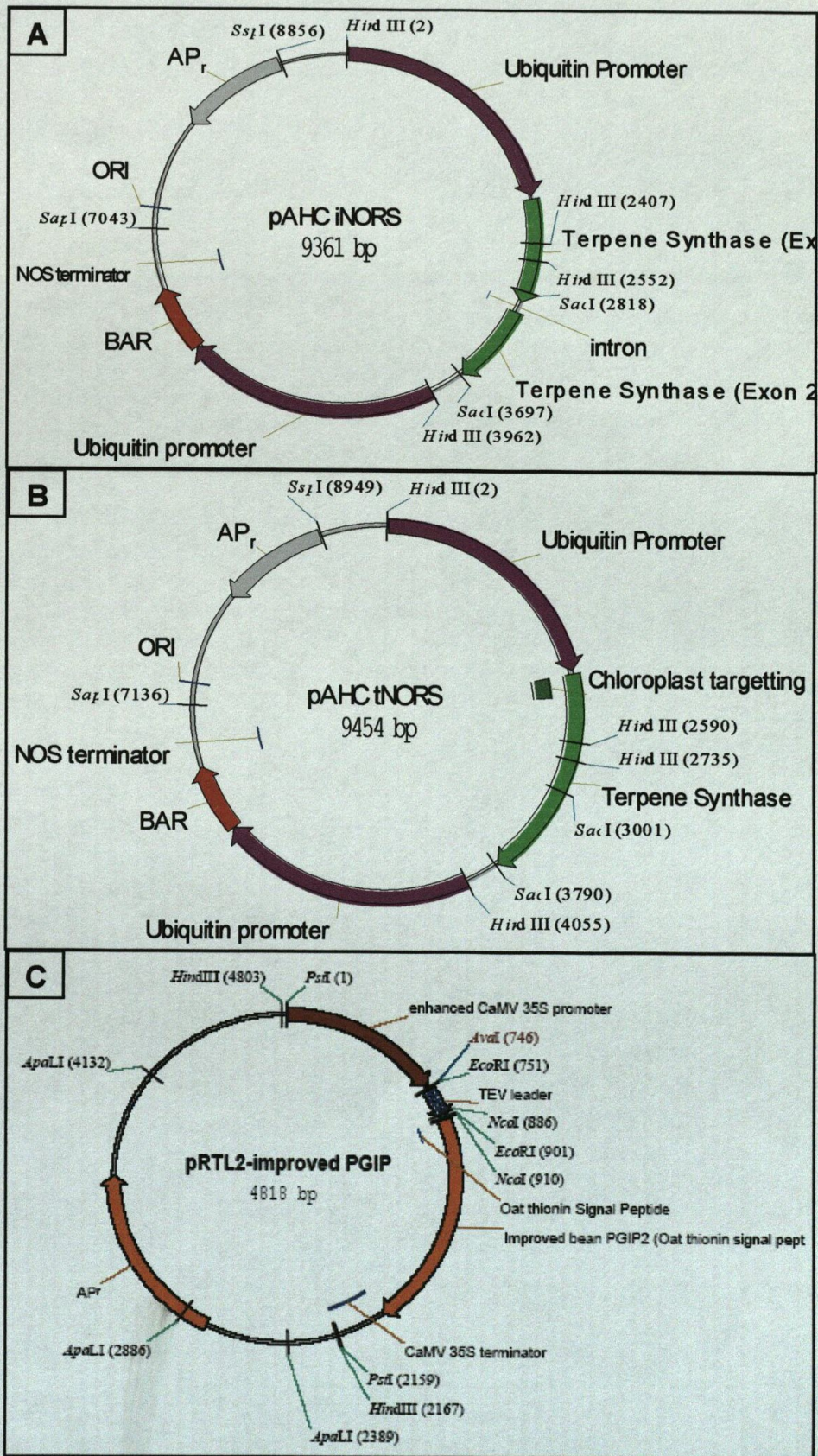
Fig 5.2 Plasmid maps of **A)** the pAHCinors (9631 bp in size); **B)** the pAHCtnors (9454 bp in size) and **C)** the pRTL2-improved PGIP vector (4818 bp in size)

5.3.3 Plasmid preparation

The pAHCinors (figure 5.2A), pAHCtnors (figure 5.2B), pSC1-mpib32 (similar to pSC1b32 in figure 3.1A, but the promoter for *b32* is the *Fusarium* inducible maize protease inhibit (mpi) promoter (Cordero *et al.*, 1994) instead of CAMV35S) and pRTL2-improved PGIP (figure 5.3C) plasmid vectors were prepared (Section 3.3.3) and used for bombardment as explained in section 5.3.4. The strawberry terpene synthase genes (*inors* and *tnors*, Duradeva *et al.*, 1996), were made available by Dr. O. Scholten (Plant Research International, Wageningen). These genes were cloned into respective vectors by Dr. E. Berger (CSIR-Bio/Chemtek, Pretoria) and the resulting pAHCinors and pAHCtnors plasmids, were made available for bombardment. The pSC1-mpib32 plasmid (containing the *Fusarium* inducible mpi promoter), was constructed by Dr. P. Chimwamurombe (University of Pretoria). The pRTL2-improved PGIP plasmid (containing oat cell-wall bound thionin (*Asthi1*) signal peptide, Iwai *et al.*, 2002), was constructed by Dr. A Slaughter (University of Pretoria).

5.3.4 Microprojectile Bombardment

Microprojectile bombardment of the above-mentioned DNA plasmids occurred as described in section 4.3.3. Bombardment of the *b32-bar* and *pgip2* minimal transgene cassettes (figure 5.1) was also performed as outlined in section 4.3.3, with the difference being the ratio of each fragment in the bombardment mix. The total amount of DNA bombarded however, was the same (0.16µg per 2µl shot). Embryogenic type II calli were used as explants. All experiments were conducted using the Particle Inflow Gun under sterile conditions at room temperature.

Table 5.1   Conditions for co-bombardment of Hi-II callus tissues with the *bar-b32* and *pgip2* linear minimal transgene cassettes

Experiment #	# plates of calli BB	Age of Hi-II calli (months)	*bar-b32* : *pgip2* fragment (ng)[a]	*bar-b32* : *pgip2* fragment (molar amounts)[b]
BBF 1	2	2 ½ months	80 : 80	1 : 3.3
BBF 2	1	3 months	80 : 80	1 : 3.3
BBF 3	2	3 ½ months	80 : 80	1 : 3.3
BBF 4	2	4 ½ months	80 : 80	1 : 3.3
BBF 5	2	5 ½ months	80 : 160	1 : 6.6
BBF 6	2	5 ½ months	120 : 240	1 : 6.5

a = Ratio of the amount (ng) of *bar-b32* fragment to the *pgip2* fragment bombarded onto Hi-II calli
b = Ratio of the number of molecules (moles) of *bar-b32* fragment to the *pgip2* fragment bombarded onto Hi-II callli

Table 5.2 Conditions for co-bombardment of Hi-II callus tissues with the pSC1mpib32 and pRTL2-improved PGIP plasmids

Experiment #	Age of Hi-II calli (months)	# Plates of Hi-II calli BB
BBM 1	3 months	3
BBM 2	4 months	2
BBM 3	4 ½ months	6
BBM 4	5 months	6
BBM 5	5 months	11

Table 5.3 Data of bombardment of Hi-II callus tissues with the respective strawberry terpene synthase genes indicated

Experiment #	Plasmid BB	Age of Hi-II calli (months)	# Plates of Hi-II calli BB
BBI 1	pAHC*inors*	3 Months	1
BBI 2	pAHC*inors*	3 ½ months	1
BBI 3	pAHC*inors*	4 months	1
BBI 4	pAHC*inors*	5 months	1
BBT 1	pAHC*tnors*	3 months	1
BBT 2	pAHC*tnors*	4 ½ months	1
BBT 3	pAHC*tnors*	5 months	1

5.3.5 Selection and regeneration of transgenics

Selection and regeneration of putative transgenics were done according to the method outlined in Section 4.3.4.

5.3.6 Germination and Pollination of Transgenic progeny

Putative transgenic seeds were germinated on ½MS media supplemented with 1 mg l^{-1} bialaphos, and incubated at 25°C under fluorescent lights (70 - 80 μmol m^{-2}s). Putative transgenics were either self-pollinated within the same event, or cross-pollinated with untransformed M37W plants.

5.3.7 Chlorophenol Red (CPR) Assay

This assay was performed as outlined in Section 3.3.6.

5.3.8 Herbicide Resistance / Basta® painting of leaves

A leaf of the putative transgenic plant or the control (untransformed) plant (3-4 leaf stage or older) was painted as outlined in Section 3.3.7. Tissue necrosis on the painted leaf surface was observed within 5 days after painting and leaves were recorded as being herbicide resistant (R) or sensitive (S).

5.3.9 DNA Extraction

Genomic DNA was extracted from putative transgenic maize leaf material using the method outlined in Section 4.3.8.

5.3.10 Polymerase Chain Reaction (PCR) Analysis

The primers and PCR protocol for amplification of the *b32*, *pgip2*, *bar* and *nad* genes are described in Section 3.3.9. T*nors* – (5'- GCA TTG CCG AAG ATA GC TTG C - 3') and (5'- GCA TTG GTT GCC ATC GTA GC -3'), and *bla* – (5' - TGCTTAATCAGTGAGGCACC - 3') and (5' - AGATCAGTTGGGTGCACG - 3') specific primers were used to amplify the 1554bp and 631bp fragment respectively, from genomic DNA preparations of putative transgenic Hi-II maize plants. The PCR program used was similar to the program described in Section 3.3.9, with the exception of the annealing temperature being 60°C for *tnors* and 62 °C for the *bla* (ampicillin resistance) gene.

5.4   RESULTS

5.4.1 BOMBARDMENT OF Hi-II CALLI WITH *bar-b32* AND *pgip2* LINEAR DNA MINIMAL TRANSGENE CASETTES

5.4.1.1 Selection and regeneration of transgenics

Six independent experiments were performed (11 plates of Hi-II calli in total were bombarded). The number of plates of Hi-II calli bombarded was dependant on the amount of Hi-II calli available at time of bombardment. Putatively transformed Hi-II calli were identified by its vigorous proliferation (producing more type-II calli) on bialaphos-containing $N6_{10}$ medium, while non-transformed tissues turned brown and watery.

Table 5.4   Data of bombardment experiments performed on Hi-II callus tissue with the *bar-b32* and *pgip2* linear minimal transgene cassettes

Experiment #	*bar-b32* :*pgip2* fragment BB (ng)	% Hi-II calli proliferating after 4 weeks on selection media [a]	% Hi-II calli proliferating after 6 weeks on selection media [b]	# T_0 events identified [c]
BBF 1	80 : 80	45 %	20 %	10
BBF 2	80 : 80	50 %	20 %	1
BBF 3	80 : 80	65%	20 %	2
BBF 4	80 : 80	60 %	50 %	0
BBF 5	80 : 160	60 %	40 %	6
BBF 6	120 : 240	45 %	30 %	5

a = $N6_{10}$ callus induction media supplemented with 3 mg l^{-1} bialaphos used as selection media
b = $N6_{10}$ callus induction media supplemented with 5 mg l^{-1} bialaphos used as selection media
c = Number of rooted and shooted plantlets on regeneration media

The percentage of proliferating Hi-II calli varied from 45 - 65 % within the first 4 weeks of selection (3 mg l^{-1} bialaphos). This percentage however, decreased to 20 - 50 % after 6 weeks of selection (5 mg l^{-1} bialaphos) on $N6_{10}$ callus induction media. Bialaphos-resistant Hi-II calli, when subcultured onto OT6S maturation media (3 mg l^{-1} bialaphos), matured to form somatic embryos. Shoots and roots were produced from these somatic embryos (Figure 5.3) when transferred to OT2S regeneration medium (supplemented with 1 mg l^{-1} bialaphos).
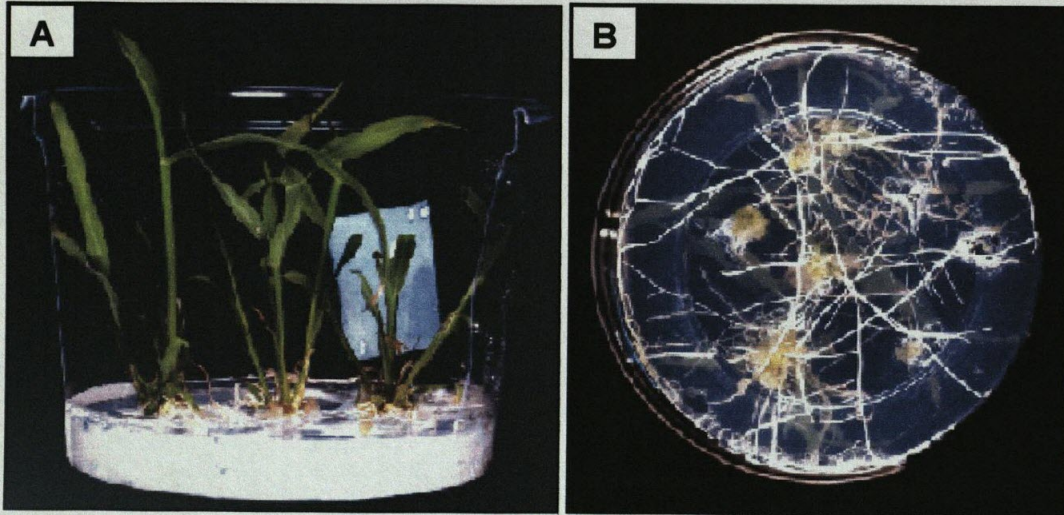
Fig 5.3 Putative Hi-II transgenic plants (BBF 1.1) regenerating on OT2S regeneration media (supplemented with 1mg l^{-1} bialaphos). Plants display: **A)** healthy shoot (greater than 3cm in height) development and **B)** strong root (greater than 1cm in length) development.

Twenty-four independent events (listed in table 5.5) were identified. For easier reference, a code will be allocated: for example, T_0 plants from experiment BBF 1 will be referred to as BBF 1.1A, BBF 1.1B, etc. and the T_1 progeny from event BBF 1.1 will be BBF 1.1A1, BBF 1.1A2, etc. Only regenerating plantlets producing shoots greater than 3 cm in height and roots greater than 1 cm in length, were counted as events. These T_0 putative transgenics were successfully hardened off (figure 5.4A) and then transferred into pots under greenhouse conditions. Many putative T_0 transgenic plants however, did not survive when grown in pots under greenhouse conditions. Plant leaves and stalks displayed tissue necrosis and browning within a week, and these plants (not exceeding 20 cm in height) died within 2 weeks of growing in pots (figure 5.4B).

Three independent T_0 putative transgenics (BBF 1.1A, BBF 1.1B and BBF 5.1A) survived in pots when grown under greenhouse conditions (exceeding heights of 20 cm). They displayed dwarfism and abnormal ear production with only one (BBF 1.1A) of these 3 surviving events, being fertile to produce four T_1 seeds.

These T_1 seeds (when cultured on ½ MS media supplemented with 1 mg l^{-1} bialaphos) resulted in 1 seed germinating. This T_1 progeny developed a normal, healthy maize phenotype with both male and female flowering parts (figure 5.5). T_2 transgenic seeds from this event were produced.
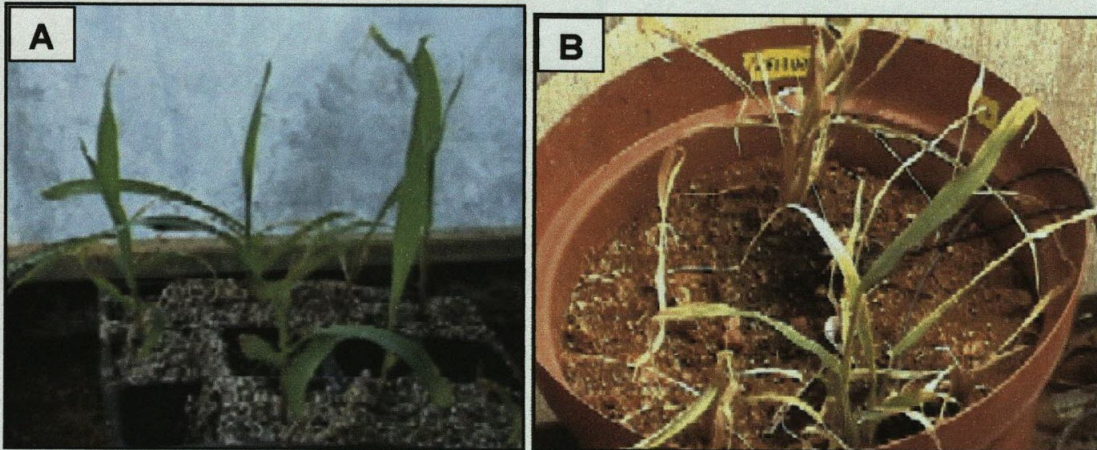
Fig 5.4 A) Putative T_0 transgenic plantlets (BBF 1.1) successfully hardened off (healthy, green shoots greater than 10 cm displayed) on a mist bed under greenhouse conditions; and **B)** Putative T_0 transgenic plants (BBF 1.1) showing extensive tissue necrosis (leaf and stalk browning) and reduced plant growth (height not exceeding 20 cm) with plant death occurring within 2 weeks after being transferred into pots under greenhouse conditions.
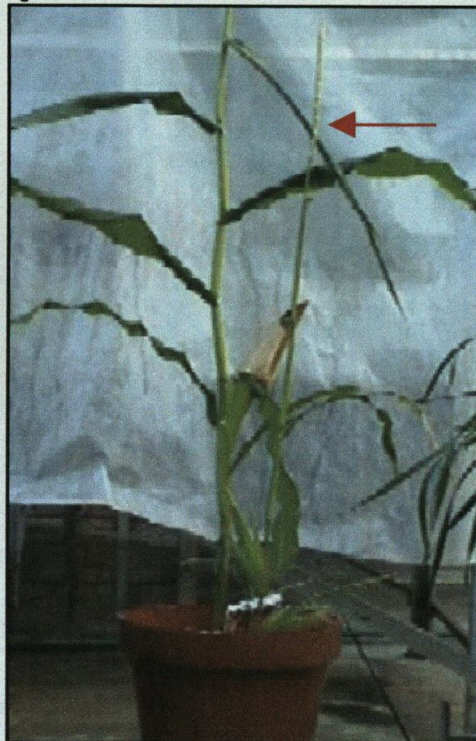
Fig 5.5 T_1 transgenic Hi-II maize plant (BBF 1.1A1, cross pollinated with untransformed M37W) displayed the normal, healthy, tall phenotype associated with maize development. The plant was fertile and showed healthy, normal ear and tassel formation (red arrow) when grown under greenhouse conditions.

5.4.1.2 Chlorophenol Red (CPR) Assay

The C PR a ssay (Wright *et al.*, 1 996) is d escribed in S ection 4 .4.2. N ineteen T_0 events tested CPR positive, while five were CPR negative (Table 5.5). These results were confirmed by PCR testing for the *bar* gene (Table 5.5).

5.4.1.3 Molecular Analysis of putative transgenics
T_0 Putative Transgenics

Ten T_0 events (from 24 independent events) were analysed by PCR for the presence or absence o f the *b 32*, *pgip2* a nd *bar* g enes (Figure 5.6A). T he positive c ontrols containing plasmid DNA (lanes 1 and 4) produced the expected amplified products of 875 bp, 731 bp and 279 bp for the *b32*, *pgip2* and *bar* genes respectively. The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA), as expected, did not produce any amplified products. Three events: BBF 1.1C (lane 7), BBF 1.1D (lane 8) and BBF 5.1A (lane 11), produced correct amplified bands for all three genes (*b32*, *pgip2* and *bar*). Five other events, BBF 1.1A, BBF 1.1B, BBF 1.1G, BBF 1.1V and BBF 1.1H (lanes 5, 6, 12, 13 and 14 respectively) displayed positive bands for the *b32* and *bar* genes only. Additionally, o ne event B BF 1.1F (lane 10) p roduced a mplified bands for *b 32* and *pgip2* genes only, while event BBF 1.1E (lane 9) produced a band for the *pgip2* and *bar* genes. A list of the PCR results for all the T_0 events tested occur in table 5.5.

PCR o f the *nad* g ene (figure 5.6B) produced positive a mplified DNA bands f or all events tested: lanes 3 to 12 (BBF1.1A, BBF1.1B, BBF1.1C, BBF1.1D, BBF1.1E, BBF1.1F, BBF5.1A, BBF1.1G, BBF1.1V and BBF1.1H respectively). Positive amplified bands also occurred for untransformed Hi-II DNA (lane 2) tested. The negative control (lane 1) did not produce any amplified DNA bands. All events tested for this gene are listed in Table 5.5.

PCR for the presence of the ampicillin resistance gene (*bla*) (figure 5.6C) was negative for lanes 1 (no template) and 2 (untransformed Hi-II). The positive control (lane 3 containing untransformed Hi-II spiked with pSC1b32 plasmid DNA) produced an amplified band of the correct size. Other events producing positive amplified bands for the *amp* gene are BBF 1.1A, BBF 1.1B, BBF 1.1F, BBF 5.1A, BBF 1.1G, BBF 1.1V and BBF 1.1H (lanes 5, 9, 10, 11, 12 and 13 respectively. Figure 5.6D shows positive amplified bands for the *bla* gene produced by the positive control (lane 1), event A1E3 (lane 2) and event H12J (lane 3). A1E3 and H12J are events bombarded with full plasmid vectors outlined in chapter 4.
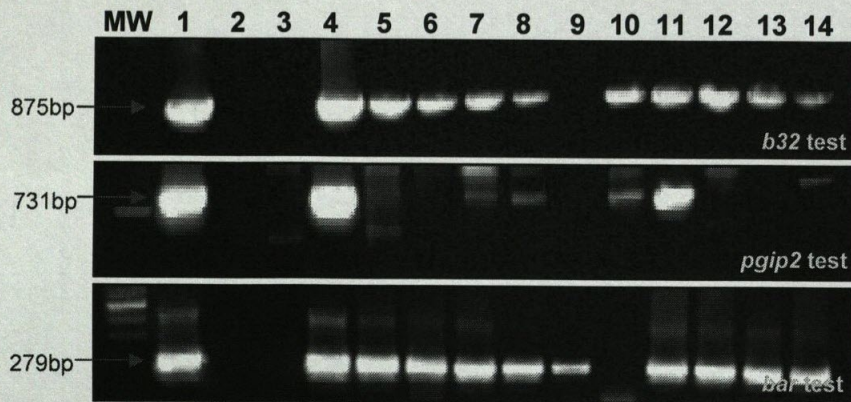
Fig 5.6A PCR of genomic DNA of putatively transgenic T_0 plants for *b32*, *pgip2* and *bar* genes. Lane M: DNA molecular weight marker (100bp ladder); Lane 1: positive control of the respective plasmid DNA (pSC1b32 for *b32* and *bar* and pGEMpgip2 for *pgip2* PCRs); Lane 2: PCR mix without DNA; Lane 3: untransformed Hi-II genomic DNA (negative control); Lane 4: untransformed Hi-II genomic DNA spiked with 1ng of the respective plasmid DNA; Lanes 5 to 14 represent genomic DNA (50ng) of the T_0 putative transgenics: lane 5: BBF 1.1A, lane 6: BBF 1.1B, lane 7: BBF 1.1C, lane 8: BBF 1.1D, lane 9: BBF 1.1E, lane 10: BBF 1.1F, lane 11: BBF 5.1A, lane 12: BBF 1.1G, lane 13: BBF 1.1V and lane 14: BBF 1.1H.
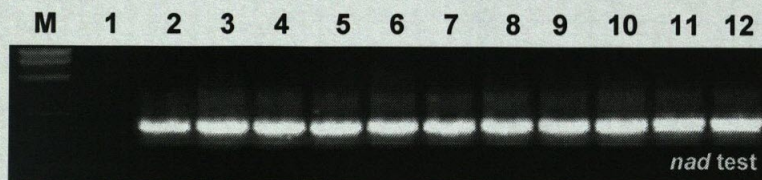
Fig 5.6 B) PCR of genomic DNA of putatively transgenic T_0 plants for the *nad* gene. Lane M: DNA molecular weight marker IV; Lane 1: PCR mix without DNA (negative control); Lane 2: 1ng untransformed Hi-II genomic DNA; Lanes 3 to 12 represent genomic DNA (50ng) of the T_0 putative transgenics: lane 3: BBF 1.1A, lane 4: BBF 1.1B, lane 5: BBF 1.1C, lane 6: BBF 1.1D, lane 7: BBF 1.1E, lane 8: BBF 1.1F, lane 9: BBF 5.1A, lane 10: BBF 1.1G, lane 11: BBF 1.1V and lane 12: BBF 1.1H.
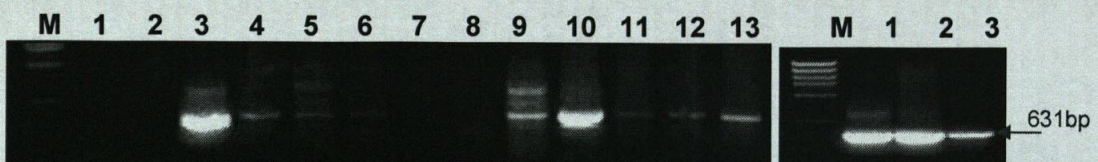
Fig 5.6 C) PCR of genomic DNA of putatively transgenic T_0 plants (minimal transgene cassettes bombarded) for the ampicillin resistance (*bla*) gene. Lane M: DNA molecular weight marker IV; Lane 1: PCR mix without DNA; Lane 2: untransformed Hi-II genomic DNA (negative control); Lane 3: untransformed Hi-II genomic DNA spiked with 1ng of pSC1b32 plasmid DNA; Lanes 4 to 13 represent genomic DNA (50ng) of T_0 putative transgenics: lane 4: BBF 1.1A, lane 5: BBF 1.1B, lane 6: BBF 1.1C, lane 7: BBF 1.1D, lane 8: BBF 1.1E, lane 9: BBF 1.1F, lane 10: BBF 5.1A, lane 11: BBF 1.1G, lane 12: BBF 1.1V and lane 13: BBF 1.1H. **D)** PCR of genomic DNA of transgenic T_3 plants (full plasmids bombarded from chapter 4) for the ampicillin resistance (*amp*) gene. Lane M: DNA molecular weight marker IV; Lane 1: positive control with 1ng of the pSC1b32 plasmid DNA; Lane 2: A1E3 and Lane 3: H12J.

Table 5.5 Summary of results obtained for T_0 putative transgenics produced from bombardment experiments with the *bar-b32* and *pgip2* linear minimal DNA transgene cassettes

Transformation Event Identified	CPR Result	T_0 PCR Results					Pollination	# T_1 seeds produced
		b32	*pgip2*	*bar*	*amp*[b]	*nad*[c]		
BBF 1.1 A[a]	+	+	-	+	+	+	M37W	4
BBF 1.1 B[a]	+	+	-	+	-	+	M37W	0
BBF 1.1 C	+	+	+	+	-	+	M37W	0
BBF 1.1 D	+	+	+	+	-	+	M37W	0
BBF 1.1 E	+	-	-	+	-	+	M37W	0
BBF 1.1 F	-	+	+	-	+	+	M37W	0
BBF 1.1 G	+	+	-	+	-	+	M37W	0
BBF 1.1 H	+	+	-	+	+	+	M37W	0
BBF 1.1 V	+	+	-	+	+	+	M37W	0
BBF 1.2 A	+	-	-	+	-	+	M37W	0
BBF 2.1B	+	+	-	+	-	+	M37W	0
BBF 3.1 D	-	+	-	-	+	+	M37W	0
BBF 3.1 G	-	-	-	-	-	+	M37W	0
BBF 5.1 A[a]	+	+	+	+	+	+	M37W	0
BBF 5.1 E	+	+	-	+	-	+	M37W	0
BBF 5.1 A	+	+	-	+	-	+	M37W	0
BBF 5.2 C	+	+	-	+	-	+	M37W	0
BBF 5.2 F	+	-	-	+	-	+	M37W	0
BBF 5.2 C	-	-	-	-	-	+	M37W	0
BBF 6.1 A	+	-	-	+	+	+	M37W	0
BBF 6.1 C	+	+	-	+	-	+	M37W	0
BBF 6.1 F	+	+	+	+	-	+	M37W	0
BBF 6.1 K	-	+	-	-	-	+	M37W	0
BBF 6.2 A	+	+	-	+	-	+	M37W	0

a = Only these events survived in pots under greenhouse conditions, with only BBF 1.1A producing T_1 seeds
b = PCR test for the presence of the ampicillin resistance gene
c = PCR test for the presence of the *nad* gene (a conserved multicopy gene of the mitochondria)

The only germinating T_1 progeny from event BBF 1.1A, displayed herbicide sensitivity (tissue browning and necrosis) when tested by Basta® painting of a leaf section. This T_1 progeny (BBF 1.1A 1) was negative for the *b32*, *pgip2*, *bar* and *amp* genes, when tested via PCR (data not shown). However, PCR for the presence of the *nad* gene tested positive (data not shown).

5.4.2 BOMBARDMENT OF Hi-II CALLI WITH THE pSC1-mpib32 AND pRTL2-improvedPGIP PLASMIDS

5.4.2.1 Selection and Regeneration of Transgenics

Five independent experiments were performed with a total of 28 plates of Hi-II callus tissues being bombarded. Bombarded calli were placed on $N6_{10}$ callus induction media supplemented with 3mg l^{-1} bialaphos for selection. Putatively transformed Hi-II calli were identified by its proliferation on selection media, while untransformed callus tissues displayed tissue necrosis (brown and watery callus formation). After 4 weeks of selection, the percentage of proliferating Hi-II calli ranged from 5 - 46 %. This percentage however, decreased upon further selection (data not included). Surviving Hi-II calli (bialaphos resistant) were maintained as individual events and allowed to proliferate on individual tissue culture plates. A part of the proliferating Hi-II callus tissue (for each event) was removed for genomic DNA extraction (molecular analysis performed in section 5.4.2.2), while remaining tissues were transferred onto OT6S (3 mg l^{-1} bialaphos) and OT2S media (1 mg l^{-1} bialaphos) for maturation and regeneration respectively. No regenerating plants however, were produced (no shoot or root production occurred).

5.4.2.2 Molecular Analysis of Hi-II calli events

The genomic DNA isolated from individually surviving calli events, were tested via PCR for the presence of the *b32*, *pgip2* and *bar* genes. Figure 5.7A shows that none of the events tested, were positive for the *b32* or *pgip2* genes (absence of amplified band for the respective genes in lanes 5 to 14). Three events however, in lanes 6 (BBM 1.1), 7 (BBM 2.1) and 8 (BBM 3.1) produced the correct amplified bands for the *bar* gene only.

The positive controls containing plasmid DNA (lanes 1 and 4) produced the expected amplified products of 875 bp, 731 bp and 279 bp for the *b32*, *pgip2* and *bar* genes respectively. The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA), as expected, did not produce any amplified products.
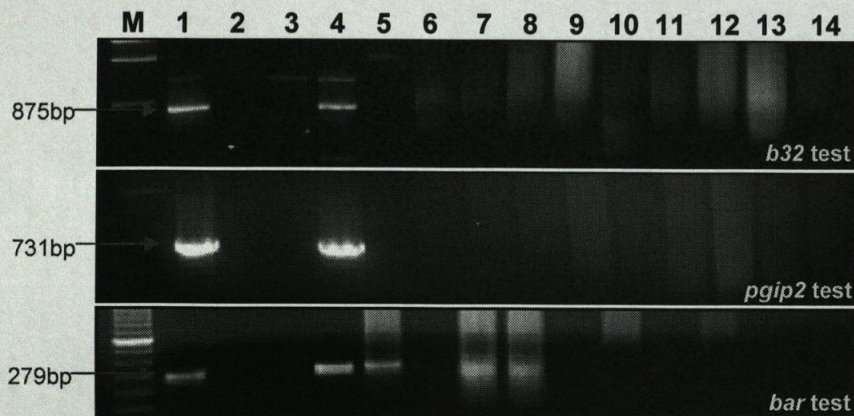
Fig 5.7 A) PCR of genomic DNA isolated from independently bombarded Hi-II callus tissues (surviving after 4 weeks on selection media) for the *b32*, *pgip2* and *bar* gene presence. Lane M: DNA molecular weight marker (100bp ladder); Lane 1: positive control of the respective plasmid DNA (pSC1b32 for *b32* and *bar,* and pGEMpgip2 for *pgip2* PCRs); Lane 2: PCR mix without DNA; Lane 3: untransformed Hi-II genomic DNA (negative control); Lane 4: untransformed Hi-II genomic DNA spiked with 1ng of the respective plasmid DNA; Lanes 5 to 14 represent genomic DNA (50ng) of the bombarded callus tissues: lane 5: BBM 1.1, lane 6: BBM 1.2, lane 7: BBM 2.1, lane 8: BBM 3.1, lane 9: BBM 4.1, lane 10: BBM 4.4, lane 11: BBM 5.3, lane 12: BBM 5.5, lane 13: BBM 5.8 and lane 14: BBM 5.11.

PCR results of the *nad* gene for the selected events tested in Figure 5.7A, occur in figure 5.7B. Positive amplified DNA bands (of the correct size) were produced for all events tested in lanes 3 to 12 (BBM 1.1, BBM 1.2, BBM 2.1, BBM 3.1, BBM 4.1, BBM 4.4, BBM 5.3, BBM 5.5, BBM 5.8 and BBM 5.11 respectively). Positive amplified bands were also produced for untransformed Hi-II DNA (lane 2) tested. The negative control (lane 1) did not produce any amplified DNA bands. Table 5.6 displays a summary of the results obtained for all Hi-II calli events tested.
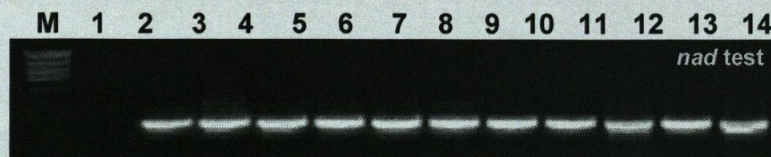
Fig 5.7 B) PCR of genomic DNA isolated from independently bombarded Hi-II callus tissues (surviving after 4 weeks on selection media) for the *nad* gene presence. Lane M: DNA molecular weight marker IV; Lane 1: PCR mix without DNA (negative control); Lane 2: 1ng untransformed Hi-II genomic DNA; Lanes 3 to 12 represent genomic DNA (50ng) of bombarded callus tissues: lane 3: BBM 1.1, lane 4: BBM 1.2, lane 5: BBM 2.1, lane 6: BBM 3.1, lane 7: BBM 4.1, lane 8: BBM 4.4, lane 9: BBM 5.3, lane 10: BBM 5.5, lane 11: BBM 5.8 and lane 12: BBM 5.11.

Table 5.6   Data of bombardments of Hi-II callus tissues with the pSC1-mpib32 and pRTL2-improvedPGIP plasmids

Experiment #	% calli proliferating after 4 weeks on selection media[a]	PCR results of Putatively transgenic Hi-II calli			
		b32	*pgip2*	*bar*	*nad*
BBM 1.1	20 %	-	-	+	+
BBM 1.2	30 %	-	-	-	+
BBM 1.3	5%	-	-	-	+
BBM 2.1	40%	-	-	+	+
BBM 3.1	30%	-	-	+	+
BBM 4.1	46 %	-	-	-	+
BBM 4.2	15 %	-	-	-	+
BBM 4.3	20 %	-	-	-	+
BBM 4.4	44 %	-	-	-	+
BBM 4.5	45 %	-	-	-	+
BBM 4.6	20 %	-	-	-	+
BBM 5.1	40 %	-	-	-	+
BBM 5.2	30 %	-	-	-	+
BBM 5.3	46 %	-	-	-	+
BBM 5.4	35 %	-	-	-	+
BBM 5.5	40 %	-	-	-	+
BBM 5.6	20 %	-	-	-	+
BBM 5.7	35 %	-	-	-	+
BBM 5.8	35 %	-	-	-	+
BBM 5.9	10 %	-	-	-	+
BBM 5.10	25 %	-	-	-	+
BBM 5.11	46 %	-	-	-	+

a = $N6_{10}$ callus induction media supplemented with 3 mg l^{-1} bialaphos used as selection media

5.4.3 BOMBARDMENT OF Hi-II CALLI WITH THE TERPENE SYNTHASE (*inors* and *tnors*) GENES

5.4.3.1 Selection and regeneration of transgenics

Four independent bombardment experiments were performed with pAHC*inors* plasmid (table 5.7). After 4 weeks on $N6_{10}$ callus induction media (3 mg l^{-1} bialaphos selection), Hi-II calli proliferation varied between 10-20%. However, these calli did not survive further selection transfers, becoming watery and brown after 6 weeks on selection media. None of these calli regenerated into plantlets. Thus no transgenics with the *inors* gene were produced.

Bombardment with the pAHC*tnors* plasmid resulted in 30-50% of the bombarded type-II calli proliferating after 4 weeks on $N6_{10}$ callus induction media supplemented with 3 mg l^{-1} bialaphos (Table 5.7).

Table 5.7 Data of bombardment of Hi-II callus tissues with the respective plasmid vectors indicated.

Experiment #	Plasmid BB	# Plates of Hi-II calli BB	% calli proliferating after 4 weeks on selection media [a]	# Events identified [b]
BBI 1	pAHC*inors*	1	20 %	0
BBI 2	pAHC*inors*	1	20 %	0
BBI 3	pAHC*inors*	1	20%	0
BBI 4	pAHC*inors*	1	10 %	0
BBT 1	pAHC*tnors*	1	50 %	18
BBT 2	pAHC*tnors*	1	40 %	8
BBT 3	pAHC*tnors*	1	30%	5

a = $N6_{10}$ callus induction media supplemented with 3 mg l^{-1} bialaphos used as selection media
b = Number of rooted and shooted plantlets produced on OT2S regeneration media (1mg l^{-1} bialaphos selection)

Bialaphos-resistant Hi-II calli were transferred onto OT6S media (3 mg l^{-1} bialaphos) for maturation, producing somatic embryos. These somatic embryos produced shoots and roots when placed onto OT2S regeneration media (1 mg l^{-1} bialaphos), as displayed in figure 5.8A. Only regenerating plantlets producing shoots greater than 3cm in height, and roots greater than 1 cm in length (figure 5.8A), were counted as an event. Thirty-one independent T_0 events were identified from the 3 independent experiments (3 plates of Hi-II callus bombarded in total).

Many putative T_0 transgenics produced a sweet, floral scent when regenerating in tubs. However, this scent was not detected when hardened off and transferred into pots under greenhouse conditions. All T_0 plants displayed dwarfism, abnormal ear and tassel formation (figure 5.98), with many plants dying within two weeks of growth in pots. Only two T_0 events survived (events BBT 1.1B and BBT 2.1A), however only event (BBT 1.1B) was fertile to produce sixteen T_1 seeds (cross-pollinated with untransformed M37W plants).
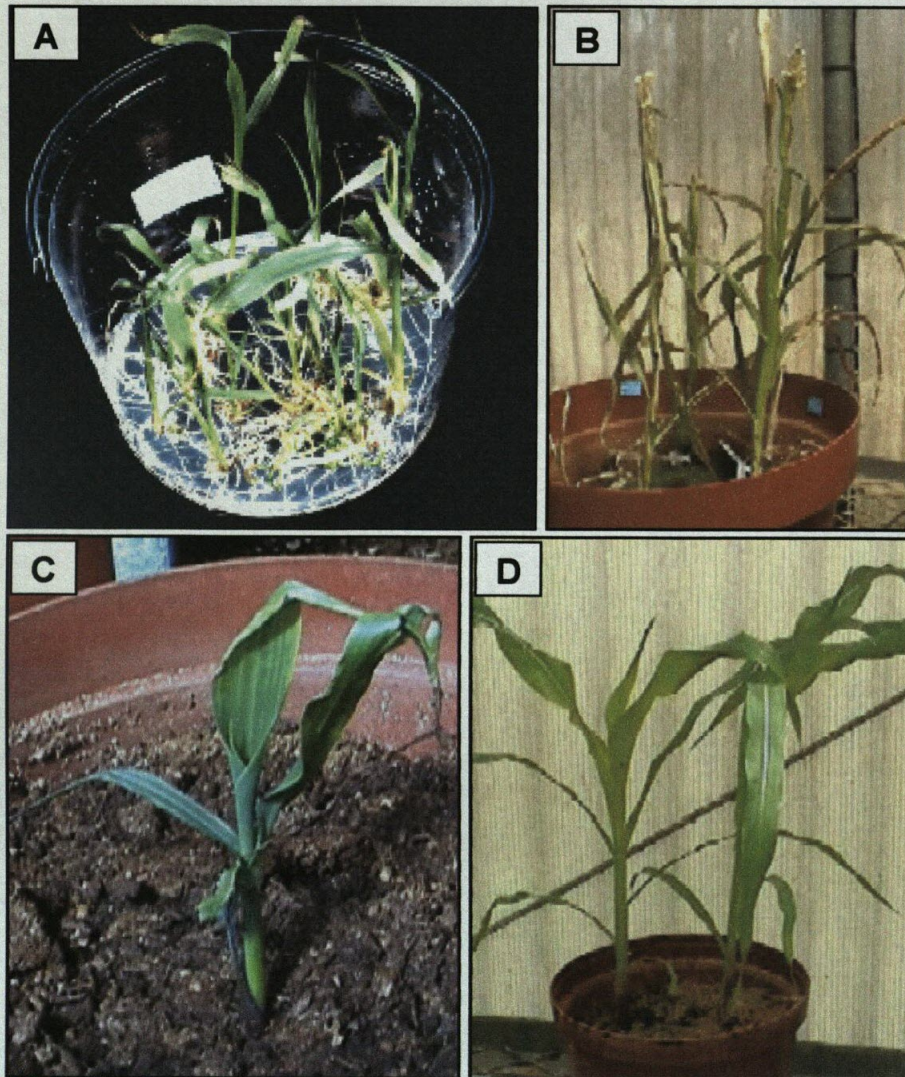
Fig 5.8   A) Putatively transgenic T_0 Hi II plantlets (BBT 1.1) displaying healthy shoot (greater than 3 cm in height) and extensive root (greater than 1 cm in length) development on OT2S regeneration medium (1 mg l^{-1} bialaphos); **B)** Putative T_0 Hi-II transgenic plants (BBT 1.1) displaying dwarfism and abnormal ear and tassel formation when grown in pots under greenhouse conditions; **C)** Putative T_1 progeny (BBT 1.1B1) displaying firm stalk and leaf development after 2 weeks of germination, and **D)** Putative T_1 transgenic progeny (BBT 1.1B1 and BBT 1.1B2) after 4 weeks of growth under greenhouse conditions, displaying the normal tall maize phenotype.

Only six of the sixteen T_1 seeds produced (BBT 1.1B), germinated on ½ MS media (supplemented with 1 mg l^{-1} bialaphos) producing shoots (greater than 3 cm in height) and roots (greater than 1 cm in length). Five of these six T_1 plantlets produced a distinct sweet, floral/lemon-like smell when germinating in tubs however, this smell was not detected when these plants were hardened off and places into pots under greenhouse conditions. These T_1 progeny plants displayed strong, healthy stalk and leaf development after 2 weeks (figure 5.8C), and after 4 weeks, tall phenotypes were observed (figure 5.8D). All T_1 progeny were Basta® painting sensitive.

5.4.3.2 Molecular Analysis of putative transgenics

PCR analysis of the T_0 and T_1 putative transgenics were performed. Figure 5.9A displays the PCR results of selected T_0 putative transgenics for the *tnors* and *bar* genes. The positive controls containing plasmid DNA (lanes 1 and 4) produced the expected amplified products of 1544 bp and 279 bp for the *tnors* and *bar* genes respectively. The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA) did not produce any amplified products. Four lanes: 6, 7, 9 and 10 (BBT 1.1B, BBT 1.1C, BBT 1.4A and BBT 1.24A) produced the correct amplified bands for the *tnors* and *bar* genes. Lanes 5, 8, 11 and 12, representing events BBT 1.1A, BBT 1.1D, BBT 2.1A and BBT 2.1B respectively, tested positive for the *bar* gene only. All events tested were positive for the PCR amplification of the *nad* gene (data not included).
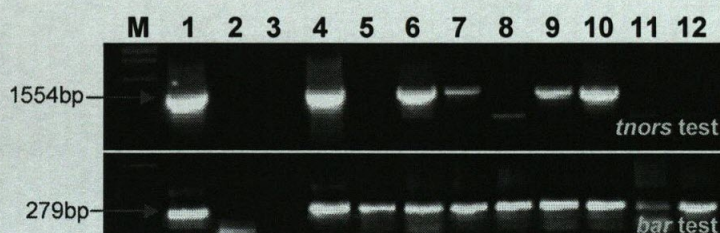
Fig 5.9 PCR of genomic DNA of putatively transgenic T_0 plants for *tnors* and *bar* genes. Lane M: DNA molecular weight marker (100bp ladder); Lane 1: positive control containing pAHCtnors plasmid DNA; Lane 2: PCR mix without DNA; Lane 3: untransformed Hi-II genomic DNA (negative control); Lane 4: untransformed Hi-II genomic DNA spiked with 1ng of pAHCtnors; Lanes 5 to 12 represent genomic DNA (50ng) of the T_0 putative transgenics: lane 5: BBT 1.1A, lane 6: BBT 1.1B, lane 7: BBT 1.1C, lane 8: BBT 1.1D, lane 9: BBT 1.4A, lane 10: BBT 1.24A, lane 11: BBT 2.1A and lane 12: BBT 2.1B.

PCR analysis of the T_1 progeny is displayed in figure 5.10A. None of the T_1 progeny produced any amplified PCR bands for the *tnors* and *bar* genes (lanes 6 to 11). Lane 5 (the T_0 parent plant, BBT 1.1B) however produced positive bands for the *tnors* and *bar* genes.

The negative controls: lane 2 (containing no template DNA) and lane 3 (containing untransformed Hi-II genomic DNA), as expected, did not produce any amplified products. Positive controls containing plasmid DNA (lanes 1 and 4) produced the expected amplified products of 1544 bp and 279 bp for the *tnors* and *bar* genes respectively.
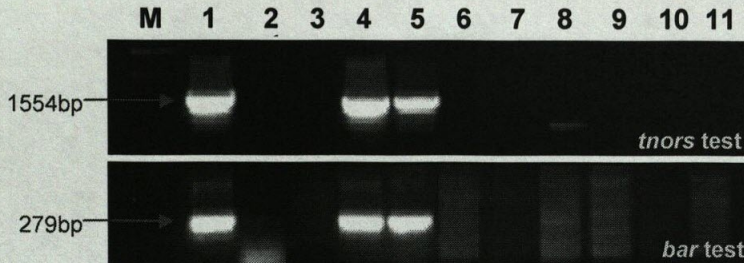
Fig 5.10 A) PCR of genomic DNA of putatively transgenic plants for *tnors* and *bar* genes. Lane M: DNA molecular weight marker (100bp ladder); Lane 1: positive control containing pAHCtnors plasmid DNA; Lane 2: PCR mix without DNA; Lane 3: untransformed Hi-II genomic DNA (negative control); Lane 4: untransformed Hi-II genomic DNA spiked with 1ng of pAHCtnors; Lane 5: 50ng genomic DNA of the T_0 parent event BBT 1.1B; lanes 6 to 11 represent genomic DNA (50ng) of the T_1 putative transgenics: lane 6: BBT 1.1B1, lane 7: BBT 1.1B2, lane 8: BBT 1.1B3, lane 9: BBT 1.1B4, lane 10: BBT 1.1B5 and lane 11: BBT 1.1B6.
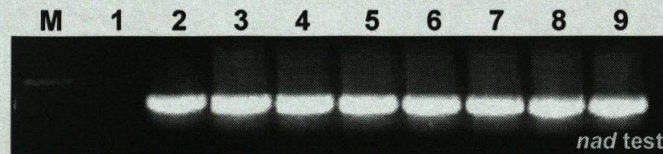
Fig 5.10 B) PCR of genomic DNA of putatively transgenic plants for *nad* gene. Lane M: DNA molecular weight marker IV; Lane 1: PCR mix without DNA (negative control); Lane 2: 1ng untransformed Hi-II genomic DNA; Lanes 3: 50ng genomic DNA of the T_0 parent event BBT 1.1B; lanes 4 to 9 represent genomic DNA (50ng) of the T_1 putative transgenics: lane 4: BBT 1.1B1, lane 5: BBT 1.1B2, lane 6: BBT 1.1B3, lane 7: BBT 1.1B4, lane 8: BBT 1.1B5 and lane 9: BBT 1.1B6.

PCR of the *nad* gene (figure 5.10B) produced positive amplified DNA bands for all events tested: lanes 3 to 9 (BBT 1.1B, BBT 1.1B1, BBT 1.1B2, BBT 1.1B3, BBT 1.1B4, BBT 1.1B5 and BBT 1.1B6 respectively). Positive amplified bands also occurred for the untransformed Hi-II DNA (lane 2). The negative control (lane 1) did not produce any amplified DNA bands. PCR results for all T_1 progeny are listed in Table 5.8.

Table 5.8 Summary of results obtained for the T_1 progeny of event BBT 1.1B (bombardment experiments with pAHCtnors)

T_1 Progeny	Basta® paint result	T_1 PCR Results			Pollination	# T_2 seeds produced
		tnors	bar	nad		
BBT 1.1B 1	S	-	-	+	M37W	> 50
BBT 1.1B 2	S	-	-	+	Self	> 50
BBT 1.1B 3	S	-	-	+	M37W	> 50
BBT 1.1B 4	S	-	-	+	Self	> 50
BBT 1.1B 5	S	-	-	+	Self	0
BBT 1.1B 6	S	-	-	+	Self	> 50

Table 5.8 shows that none of the T_1 progeny were PCR positive for the *tnors* and *bar* genes. The number of T_2 seeds produced for the progeny are also listed.

5.5 DISCUSSION

5.5.1 BOMBARDMENT OF Hi-II CALLI WITH *bar-b32* AND *pgip2* LINEAR MINIMAL TRANSGENE CASETTES

Whole plasmids are used in both *Agrobacterium*-mediated transformation and direct DNA transfer, generally leading to the integration of undesired vector backbone sequences into the host genome along with the transgene(s). These vector backbone sequences often have negative effects on the expression of the transgene or an endogenous gene, and can promote transgene rearrangements (Matze *et al.*, 1996).

Therefore, in this study maize Hi-II callus tissues were co-bombarded with two linear DNA fragments (*bar-b32* and *pgip2* linear minimal transgene cassettes) using biolistics. There have been no data published to date on the optimal bombardment ratio of minimal transgene cassettes for maize. Thus, different ratios of 1:1 and 1:2 of the *bar-b32* to *pgip2* minimal transgene cassette (Table 5.4) were examined. Hi-II maize callus tissues were previously transformed using a total DNA mass of 160 ng per bombardment shot (O'Kennedy *et al.*, 1998). This total concentration per shot was maintained in most experiments even though the mass of the individual fragments varied.

The bombardment conditions used for bombardment experiments were according to those found optimal for Hi-II maize callus transformation (O'Kennedy et al., 1998) and are explained in Section 4.5.

Bombarded Hi-II callus tissue was selected on bialaphos-containing callus induction media ($N6_{10}$). Bialaphos concentrations were increased on further selection in order to eliminate possible "escape" plants. This possibly explains the decrease in percentage of proliferation calli from 45 – 65 % (after 4 weeks of selection) to 20 – 50 % (after 6 weeks of selection). However, not all of the proliferating callus tissue formed somatic embryos and regenerating plantlets: only 24 independent T_0 events produced were successfully rooted and shooted plantlets on regeneration media. This can be attributed to either tissue culture stress (preventing root or shoot development) or that some of the surviving calli were actually "escapes" and were thus, not able to regenerate in the presence of bialaphos on the regeneration medium. The CPR results of all leaf pieces of regenerating putative transgenics tested, correlated with the presence or absence of the bar gene by PCR analysis.

T_0 putative transgenics displayed abnormal phenotypes, which can be attributed to the tissue culture stress, as the respective T_1 plants (figure 5.6, germinating directly from seeds) were of normal, tall maize phenotypes. Few events however, were fertile. This can be explained by either the tissue culture stress and selection conditions that transformed callus tissues were subjected to, or that the transgenes introduced were unstable and negatively affected the plant growth.

The nad gene is a conserved multicopy gene of the mitochondria (Mannerlof & Tenning, 1997), and thus occurs in all plants. The positive testing for the nad gene presence by PCR for all putative transgenics, indicated that the genomic DNA extracted for molecular analysis was of good quality. Thus, the negative PCR testing for some genes of the putative transgenics, were indeed negative (no gene integration) and not due to poor genomic DNA quality.

PCR analysis for the presence of the ampicillin resistance gene (bla), which normally occurs in the plasmid vector backbone, is expected to be positive for many transgenics bombarded with the full plasmid constructs. This positively amplified band for the bla gene presence occurred in events indicated in figure 5.6D.

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Contrarily, putative transgenics (from bombardment with only minimal transgene cassettes) was expected to be negative as these cassettes lack the plasmid vector backbone, which harbours the antibiotic resistance gene. However in figure 5.7C, many T_0 events produced a positive amplified band for the *bla* gene. This result is not due to contamination of the PCR sample mix as the negative control (PCR mix with no template) was negative. The only possible reason for this could be that the initial minimal transgene cassettes isolated, had some plasmid backbone contamination. These cassettes were digested from the respective plasmids and size fractionated on an agarose gel (as explained in Section 5.3.2). Fragments were then excised and purified from the gel. Fragments of the backbone (containing the *bla* gene) could have occurred in close proximity to the minimal transgene cassette DNA band of interest on the gel, and thus, was possibly excised with the DNA cassette bands from the gel. Future suggestions to eliminate this problem would be to include a re-purification step (size fractionating and re-purifying the DNA cassettes isolated initially). However, this process may cause loss of some of the DNA cassettes resulting in a lower concentration yield of the linear DNA minimal transgene cassettes.

The T_1 progeny produced (BBF 1.1A 1) was negative for the *b32, bar, pgip2* and *bla* genes via PCR analysis (data not shown). This result cannot be explained by poor genomic DNA quality as the *nad* gene PCR tested positive. The T_0 parent of this event (BBF 1.1A in Table 5.5) was *b32* and *bar* PCR positive. This loss of genes in the T_1 generation can be explained by transgene instability in the second generation. Transgene instability has been previously reported to change over plant generations (Cherdshewasart *et al.*, 1993). These effects have been linked to multiple copy integration, positional effects and methylation patterns (Jorgensen, 1990; 1993). An example of transgene instability due to methylation was observed in field trials of petunia with altered flower colour (Meyer *et al.*, 1992).

Thus, this study indicates that Hi-II maize callus tissue can be successfully transformed with linear minimal DNA transgene cassettes however the introduced transgenes display instability and are lost in the second generation.

5.4.2 BOMBARDMENT OF Hi-II CALLI WITH pSC1-mpib32 AND pRTL2-improvedPGIP CONSTUCTS

The pSC1-mpib32 plasmid contains the maize *b32* gene under control of the Mpi promoter (*Fusarium* inducible). The pRTL2-improvedPGIP plasmid contains bean *pgip2* gene and the signal peptide from an oat well-wall bound thionin (*Asth1*), instead of the *pgip2* signal peptide. Bombardment of these plasmids onto Hi-II callus tissues yielded a low percentage of calli proliferation within the first 4 weeks of selection. All Hi-II calli became necrotic and died on further selection, and no transgenics (rooted and shooted plants) were produced. This could possibly be due to no callus tissues being transformed, thus they were unable to survive under herbicide selection conditions.

PCR analysis of callus tissues (figure 5.7A) revealed no transgenic tissues containing either of the antifungal genes. The *bar* gene however, was incorporated in few calli tissues. The PCR analysis of *nad* gene for all Hi-II calli used, tested positive (figure 5.7B) indicating that genomic DNA used, was of good quality with no specific inhibitors for PCR amplification.

A possible reason why no transgenics were produced could be that most of the bombarded plasmids were fragmented during physical delivery, and thus only the antifungal genes (not the *bar* selectable marker gene) were incorporated. Therefore, the transformed tissues were sensitive to the bialaphos selection, and did not survive the first 2 weeks of selection. Those tissues that did survive were either 'escapes' or those that contained the *bar* gene only. A possible suggestion for future experiments would be to use a lower bialaphos concentration for initial selection, which can be increased later to eliminate escape tissues.

5.4.3 BOMBARDMENT OF Hi-II CALLI WITH THE TERPENE SYNTHASE (*inors* and *tnors*) GENES

The b iosynthesis o f t erpenoids (illustrated i n f igure 2 .4) i nvolves p roduction of the higher building blocks such as geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP). The linalool (monoterpene) synthase enzyme (encoded for by the *tnors* gene) is responsible for the conversion of GPP to form linalool in the plastid (chloroplast). This gene has a plastid-targeting signal, which results in the enzyme being targeted to the chloroplast. The nerolidol synthase enzyme (encoded for by the *inors* gene) lacks a plastid-targeting signal, and is responsible for the conversion of FPP to form nerolidol in the cytosol (reviewed in Section 2.6.3).

Scholten and co-workers (unpublished) recently reported that linalool and nerolidol showed inhibition of mycelium growth of several *Fusarium* isolates. Thus the pAHCinors (containing *inors* gene) and pAHCtnors (containing *tnors* gene) plasmids were bombarded onto Hi-II callus tissues in this study.

5.4.3.1 Bombardment with the *inors* gene

Bombardment with the pAHCinors plasmid (harbouring the *i nors* g ene) resulted i n 10-20% of the bombarded callus tissues proliferating after 4 weeks on selection media. This percentage decreased on further selection. After 6 weeks, all Hi-II calli became necrotic and no transgenics were produced. A few possible suggestions for the lack of transformants are explained. Firstly, integration of the *bar* gene was probably unsuccessful therefore, Hi-II calli did not survive the herbicide selection and no transgenics were produced.

The *inors* gene lacks the N-terminal signal peptide for chloroplast targeting (similar to *tps1* maize terpene synthase gene, Schnee *et al.*, 2002) and thus FPP in the cytoplasm (present in higher concentrations that GPP) is used to synthesise nerolidol (Gershenzon & Kreis, 1999; Newman & Chapell, 1999). However, nerolidol was observed to be rapidly converted to (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Donath & Boland, 1994). This conversion (studied in a number of plant species) was achieved using exogenous nerolidol (Gäbler *et al.*, 1991; Donath & Boland, 1995), indicating that conversion was achieved without herbivory or elicitor treatment. The enzymes required for converting nerolidol to DMNT, were thus constitutively expressed, and specific release of DMNT was regulated upstream of nerolidol

production (Parè & Tumlinson, 1997). Therefore, if bombarded Hi-II calli in this study was s uccessfully t ransformed w ith t he *inors* g ene, nerolidol was produced b ut this was probably rapidly converted to DMNT. Since nerolidol production occurred constitutively (ubiquitin p romoter u sed in pAHCinors), D MNT was constantly being produced and probably reached toxic concentrations in the calli tissues. This could be a second reason why some calli proliferated after 4 weeks of selection, but they became necrotic and did not survive to produce any transgenics later on.

Several nutritionally important metabolites are synthesized via the terpenoid pathway: plant hormones, pigments and vitamins such as, gibberellins, lycopene and tocopherols (Croteau *et al.*, 2000). The results of manipulation of terpenoid pathways might have negative effects on related terpenoid accumulation, and on growth and development of plant tissues, due to hormonal imbalances (Fray *et al.*, 1995). Thus, a third possible reason for no transgenics produced, could be that integration of this gene interfered with normal terpenoid metabolism causing a negative, toxic effect to tissue growth.

5.4.3.2 Bombardment with the *tnors* gene

The most common terpenes emitted by maize are the monoterpene linalool and the sesquiterpenes, (*E*)-bergamotene, (*E*)-β-farnesene and β-caryophyllene (reviewed in Schnee *et al.*, 2002). However, these emitted volatiles are released only during herbivory and cannot be stimulated by mechanical wounding of the leaves (Turlings *et al.*, 1991). Bombardment with the pAHCtnors plasmid was aimed to constitutively express linalool in Hi-II maize tissues by using the maize ubiquitin promoter. A linalool synthase gene (from the Californian flower, *Clarkia breweri*) has been described to contain a putative plastid-targeting signal (Cseke *et al.*, 1998) and results in localization of the active linalool synthase enzyme in the plastids.

Table 5.7 indicates that 30-50% of the bombarded Hi-II callus tissue proliferated on 3 mg l^{-1} bialaphos-containing selection media (after 4 weeks). Selection was further increased to 5 mg l^{-1} bialaphos to eliminate "escape" tissues that lacked the *bar* selectable marker gene. Surviving calli matured to form somatic embryos and regenerated to form plantlets. Thirty-one independent events were identified, however many of these putative transgenics did not survive under greenhouse conditions. This is possibly due to tissue culture stress, or to transgene instability (Cherdshewasart *et al.*, 1993), which somehow negatively affected plant growth and development.

Linalool is an acyclic monoterpene alcohol having a peculiar creamy, floral, sweet taste and scent (Arctander, 1969). Thus the sweet, floral scent detected when putative transgenic plantlets were regenerating under tissue culture conditions (limited air space) in tubs (T_0 and T_1 generations), could be the linalool aroma. This scent was absent when plants were transferred to the greenhouse. This was probably due to the emitted scent being dissipated and diluted in the open atmosphere (greenhouse), and thus could not be smelt. Additionally, the transgene displayed instability when exposed to the new greenhouse environment, and linalool production was decreased or halted.

The surviving T_0 putative transgenics displayed dwarfism and abnormal ear and tassel development under greenhouse conditions. This once again can be attributed to tissue culture stress because the T_1 generation plants (germinating directly from seeds) displayed normal, tall and fertile phenotypes (figure 5.8C). An additional reason for the dwarf phenotype could be due to depletion of gibberellins. Previous results indicated that the introduction of linalool synthase in petunia may have resulted in the redirection of the flux of the isoprenoid precursors, thus affecting the production of sesquiterpenes, sterols, carotenoids, diterpenes, gibberellins and other higher terpenes (Lucker et al., 2001). Furthermore, a phytoene synthase gene when over-expressed in tomato, caused depletion of the gibberellin pathway resulting in a dwarf phenotype (Fray et al., 1995). Thus, manipulation of the terpenoid pathway by introduction of the tnors gene into Hi-II maize, could explain the dwarf phenotypes observed in T_0 plants. The T_1 progeny tested negative for tnors via PCR, and the terpenoid pathway was probably restored, thus explaining the normal tall phenotypes displayed.

Many T_0 events tested via PCR (section 5.4.3.2) were positive. PCR amplification of the nad gene was positive for all events indicating that genomic DNA was of good quality. PCR analysis of BBT 1.1B tested positive for the tnors and bar genes, however all T_1 progeny were negative for these genes. Basta® painting of leaves of all T_1 progeny were negative, displaying herbicide sensitivity, which correlated with PCR negative testing for the bar gene. The negative PCR results obtained for all T_1 progeny cannot be attributed to poor DNA quality as nad PCR for all progeny tested positive (figure 5.11B). This "loss" of gene in the second generation can be possibly attributed to DNA shuffling by the process of recombination.

Evolutionary conservation of functional domains in plant isoprenoid synthases (Mau *et al.*, 1994) suggests that chimeric enzymes with different functional domain sequences can be created to synthesize new products. Thus, some DNA shuffling might have occurred and the PCR primers could not specifically bind to the template DNA for amplification.

Therefore, this study has shown that the *tnors* gene was successfully transformed into Hi-II maize in the first generation. However, due to transgene instability or manipulation of the terpenoid pathway, this gene was "lost" or not detected in the second generation.

5.6   CONCLUSION

The study in this chapter reports on the successful transformation of the linear DNA minimal transgene cassettes (*bar-b32* and *pgip2* cassettes) and the *tnors* gene onto Hi-II maize callus tissue, producing independent T_0 trangenics. However, these integrated genes were absent in the T_1 progeny plants indicating transgene instability between generations. Bombardment with the pSC1-mpib32 (*Fusarium* inducible promoter) and pRTL2-improved PGIP (containing the oat cell-wall bound thionin signal peptide) did not yield any proliferating Hi-II callus and thus no transgenics were produced.

CHAPTER SIX:

CONCLUDING REMARKS AND FUTURE PROSPECTS

Fusarium verticillioides causes ear- and stalk-rot infection in maize and dramatic decreases in maize yield. This fungus also produces harmful mycotoxins which pose as health hazards to both humans and animals. Thus, this study aimed at introducing antifungal resistance to *F. verticillioides* by expressing selected plant-derived antifungal genes via particle bombardment into selected elite white maize and the Hi-II (laboratory maize strain) genotypes. The anti-fungal defense genes chosen to engineer resistance to *F. verticillioides* were (i) bean *pgip2* gene (encoding polygalacturonase-inhibitor protein (PGIP), (ii) maize *b-32* gene (encoding a ribosome-inhibiting protein (RIP)) and (iii) strawberry terpene synthase gene (encoding nerolidol (no plastid targeting), or linalool (with chloroplast targeting)). The *bar* selectable marker gene was used within this study.

High frequency plant regeneration from cultured explant material is considered a prerequisite for successful transformation of crops. Therefore, the first step of this study was to identify highly regenerable genotypes by screening six elite white maize genotypes on three different callus initiation media. A483-1 and A483-4 (CIMMYT elite white maize genotypes) and M37W (South African elite white maize genotype), were found to produce the highest number of regenerants per explants (3.6, 2.8 and 0.8 respectively) on $N6_{10}$ callus induction media. These three genotypes, when co-bombarded with the *b32, bar* and *pgip2* genes produced a 0.17% transformation efficiency. Stable integration (1-2 copies) of *b32* gene was obtained in two events. Only one event however, was fertile and future work (northern and western blotting) will be performed on the progeny of this event.

Co-bombardment of Hi-II callus with the *b32, bar* and *pgip2* genes yielded five fertile events (containing at least one antifungal gene) and resulted in a transformation efficiency of 7.5% (per Hi-II callus plate bombarded). Stable integration (2-10 copies) of *b32* and *pgip2*, and production of both mRNA transcripts was confirmed for selected events. A reduction of seed surface infection (up to 50%) was obtained for transgenic Hi-II seeds when compared to untransformed Hi-II control seeds during fungal infection studies with *Fusarium verticillioides* (MRC826).

T$_4$ transgenic seeds produced in this study will be used for future field trial analysis. Future work with these transgenics will include western blotting (protein expression), segregation studies (to produce a homozygous line) and pathogenicity tests. The long term aim will be to produce commercially viable, fungal resistant maize.

Other co-bombardment experiments with the *bar-b32* and *pgip2* linear DNA minimal transgene cassettes (lacking plasmid vector backbone), produced only one fertile event. However, the introduced transgenes were absent in the T$_1$ generation. Future work will include Southern blotting of this event, and more bombardment experiments being performed, to possibly stably introduce these linear DNA fragments into maize.

Bombardment experiments with the improved constructs: pSC1-mpib32 (*b32* under control of the *Fusarium* inducible maize protease inhibitor (mpi) promoter) and pRTL2-improved PGIP2 (encoding an oat cell wall bound thionin signal peptide fused to PGIP2), did not produce any transgenics. Future work will therefore focus on introducing these constructs individually in maize, and testing integration patterns in the transgenics produced.

No transgenics were produced when the construct containing the *inors* gene (no plastid targeting) was bombarded. However, introduction of the *tnors* gene (with chloroplast targeting), was achieved in primary transgenic plants, but this gene was not detected via PCR analysis in the T$_1$ progeny. Future work will thus focus on more bombardment experiments being conducted with the *tnors* gene, to possibly demonstrate stable integration in secondary generations.

This study formed part of the European Union funded project, Safemaize (ICA4-CT2000-30033), and is thus a preliminary study towards terminating fungal infection at an early stage. Results of this study demonstrated that selected antifungal genes were introduced into maize, with some being stably integrated and producing mRNA transcripts. Fungal infection studies showed that some resistance to seed surface infection occurred. Thus, this study supports the hypothesis that combinatorial expression of selected antifungal genes is possible using biolistics, and that expression of these genes could introduce resistance to *F. verticillioides*. A long term benefit will be to increase maize food security through improved maize harvests.

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APPENDICES

Appendix 1: Summary of results obtained for the selected T_3 progeny of putative transgenics (grown under glasshouse trial conditions at the Agricultural Research Council-Grains Institute in Potchefstroom, South Africa)

Code	Basta® Paint Results	PCR tests b32	pgip2	bar
A1E 3	R	+	+	+
A1E 5	R	+	+	+
A1E 7	R	+	+	+
A1E 11	R	+	+	+
A2E 3	R	+	+	+
A2E 6	R	+	+	+
F1A 5	R	+	-	+
F1A 6	R	+	-	+
F1A 7	R	+	-	+
F1A 8	R	+	-	+
F1A11	R	+	-	+
F1A 14	R	+	-	+
F1A 15	R	+	-	+
F1A 16	R	+	-	+
F1F 1	R	+	-	+
F1F 6	R	+	-	+
F1F 9	R	+	-	+
F1F 13	R	+	-	+
F1F 15	R	+	-	+
F1F 18	R	+	-	+
F1G 5	R	+	-	+
F1G 6	R	+	-	+
F4A 3	R	+	-	+
F4A 4	R	+	-	+
H3C 2	R	+	-	+
H3C 5	R	+	-	+
H3C 10	R	+	-	+
H7G 1	R	+	-	+
H7G 3	R	+	-	+
H7G 4	S	+	-	-
H7G 5	R	+	-	+
H7H 3	R	+	-	-
E9A 5	S	+	-	+
H8C 2	R	+	-	+
H8C 4	R	+	-	+
H8C 6	R	+	-	+
H8C 7	R	+	-	+
H8C 8	R	+	-	+
H8C 9	R	+	-	+
H8C 10	R	+	-	+
H8E 1	R	+	-	+
H8E 3	R	+	-	+
H8E 7	R	+	-	+
H8E 8	R	+	-	+
H8E 9	R	+	-	+
H12I 1	R	+	-	+
H12I 2	R	+	-	+
H12I 3	R	+	-	+
H12I 4	R	+	-	+
H12I 7	R	+	-	+
H12J 2	R	+	-	+
H12J 3	R	+	-	+
H12J 8	R	+	-	+
H12J 9	R	+	-	+
H12J 10	R	+	-	+
H12J 16	R	+	-	+

| Code	Basta® Paint Results	PCR tests b32	pgip2	bar
A1B 2	S	-	-	-
A1B 4	S	-	-	-
A1B 7	S	-	-	-
A1B 8	S	-	-	-
A1B 10	S	-	-	-
A2E 8	S	-	-	-
C3D 1	S	-	-	-
C3D 7	S	-	-	-
C3D 8	S	-	-	-
D9A 1	S	-	-	-
E9A 4	S	-	-	-
E9A 5	S	+	-	-
E9A 6	S	-	-	-
E9A 8	S	-	-	-
F1A 10	S	-	-	-
F1A 17	S	-	-	-
F1A 18	S	-	-	-
F1F 2	S	-	-	-
F1F 4	S	-	-	-
F1F 7	S	-	-	-
F1F 8	S	-	-	-
F1F 9	S	+	-	-
F1F 10	S	-	-	-
F1F 16	S	-	-	-
F1F 17	S	-	-	-
F1G 1	S	-	-	-
F1G 2	S	-	-	-
F1G 3	S	-	-	-
F1G 10	S	-	-	-
F4A 1	S	-	-	-
F4A 2	S	-	-	-
F4F 6	S	-	-	-
F4F 11	S	-	-	-
F4J 1	S	-	-	-
F4J 5	S	-	-	-
F4J 6	S	-	-	-
F4J 8	S	-	-	-
G1H 6	S	-	-	-
H3C 7	S	-	-	-
H7H 2	S	-	-	-
H7H 4	S	-	-	-
H8C 1	S	-	-	-
H8C 3	S	-	-	-
H12C 4	S	-	-	-
H12C 6	S	-	-	-
H12C 7	S	-	-	-
H12C 8	S	-	-	-
H12C 9	S	-	-	-
H12J 5	S	-	-	-
H12J 6	S	-	-	-
H12J 13	S	-	-	-
H12J 14	S	-	-	-
H12J 15	S	-	-	-
H12J 17	S	-	-	-
H12J 19	S	-	-	-

Appendix 2:
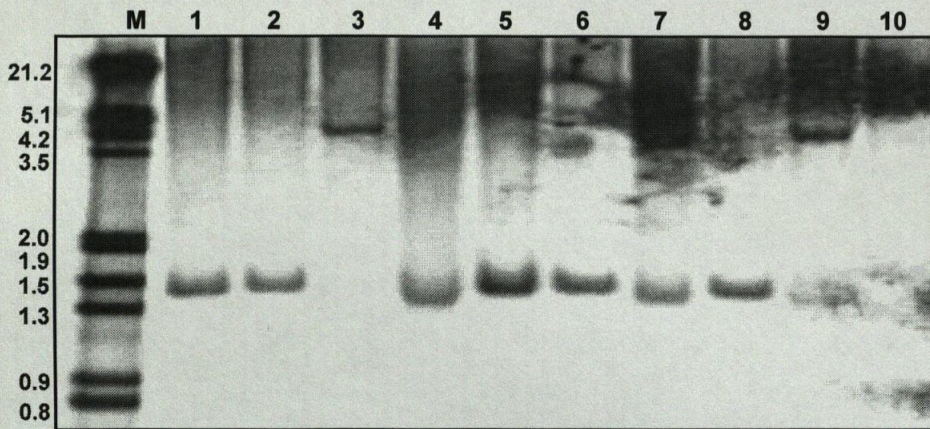
Southern blot analysis (*pgip2*) of T$_3$ transgenic Hi-II maize progeny of event A (*pgip2* positive as shown by PCR). Five microgram of genomic DNA (purified from leaf material) were digested with the *Hind*III (lanes1,2,4,5,6,8,9,10) and *Eco*RI (lanes 3,7,9) restriction enzymes and resolved on a 0.8% agarose gel, transferred onto a nylon membrane and then probed with the *pgip2* probe outlined in Section 3.3.10. Lane M: DIG-labelled molecular weight marker III (lambda DNA digested with *Eco*RI and *Hind*III). The lanes represent the following transgenic events: Lane 1: A1E3, lane 2: A1E5, lane 3: A1E5, lane 4: A1E7, lane 5: A1E11, lane 6: A2E3, lane 7: A2E3, lane 8: A2E6, lane 9: A2E6 and lane 10: untransformed Hi-II. Marker sizes are indicated in kb.

Appendix 3: Summary of T$_3$ transgenic plants tested via Southern Blots

Code	PCR Results			Southern Blot	
	b32	*pgip2*	*bar*	*b32*	*pgip2*
A1E 3	+	+	+	+	+
A1E 5	+	+	+	+	+
A1E 7	+	+	+	+	+
A1E 11	+	+	+	+	+
A2E 3	+	+	+	+	+
A2E 6	+	+	+	+	+
F1A 7	+	-	+	+	
F1A 8	+	-	+	+	
F1A 16	+	-	+	+	
F1F 1	+	-	+	+	
F1F 15	+	-	+	+	
F1G 5	+	-	+	+	
H3C 2	+	-	+	+	
H3C 10	+	-	+	+	
H7G 1	+	-	+	+	
H7G 3	+	-	+	+	
H7G 5	+	-	+	+	
H8C 4	+	-	+	+	
H8C 6	+	-	+	+	
H8C 7	+	-	+	+	
H8C 8	+	-	+	+	
H8E 7	+	-	+	+	
H8E 8	+	-	+	+	
H8E 9	+	-	+	+	
H12I3	+	-	+	+	
H12I 4	+	-	+	+	
H12I 7	+	-	+	+	
H12J 2	+	-	++	+	
H12J 3	+	-	+	+	
H12J 4	+	-	+	+	
H12J 8	+	-	+	+	
H12J 10	+	-	+	+	

Appendix 4:
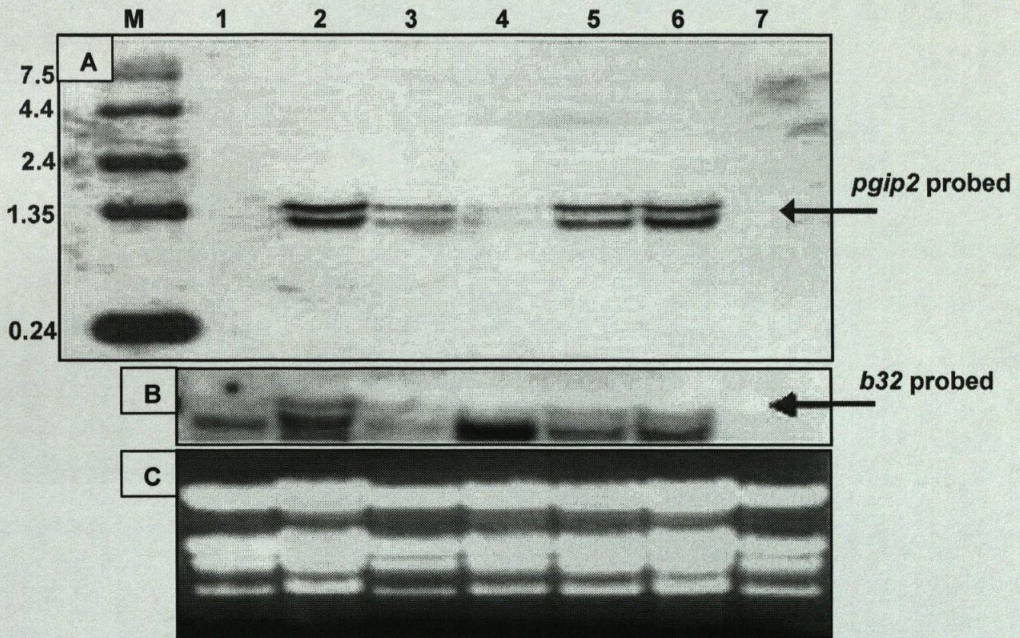
Northern blot analysis for the expression of **(A)** the introduced bean *pgip2* and **(B)** the maize *b32* transgenes in T_3 transgenic maize plants. Total RNA (extracted from leaf tissues) was probed with the DIG-labelled *pgip2* fragment (A) and *b32* fragment (B). Ethidium bromide staining of the formaldehyde agarose gel is shown below **(C)**. Lane M: RNA mass ladder (GibcoBRL). The numbered lanes represent the following: lane 1: A1E3, lane 2: A1E5, lane 3: A1E7, lane 4: A1E11, lane 5: A2E3, lane 6: A2E6 and lane 7: untransformed Hi-II maize.

Appendix 5:
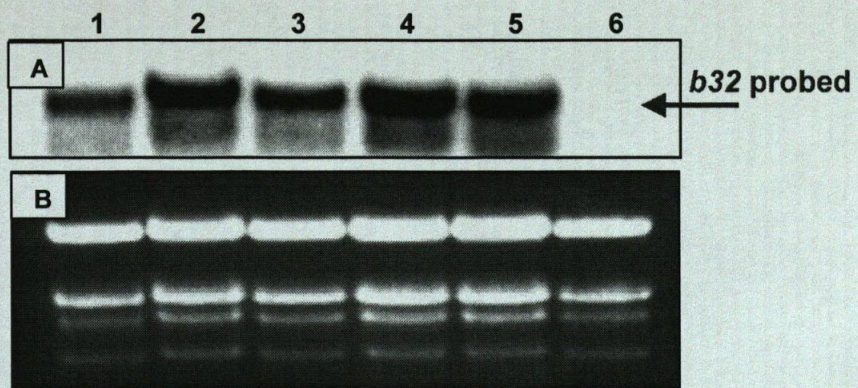
Northern blot analysis for the expression **(A)** of the introduced maize *b32* transgene in T_3 transgenic maize plants. Total RNA (extracted from leaf tissues) was probed with the DIG-labelled *b32* fragment. Ethidium bromide staining of the formaldehyde agarose gel is shown below **(B)**. The numbered lanes represent the following: lane 1: H12J2, lane 2: H12J3, lane 3: H12J8, lane 4: H12J10, lane 5: H12J16, lane 6: untransformed Hi-II maize

Appendix 6: Summary of T$_3$ transgenic plants tested via northern blots

Code	PCR Results			Northern Blot	
	b32	*pgip2*	*bar*	*b32*	*pgip2*
A1E 3	+	+	+	+	-
A1E 5	+	+	+	+	+
A1E 7	+	+	+	+^{L}	+
A1E 11	+	+	+	+^{H}	+
A2E 3	+	+	+	+	+
A2E 6	+	+	+	+	+
F1A 5	+	-	+	-	
F1A 6	+	-	+	+	
F1A 7	+	-	+	+	
F1A 8	+	-	+	+	
F1A 11	+	-	+	-	
F1A 14	+	-	+	-	
F1A 15	+	-	+	+	
F1A 16	+	-	+	+	
F1F 1	+	-	+	+	
F1F 3	+	-	+	+	
F1F 6	+	-	+	+	
F1F 9	+	-	+	+	
F1F 13	+	-	+	+^{L}	
F1F 15	+	-	+	+	
F1F 18	+	-	+	-	
F1G 5	+	-	+	+	
F1G 6	+	-	+	+	
F4A 3	+	-	+	+	
F4A 4	+	-	+	+	
H7G 1	+	-	+	+	
H7G 3	+	-	+	+	
H7G 5	+	-	+	+	
H8C 2	+	-	+	+	
H8C 4	+	-	+	+^{L}	
H8C 6	+	-	+	+	
H8C 7	+	-	+	+^{L}	
H8C 8	+	-	+	+^{H}	
H8C 9	+	-	+	+	
H8C 10	+	-	+	+	
H8E 1	+	-	+	+	
H8E 3	+	-	+	+^{H}	
H8E 7	+	-	+	+^{H}	
H12I 1	+	-	+	+	
H12I 2	+	-	+	+	
H12I 3	+	-	+	+	
H12I 4	+	-	+	+	
H12I 7	+	-	+	+	
H12J 2	+	-	+	+	
H12J 3	+	-	+	+	
H12J 8	+	-	+	+	
H12J 10	+	-	+	+	
H12J 16	+	-	+	+	

L = Low expressors on northern blot (light hybridising bands produced)
H = High expressors on northern blot (dark hybridising bands produced)

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