Genotype variation in regeneration and transformation efficiencies of South African wheat cultivars

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Preface

Crops have been of essence to man since the beginning of time. The Bible tells us many tales of harvesting and the joys that reaping time brought to the nations. Specific events in the history of the Israelites have been linked to the production of crops. The arrival of Naomi and Ruth in Bethlehem was placed in context as they arrived during reaping time (Ruth 1:22). The Israelites devoted the first portion of a new grain harvest to God during the feast of first-fruits, the feast of weeks and the feast of trumpets as a way of expressing their joy and gratitude. The poor, orphans and strangers also benefited at harvesting time in that they were allowed to collect the surplus of each harvest that was left on the fields (Lev. 19:9–10, Deut. 24:19-22).

Symbolically, crops and reaping time have been used to sketch the judgement of God (Jer. 51:33), the end of the world (Rev. 14:14-19) and the love of God for his nation (Gal. 6:8-9). God says in Deut 8:7-10: "For the Lord your God is bringing you into a good land – a land with streams and pools of water, with springs flowing in the valleys and hills; a land with wheat and barley, vines and fig trees, pomegranates, olive oil and honey; a land where bread will not be scarce and you will lack nothing; a land where the rocks are iron and you can dig copper out of the hills. When you have eaten and are satisfied, praise the Lord your God for the good land he has given you".

God says in Genesis 1:29: "I give you every seed-bearing plant on the face of the whole earth and every tree that has fruit with seed in it. They will be yours for food". It is in this context that we, as cultivators of this earth, should consume and protect the valuable resources given to us.

The results presented in this thesis are original and were obtained from research carried out at the University of the Free State, Bloemfontein, as well as at the University of Pretoria, Pretoria, under the supervision of Prof. A-M Oberholster.

The following articles have been published/submitted from the results of this study:

1. **Lacock L and Botha A-M** 1998 Transient expression of marker genes in wheat callus using particle bombardment. Proceedings of the 9th International Wheat Genetics Symposium 3: 20 - 23.

2. **Lacock L and Botha A-M** 1999 Genotype variation in regeneration and transformation efficiencies of 11 South African wheat cultivars. Plant Cell, Tissue and Organ Culture (In Press).

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Completing my study was only possible with the help of the Lord and for that I will be forever thankful.

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AFP	Antifungal protein
BA	Benzyladenine
BAP	Benzylaminopurine
BYMV	Barley yellow mosaic virus
°C	Degree celsius
cm	Centimetre(s)
2,4-D	2,4 Dichlorophenoxyacetic acid
Dn	*Duiraphis noxia*
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ec	Embryogenic callus
EDTA	Ethylenediamine tetra acetic acid
e.g.	*Exempli gratia* (for example)
EtOH	Ethanol
et al.	*Et alii* (and others)
g	Gram(s)
GNA	*Galanthus nivalus*
HMW	High-molecular weight
HR	Hypersensitive response
IAA	Indoleacetic acid
kb	Kilo base pairs
kPa	Kilo Pascal
L	Litre
LB	Luria Bertani
Lr	Leaf rust
LRR	Leucine-rich repeats
M	Molar

MAS	Marker-assisted selection
mg	Milligram(s)
mL	Millilitre
ML3	Modified MS medium
Mm	Millimetre
mM	Millimolar
MS	Murashige and Skoog medium
m/v	Mass/volume
NAA	Naphthaleneacetic acid
ne	Non-embryogenic callus
ng	Nanogram(s)
NW	North western
PEG	Polyethylene glycol
PIG	Particle inflow gun
PR	Pathogenesis related
R	Resistance
RAPD	Random amplified polymorphic DNA
RIP	Ribosome inactivating protein
RFLP	Random fragment length polymorphism
Rpm	Revolutions per minute
RNA	Ribonucleic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SDS	Sodium dodecyl sulphate
se	Somatic embryo
TEN	Tris-EDTA-sodium chloride
Tlp	Thaumatin-like protein
TMV	Tobacco mosaic virus
USA	United States of America
UV	Ultraviolet
V	Volts

v/v	Volume/volume
WSMV	Wheat scab mosaic virus
µg	Microgram(s)
µL	Microlitre(s)

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Chapter 1

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world. It is an easily accessible source of food (Brettell and Murray, 1995) and nutrition (Vasil *et al.*, 1992), and wheat end products, e.g. flour, have a wide range of applications (Snape, 1998). Presently, the annual global wheat production is 560 million tons (Braun *et al.*, 1998). The global demand for wheat production, however, will increase dramatically over the next twenty years. Braun and his colleagues stated that as much as 1050 million tons of wheat would be required per year for the ever-increasing world population. In order to meet this demand, wheat grain-yield should be increased. Plant breeders are already working towards this goal. Wheat transformation, however, will greatly aid breeders in their quest for higher and improved grain yield (Braun *et al.*, 1998).

The improvement of wheat depends on our understanding of the plant as a living organism. Plants are sessile and, thus, exposed to various environmental conditions and stress factors. Plants, therefore, have to possess a protection mechanism against such factors that could potentially harm them or endanger their survival (Lamb *et al.*, 1992). One factor influencing the plant's well being is attack by disease-causing agents such as viruses, bacteria and fungi. Most plants exhibit a natural resistance against such pathogens. Some pathogens, however, have evolved specific mechanisms enabling them to successfully parasitise their hosts (Kombrink and Somssich, 1995). The infected plants will then respond by employing various defence mechanisms as a means of protection (Lamb *et al.*, 1992).

Unfortunately, not all plants are resistant to pathogen infestation and scientists worldwide strive to improve the resistance mechanisms of various plant species against pathogens. This is also true for several cereal crops, including wheat, since many wheat cultivars are vulnerable to attack by pathogens. The solution to this problem lies in manipulating wheat in order to render the plant more resistant to pathogenic diseases.

Conventional breeding programs have been the main method of improving crop resistance against pests and pathogens (Johnson, 1992). During the past three decades an additional method, namely marker-assisted selection (MAS), has been employed to aid the plant breeder in growing more resistant crops (Melchinger, 1990). Biotechnological progress during the past fifteen years, however, has led to the emergence of plant manipulation through genetic engineering. Genetic engineering has, thus, been applied during the past decade in order to improve yield, productivity and resistance against diseases of various plant species. Many records are available on the production of transgenic plants (Gambley *et al.*, 1993; Weeks *et al.*, 1993; Nehra *et al.*, 1994) and genetically engineered plants have already been introduced into the food and fibre market (Strauss *et al.*, 1997). Wheat has become an important target for genetic engineering (Nehra *et al.*, 1994; Brettell and Murray, 1995), although it was the last among the economically important crops to be transformed (Weeks *et al.*, 1993).

Wheat is being transformed with two main objectives in mind: firstly, the improvement of economically important qualities (Snape, 1998) and, secondly, the increase in resistance against various pathogens and insects (Brettell and Murray, 1995; Barcelo *et al.*, 1998). Improving economically important traits of wheat includes the transformation of high-molecular-weight (HMW) glutenin sub-units (Barro *et al.*, 1997; Blechl and Anderson, 1998; Blechl *et al.*, 1998; Murray *et al.*, 1998) and the modification of starch-branching enzymes (Chibbar *et al.*, 1998). High-molecular-weight glutenin sub-units contribute to the mixing strength, visco-elasticity and bread-making qualities of the wheat dough, which is an important industrial quality (Snape, 1998).

Resistance of wheat against pathogens is enhanced by introducing pathogenesis-related (PR) genes such as the rice thaumatin-like protein, chitinase, β-1,3-glucanase and ribosome inactivating protein (RIP) into wheat (Chen *et al.*, 1998; Fennel *et al.*, 1998). In order to obtain aphid resistance, the

lectin *Galanthus nivalus* (GNA) gene has been transformed into wheat. This gene exhibits activity against sap-sucking insects (De la Vinã *et al.*, 1998). Transformation as a means to improve resistance is gaining more popularity since chemical control of diseases is not readily available (Grumet and Lanina-Zlatkina, 1996) and is quite expensive (Krebs and Grumet, 1993). Furthermore, chemical control is environmentally hazardous and pathogens may become tolerant to the fungicides (Grumet and Lanina-Zlatkina, 1996).

Several techniques are available for the transformation of plants. The transformation of dicotyledonous plants such as sweet potato (Gama *et al.*, 1996), poplar (Kajita *et al.*, 1994) and citrus (Peña *et al.*, 1995) has been achieved by the use of *Agrobacterium tumefaciens*. Reports are also available on the successful transformation of several cereal species using *A. tumefaciens* as a vector, e.g. barley (Tingay *et al.*, 1997) and rice (Chan *et al.*, 1992; Hiei *et al.*, 1994; Ishida *et al.*, 1996; Park *et al.*, 1996; Rashid *et al.*, 1996). Recently, Cheng and his colleagues (1997) reported on the transformation of wheat using *A. tumefaciens*. The transformation efficiency obtained during their studies, however, was very low.

Another technique employed during transformation is particle bombardment (Brettell and Murray, 1995; Barcelo *et al.*, 1998). Particle bombardment allows the direct transfer (Gasser and Fraley, 1989) of exogenous DNA into the genome of the target plant (Brettell and Murray, 1995). The foreign DNA is carried on microscopic metal particles that are accelerated towards the target tissue (Vain *et al.*, 1993; Sanford *et al.*, 1987). In this way, the DNA can penetrate several cell layers of the target tissue (Gasser and Fraley, 1989). Once the DNA has reached the cells' nuclei, it is integrated into the genome (Brettell and Murray, 1995) and is subsequently expressed (Zhang and Punja, 1994).

Particle bombardment is regarded as a simple, rapid transformation method that is applicable to a wide range of genetic material (Sanford, 1988). This method allows the transformation of intact plant tissue, as well as suspension culture cells (Vasil *et al.*, 1991). Physical barriers, such as cell walls, are thus no longer obstacles in the process of DNA delivery to the cells (Hamilton *et al.*, 1992). Further, the same, basic protocol can be applied during all bombardment procedures (Sanford, 1988).

A. tumefaciens-mediated transformation, on the other hand, does not allow rapid and reliable production of regenerable callus (Park *et al.*, 1996). The fact that monocotyledonous crop species are difficult to transform using *Agrobacterium* can be attributed to improper wound response of these plants (Potrykus, 1991). A further limitation is the unreliable transformation efficiency obtained during *Agrobacterium* transformation since frequencies range from 0.1% to as high as 20% (Barcelo *et al.*, 1998).

Two specific genes have been identified as very attractive candidates to introduce into wheat in order to amplify the battle of wheat against pathogen infestation. They are chitinase and ß-1,3-glucanase (Lamb *et al.*, 1992; Kemp *et al.*, 1998). Kemp and his co-workers demonstrated that these two enzymes are able to degrade the germtubes of invading pathogens, especially that of *Puccinia recondita*. The activity of chitinase and ß-1,3-glucanase caused the cytoplasm of the germtube cells to separate from the cell walls (Kemp, 1996). This in turn resulted in the total destruction of the tubes, as well as the urediospores. This is a good indication that chitinase and ß-1,3-glucanase could act in a similar way against other pathogens infecting wheat. Lamb *et al.* (1992) also demonstrated that these two enzymes are active *in vitro* against pathogens and have antimicrobial characteristics. Further, it has been proposed that chitinase and ß-1,3-glucanase have to be transformed simultaneously in order to act as efficient defence response genes (Stintzi *et al.*, 1993) since they act synergistically to inhibit fungal pathogens (Punja and Zhang, 1993).

During my study, I aimed to test various wheat cultivars for their susceptibility to foreign genes. This will later enable me to increase the resistance of several South African wheat cultivars against pathogens by introducing the chitinase and ß-1, 3-glucanase genes into wheat tissue. The constitutive expression of these genes will then enable the plant to degrade infecting pathogen cell walls and prevent further spreading of the pathogen (Susi *et al.*, 1995). In turn, this will ensure a more vigorous plant and result in improved grain yield.

The wheat cultivars I will investigate are hard-red winter and spring wheat cultivars. Until now, wheat transformation studies have mostly been carried out on soft white cultivars (Demeke *et al.*, 1998; Blechl, *et al.*, 1998; Hansen *et al.*, 1998). I will, therefore, investigate the susceptibility of South African hard-red wheat cultivars for regeneration and transformation efficiencies. I will establish viable tissue cultures from immature wheat embryos and test the regeneration thereof. The transformation method I will employ is known as particle bombardment. This method is used, since, as stated previously, it has been applied successfully to accomplish transformation of several plant species. I will optimise the particle bombardment process by introducing a reporter gene into the target tissue. Once this is accomplished, I aim to introduce the chitinase and ß-1,3-glucanase genes into selected wheat cultivars. This will be followed by the testing for stable, constitutive expression of these genes in the F_1 and F_2 progeny. However, due to the time constraint, the latter objectives do not fall within the scope of this study, but will be addressed during further studies.

Chapter 2

Literature Review

2.1 Plant-Pathogen Interactions

2.1.1 The communication between plants and pathogens

Plants have been able to persist in nature for as long as man can remember. Fungi, bacteria and viruses, however, frequently attempt to infect weaklings. Only a few of these encounters lead to infection and subsequent disease formation (Delaney, 1997). The survivors are thus resistant to the attacking pathogen (Greenberg *et al.*, 1994). This may be due either to the fact that the pathogen is unable to recognise and infect the host or that the host defends itself by activating various defence mechanisms (Kombrink and Somssich, 1995). Some attacks by pathogens, however, do result in disease development in susceptible plants (Delaney, 1997).

When these pathogens are examined, we find that they are specialised in such a way that they are able to successfully parasitise host plants (Kombrink and Somssich, 1995). Heath (1981), and Bushnell and Rowell (1981) have proposed that such parasites are able to overcome the host's defence mechanisms genetically. These assumptions are based on Flor's (1956) gene-for-gene hypothesis in which he concluded that host-parasite interactions result from complementary gene systems. For each host gene that causes resistance, a matching pathogen gene exists that causes pathogenicity. Resistance only occurs when both these genes are dominant. When these genes do not correspond, a basic incompatibility between the host and pathogen develops (Tepper and Anderson, 1984). Such incompatibility enables the pathogen to activate the host's defence responses (Kombrink and Somssich, 1981).

A few other aspects of pathogen virulence enable them to infect their host plants. Firstly, evolution of pathogen virulence may enable a pathogen to change the structure of its elicitors in order to evade the host's defence reactions (Tepper

and Anderson, 1984). Secondly, invading pathogens produce suppressors of the host plant's elicitors that recognise the invading pathogens (Heath, 1981; Bushnell and Rowell, 1981). Thirdly, pathogens produce enzymes, toxins or plant growth regulators that damage or change plant cells in order to weaken the plant (Kombrink and Somssich, 1995).

Mutations in once resistant host plants can also cause them to become susceptible to pathogens. These plants are unable to detect invading pathogens or to induce defence mechanisms (Delaney, 1997). Mutant plants that are not able to express resistance against specific pathogens with avirulence genes, contain inactivated resistance (R) genes.

Plant pathogens exhibit varying degrees of host specificity. Some pathogens are able to infect different hosts, whereas other pathogens are much more specific (Tepper and Anderson, 1984). Pathogens are, therefore, completely dependent on their hosts in order to carry out the metabolic processes necessary to complete their life cycles (Grumet and Lanina-Zlatkina, 1996). Pathogens utilise their host's resources for their own development, growth and multiplication (Delaney, 1997). The effect of this on the host plant is tissue damage and immediate or delayed symptoms of the specific disease.

Plants that are resistant to invading pathogens are able to sense these invading pathogens when they contain R-genes. It is these genes that recognise the corresponding avirulence genes of the pathogen (Delaney, 1997). The defence response of the host that is subsequently activated usually coincides with the activation of pathogenesis-related (PR) proteins (Nielsen *et al.*, 1994). Various hydrolases, especially chitinase and ß-1,3-glucanase, are also produced since they hydrolyse chitin and ß-1,3-glucans that are constituents of fungal cell walls (Susi *et al.*, 1995). The expression of these transgenes in tobacco raised the idea that host plants develop an effective defence mechanism before pathogens activate the natural inducible defences (Lamb *et al.*, 1992).

An interesting discovery has been made concerning genes conferring resistance to pathogens. These resistance genes contain a relatively large amount of leucine-rich repeats (LRR) (Jones and Jones, 1997). The *Cre3* gene in wheat that confers resistance to *Heterodera avenae*, a root endoparasitic nematode, contains such a leucine-rich region (Lagudah *et al.*, 1997). Frick and his co-workers (1998) found LRR in the *L6* and *N* rust resistance genes. Further, two *Cf*-2 loci in tomato, that confer resistance to tomato leaf mould, contain 38 LRR. The *Cf*-9 gene of tomato is also involved in resistance against leaf mould and the homology between the LRR of the *Cf*-2 and *Cf*-9 genes are quite high (Dixon *et al.*, 1996). It is suggested that all above-mentioned genes are involved in the signal-transduction pathway of protein-protein interactions (Lagudah *et al.*, 1997). Bent and his co-workers (1994) studied the *Arabidopsis thaliana* gene *RPS2* that results in resistance against the bacterium *Pseudomonas syringae*. This gene contains several LRR and these regions are thought to be involved in ligand binding of proteins. Thus, leucine-rich repeats seem to play an important role in the plant's defence against nematodes, bacteria, fungi and viruses (Jones and Jones, 1997).

2.1.2 Plant resistance mechanisms

Plants defend themselves by administering two main resistance mechanisms. One of these mechanisms can be restricted to the area of infection, while the other can be induced systemically (Nielsen *et al.*, 1994). These processes are the hypersensitive response (HR) and systemic acquired resistance (SAR), respectively.

2.1.2.1 Hypersensitive response (HR)

The hypersensitive response in infected plants is a strong, effective defence mechanism associated with general plant resistance (De Wit, 1995). This response is explained as a gene-for-gene complementarity phenomenon that occurs between the host and pathogen (Flor, 1956; Stinzi *et al.*, 1993; Delaney, 1997). HR is induced in the plant as soon as it recognises the infecting pathogen (De Wit, 1995). The avirulence genes of the pathogen produce race-specific elicitors that bind to plant receptors that are products of the host's resistance genes (De Wit, 1995). Recognition then occurs due to matching alleles of the pathogen avirulence genes and the host R genes (Keen, 1990). The recognition triggers the activation of a signal transduction cascade (Stinzi *et al.*, 1993) and, thus, the HR is induced (De Wit, 1995).

During the hypersensitive response, the host plant produces proteins that seem to be involved in at least three types of defence reactions (Bol *et al.*, 1990): First is the direct attack of the infecting pathogen by e.g. hydrolytic enzymes. Secondly, the pathogen is restricted to the site of infestation by e.g. lignifying enzymes. Thirdly, the host's metabolism adapts to the stress condition by producing e.g. superoxide-dismutase.

The first characteristic of the hypersensitive response in plants, is necrosis (Stinzi *et al.*, 1993). Necrotic lesions form rapidly at the site of infection and consist of areas of dead plant cells (Greenberg *et al.*, 1994). Necrosis is also accompanied by the transcriptional activation of several defence genes in the area of infection (De Wit, 1995; Delaney, 1997). The above mentioned reactions enable the plant to restrict the pathogen to the area of infestation (Stinzi *et al.*, 1993; De Wit, 1995; Delaney, 1997). This, in turn, limits pathogen growth and prevents further colonisation (Dixon and Lamb, 1990).

The hypersensitive response further involves several metabolic changes within the host plant (Stinzi *et al.*, 1993). These changes can occur at the site of infection or at distant areas in the plant (Malany and Klessig, 1992). Other changes that occur are an increase in plasmalemma permeability, the production of phenols (Tepper and Anderson, 1984), the synthesis of ethylene (Bol *et al.*, 1990) and the accumulation of antimicrobial molecules (Greenberg *et al.*, 1994).

2.1.2.2 Systemic acquired resistance (SAR)

In the early twentieth century, scientists and naturalists discovered that plants become resistant to pathogen attack after a previous infection by a pathogen (Ryals *et al.*, 1994). The first investigation of this systemic phenomenon was carried out by Ross (1961). He demonstrated that a plant becomes more resistant to tobacco mosaic virus (TMV) infection after a previous infection by the same, as well as other necrotising, pathogens. It was only during 1982 that Kees van Loon found the accumulation of a group of proteins – now known as pathogenesis-related (PR) proteins – during this systemic defence response (Van Loon and Antoniw, 1982).

This response is now known as systemic acquired resistance (Stinzi *et al.*, 1993) and results in a long-term, broad-spectrum systemic resistance after previous infection (Ryals *et al.*, 1994). Stinzi *et al.* (1993) explains that after a plant has activated the HR during pathogen attack, the uninfected plant parts exhibit a higher level of resistance to this pathogen. This is evident in the formation of necrotic lesions in the uninfected plant parts. The plant further exhibits an increased level of resistance against other necrotising pathogens (Delaney, 1997).

Various proteins were found to accumulate during the SAR (Delaney, 1997). Kombrink and Somssich (1995) demonstrated that the initiation of SAR in tobacco is associated with the production of extra-cellular PR-proteins. Several of the SAR-associated proteins exhibit direct antimicrobial activities (Ryals *et al.*, 1994). Chitinases and ß-1,3-glucanases, that are known to exhibit antifungal activities, were found to be encoded by SAR genes (Linthorst, 1991). Other SAR genes encode thaumatin-like proteins that are also involved in antifungal processes (Vigers *et al.*, 1991; Woloshuk *et al.*, 1991). The PR-1 proteins that exhibit antifungal activity, are also closely related to another group of SAR genes (Ryals *et al.*, 1994).

2.1.3 Pathogenesis-related proteins

In the early 1970s the synthesis of several proteins was observed in tobacco plants when the plants reacted hypersensitively to tobacco mosaic virus infection. Characteristics of these proteins include an acidic nature, indigestibility by proteases, and an apoplastic location (Bol *et al.*, 1990). The name pathogenesis-related proteins or PR-proteins have been given to these proteins. Today a large number of PR-proteins have been identified and they have been classified into five main groups (Table 2.2.3.1).

Table 2.1.3.1 Summarised characteristics of the five pathogenesis-related protein groups (Kauffmann *et al.*, 1990; Stinzi *et al.*, 1993).

PR-group	Name	Molecular weight (kDa)	Acidic/basic nature	Function
1	Tobacco PR-proteins	15 - 17	Acidic	Viral resistance Virus localisation
2	ß-13,-glucanase PR-2 PR-O PR-N PR-Q	25 - 35	Acidic	Fungal and bacterial resistance
3	Chitinase Lysozyme	25 - 35	Acidic Basic	Fungal resistance Lysozyme activity
4	r1, r2 s1, s2	13 – 14.5	Neutral	Unknown
5	"Thaumatin"-like proteins	22 - 24	Acidic Neutral	Fungal resistance

The induction of PR-proteins has been studied in a number of plant species. It was found that these proteins are induced by a whole range of pathogenic plant offenders, including viruses, viroids, fungi, bacteria and insects (Stinzi *et al.*, 1993). Tomato plants infected with *Cladosporium vulvum*, e.g., produced PR-proteins during the hypersensitive reaction. The PR-proteins are produced soon after infection and accumulate in the vacuoles of the mesophyll and are, thus, available during the HR. This could directly facilitate the defence response of the host plant (Danhash *et al.*, 1993). In tobacco, the induction of PR-proteins leads to an acquired resistance to viruses, fungi and bacteria other than the infecting bacteria (Bol *et al.*, 1990). Some PR-proteins are induced during treatment with various chemicals, treatment with phytohormones and especially treatment with ethylene. Abiotic factors such as salt or osmotic stress are also thought to be involved in PR-protein induction (Stinzi *et al.*, 1993) that

lead to an acquired resistance (Bol *et al.*, 1990). Therefore, the production of PR-proteins can be seen as an adaptation of the plant to stress conditions (Badur *et al.*, 1994).

2.1.4 The defending molecules: chitinases and ß-1,3-glucanases

Molecular reagents that are involved in the defence reactions of a plant during stress conditions should have the following characteristics (Kombrink and Somssich, 1995): 1) antimicrobial activity, 2) induction or accumulation during pathogen attack and 3) differential expression during incompatible interactions. The two enzymes that supposedly adhere to these traits and that are involved in plant protection reactions are chitinase and ß-1,3-glucanase (Lamb *et al.*, 1992).

2.1.4.1 Identification

The chitinase [poly {1,4-(N-acetyl-ß-D-glucosamide)} glycohydrolase, E.C. 3.2.1.14] and ß-1,3-glucanase (1,3-ß-D-glucan glycanohydrolase, E.C. 3.2.1.29) enzymes are present in all plant tissues (Kragh *et al.*, 1993). Bol *et al.* (1990) found high concentrations of chitinases and ß-1,3-glucanases and their respective mRNAs in roots of healthy plants. Healthy plants also produce chitinases and ß-1,3-glucanases in their stems and flowers (Memelink *et al.*, 1990), as well as in their lower leaves and in *in vitro* cultures (Kragh *et al.*, 1993).

2.1.4.2 Induction of chitinase and β-1,3-glucanase

Chitinases and ß-1,3-glucanases are induced by various conditions. These enzymes are mostly synthesised when the plant experiences stress (Ignatius *et al.*, 1994), e.g. during fungal or viral infections or when the plant is

exposed to ethylene and insects (Bol *et al.*; Botha *et al.*, 1998). Exposure to ozone also leads to the synthesis of these two hydrolases (Ignatius *et al.*, 1994). During a study performed on cucumber, chitinases were induced by salicylic acid, wounding, chitosan application and by non-pathogenic infection that lead to the hypersensitive reaction (Zhang and Punja, 1994). Tweddell *et al.* (1994) suggested that chitinase and ß-1,3-glucanase production occur during metabolic inhibition.

2.1.4.3 Chitinase and β-1,3-glucanse activity

The first suggestions that chitinases and β-1,3-glucanases are involved in the plant's defence mechanisms had been made as early as 1971 (Stinzi *et al.*, 1993). These suggestions were based on the fact that the substrates of these two enzymes, namely chitin and ß-1,3-glucan, are important constituents of fungal cell walls (Mauch *et al.*, 1988). Thus, these two enzymes hydrolyse their substrates in the invading pathogen's cell wall (Arlorio *et al.*, 1992; Susi *et al.*, 1995). Proof for the activity of these two enzymes has been discovered worldwide. Broglie and Broglie (1993) and Vierheilig *et al.* (1993) demonstrated the activity of chitinases against pathogenic fungi by enhancing the expression of chitinase in tobacco roots. Chitinases also inhibit the growth of fast growing saprophytes (Schlumbaum *et al.*, 1986; Roberts and Selitrennikoff, 1990; Broekaert *et al.*, 1988, 1989). Evidence of its antifungal properties was further shown using agar plates with *Trichoderma viride* (Stinzi *et al.*, 1993) and *Puccinia recondita* (Kemp, 1996). The growth of *Fusarium solani* germlings is also inhibited by specific chitinases and ß-1,3-glucanases, since these enzymes cause the lysis of the pathogen's hyphal tips (Stinzi *et al.*, 1993). Various other scientists demonstrated the antifungal activities of these two enzymes on agar plates (Mauch *et al.*, 1988; Arlorio *et al.*, 1992).

Chitinases and ß-1,3-glucanases aid plants in the battle against pathogens in two main ways. Firstly, they act as hydrolytic enzymes (Tweddell

et al., 1994). During fungal infections, the synthesis of chitinases and ß-1,3-glucanases in the plant increases (Susi *et al.*, 1995). These enzymes are then able to partially degrade the fungal cell walls by hydrolysing the structural components of these walls (Kombrink and Somssich, 1995). They execute their task by causing the tips of the hyphae and germtubes to swell (Mauch *et al.*, 1988; Broekaert *et al.*, 1989; Kemp, 1996). The chitin strands in the cell walls are then loosened and, as a result, the cell walls become weaker and thinner, and then finally disappear (Arlorio, 1992). The end result is a complete destruction of the germtubes and fungal hyphae (Kemp *et al.*, 1998).

The second factor influencing the plant's defence response against pathogens is the products released by the hydrolytic enzymes. Chitinase and ß-1,3-glucanase release oligosaccharides that the plant perceives as signals or elicitors (Stinzi *et al.*, 1993) during the initial stages of pathogen attack (Dixon and Lamb, 1990). These elicitors are chitins, chitosan oligomers (Stinzi *et al.*, 1993) and ß-1,3-glucans (Ignatius *et al.*, 1994) that induce metabolic changes leading to the defence response of the plant (Stinzi *et al.*, 1993). It has been demonstrated that wheat and carrot chitinases act as signal enhancers, by generating elicitors from the fungal cell walls that they degrade (Salzer *et al.*, 1997).

Another aspect of the functioning of chitinases and ß-1,3-glucanases is that they operate synergistically (Wyatt *et al.*, 1991). Individually, these enzymes affect very few fungi (Mauch *et al.*, 1988), but together they strongly inhibit the growth of various pathogens (Wyatt *et al.*, 1991). Restriction of mycelial growth *in vitro* is most effective when both enzymes are administered (Maher *et al.*, 1993; Kemp *et al.*, 1998). The regulation and expression of chitinases and ß-1,3-glucanases also take place co-ordinately (Mauch *et al.*, 1988). This has been demonstrated by Punja and Zhang (1993), when it was shown that the accumulation of chitinases mimic the accumulation of ß-1,3-glucanases in infected or abiotically stressed tissue.

2.2 Conferring Disease Resistance in Plants

Various methods exist to protect wheat plants against the symptoms of infecting pathogens. One option for restricting such infestations, is the application of fungicides. Chemical control, however, is expensive in developing countries and is an environmental hazard (Daniel, 1994). Alternative methods of effectively controlling diseases in wheat are conventional breeding, marker-assisted selection and genetic manipulation.

2.2.1 Conventional breeding

The age-old process of crossing one plant with a close relative containing certain superior characteristics is still used as the basis of current plant, especially crop, improvement. This process, known as conventional breeding, aims toward general crop improvement, including an increase in yield, quality and disease resistance (Lörz *et al.*, 1998). This is accomplished by crossing parental lines possessing the trait(s) of interest (Winter and Kahl, 1995) and continued growing and screening of the offspring for the desired trait(s) (Johnson, 1992).

Several prerequisites have to be met in order to establish a successful breeding program. Parental lines possessing the best sources of the trait(s) of interest have to be selected (Fedak, 1998). This selection process is influenced by the availability of e.g. sources of resistance, since resistance for some diseases are not readily available (Johnson, 1992). A continued supply of resistance sources is further necessary in order to counteract the continued pathogenic mutation and recombination rates (Riley *et al.*, 1968). Another factor influencing a successful breeding program is an effective procedure for selecting the traits of interest (Johnson, 1992).

Conventional breeding is a relatively simple method of plant improvement and it reduces the need for additional methods, such as chemical control, for crop protection (Johnson, 1992). A recent example of transferring disease resistance genes into a common wheat cultivar was described by Aung and Kerber (1998). Stem and leaf rust resistance were transferred form a wild relative of wheat, *Aegilops triuncialis* L., to wheat through back-crossing and embryo rescue.

The method of conventional breeding, however, encompasses a few disadvantages (Winter and Kahl, 1995). The process of generating offspring via repeated back-crossings, selfing procedures and screening of the offspring for the desired traits, are very time-consuming (Winter and Kahl, 1995). The complexity of the wheat genome further contributes to this drawn-out breeding process (Langridge and Chalmers, 1998; Lu *et al.*, 1998). The fact that conventional breeding is such a long-term process and involves so many back-crossing and screening procedures, cause it to be a very costly method (Johnson, 1992; Braun *et al.*, 1998). Combining complex characters in one individual is difficult since these characters are most probably controlled by separate genetic systems (Johnson, 1992). Johnson further stated that a characteristic such as yield could be negatively influenced when breeding for another advantageous trait. A concern raised by Braun and his colleagues (1998) is the fact that access to wheat germplasm may become limited due to plant and gene patents.

2.2.2 Marker-assisted selection

Until 1990, the progress made in breeding programs by using marker-assisted selection (MAS), was limited due to the lack of suitable molecular markers. Today, the application of MAS in such programs are becoming more possible since genetic linkage maps for important crop species are increasingly becoming available (Melchinger, 1990).

Marker-assisted selection is based on the production of molecular markers by using molecular techniques (Johnson, 1992). These markers are genetically linked to genes of interest, e.g. resistance genes, and permit the breeder to connect the phenotypic expression of these genes with specific regions of the plant's genome. The marker genotype is, therefore, used during subsequent screening procedures to select for a specific trait (Winter and Kahl, 1995). The individuals exhibiting the marker phenotype are then used as parents in subsequent crossings (Melchinger, 1990).

Isozymes and restriction fragment length polymorphisms (RFPLs) were initially employed in the development of molecular markers (Melchinger, 1990). Random amplified polymorphic DNA (RAPD) is also applied in marker technology, but the use of these systems proved problematic in the complex wheat genome (Balyan *et al.*, 1998). New techniques employed in generating markers and linkage maps include amplified fragment length polymorphisms (AFLPs) and micro-satellites (Fedak, 1998). At present, more that 50 loci on the wheat genome have been labelled with molecular markers (Langridge and Chalmers, 1998) and well developed maps for wheat are becoming increasingly more available (Clarke *et al.*, 1998).

Marker-assisted selection is a useful tool in accessing characteristics that are of potential use in crop improvement (Winter and Kahl, 1995). Other advantages of MAS include: the tagging of a large number of genes; the fact that

molecular markers are usually co-dominantly inherited; and that early screening for the marker genotype can be performed (Melchinger, 1990). Time-saving is a further consideration in applying MAS. The use of molecular markers can eliminate three back-crossing procedures of a breeding program during screening processes (Tanksley *et al.*, 1989).

The development of molecular markers is, however, only starting to help breeders understand the structure of the wheat genome. The genome's complexity is inhibiting rapid development and application of molecular markers (Langridge and Chalmers, 1998). It is further debatable whether all the time, labour and costs being spent on the development of marker technology can be justified in breeding programs (Melchinger, 1990).

2.2.3 Biotechnological engineering

Genetic manipulation of wheat plants is a recent innovation in the combat against plant pathogens (Brettell and Murray, 1995). It is a very forthright method of transferring certain traits to crop plants (Jähne *et al.*, 1995) and allows the insertion of foreign genes into plants. Genetic manipulation can also be applied in triggering endogenous genes with the aim of expressing these genes at different levels or in different tissue (Barcelo *et al.*, 1998).

Cereals are important crop plants and, thus, increasingly becoming targets for gene transfer (Nehra *et al.*, 1994; Jähne *et al.*, 1995). Scientists in the USA have experimented with more than 2 300 field trials of transgenic crops over the past decade (Strauss *et al.*, 1997). The main objectives of cereal transformation are the betterment of agronomic or quality traits such as disease resistance and improvement of grain quality and yield. The ability to transfer genes into plants enables scientists to study the behaviour of transgenes in plants and to increase the transformation efficiency. The ultimate goal is to obtain continued expression of the transgene and its stable inheritance to change the plant's phenotype (Brettell and Murray, 1995). Applying genetic engineering as tool for cereal improvement, however, involves the establishment of an efficient tissue culture system (Viertel and Hess, 1996; Tiwari *et al.*, 1997; Özgen *et al.*, 1998).

2.2.3.1 Wheat tissue culture

The concept of *in vitro* cell culture arose during the early 1900s (Dodds and Roberts, 1985). The world-renown botanist, Gottlieb Haberlandt, experimented with isolated, fully differentiated palisade and pith cells, which he placed on an artificial nutrient medium. These cells, however, only managed to survive on the medium, without any further cell proliferation (Razdan, 1993). Since these early attempts, the importance of establishing an optimum micro-

environment for isolated cells, has been realised. In 1939 Gautheret, White and Nobécourt announced the importance of auxin enriched media in the cultivation of plant tissue for sustained growth (Razdan, 1993). Cytokinins were discovered by several workers during the late 1940s and early 1950s (Dodds and Roberts, 1985). Tissue placed on media enriched with cytokinins exhibited more proliferation than on media containing auxin (Razdan, 1993). In 1957 Skoog and Miller introduced the idea of using these hormones to induce and control the development of shoots and roots of tissue culture material. During 1958 to 1959, Reinert and Steward first reported the formation of somatic embryos from carrot tissue (Bhojwani and Razdan, 1983). Another breakthrough in plant tissue cultures was achieved during the 1970s with *in vitro* clonal propagation. This contributed greatly to various agricultural industries (Dodds and Roberts, 1985). The above-mentioned milestones in establishing and developing plant tissue cultures represent only a few steps in the process of plant regeneration. Today, plant tissue cultures are applied widely, especially as basis for biotechnology (Razdan, 1993).

Establishing tissue cultures are made possible by a trait possessed by all plant cells: totipotency. Totipotency is defined as the ability of a single cell to grow and develop into a complete organism (Dodds and Roberts, 1985; Razdan, 1993). This phenomenon implies that each cell within an individual contains all the genes necessary for cell division and differentiation (Razdan, 1993). Tissue cultures are based on two *in vitro* propagation methods: somatic embryogenesis and micropropagation. Micropropagation refers to the asexual or vegetative propagation of plants (Bhojwani and Razdan, 1983). Somatic embryogenesis, on the other hand, involves the development of regenerable, non-zygotic embryos. These embryos possess the ability to germinate and develop into complete plants (Razdan, 1993).

The efficient generation of plants from tissue culture is subjected to a few prerequisites. First, the systematic regeneration of sufficient numbers of plants (Ahloowalia, 1992) should be obtainable from such *in vitro* cultures (Özgen *et al.*, 1998). Further, the establishment of an efficient tissue culture system involves the selection of an adequate explant, as well as efficient callus induction, maintenance and regeneration procedures (Özgen *et al.*, 1996; Tiwari *et al.*, 1997).

2.2.3.1.1 Choice of explant

Wheat tissue cultures are being established using various explant sources. These include mature and immature embryos, immature inflorescences, immature leaves, mesocotyls, seeds and apical meristems (Özgen *et al.*, 1996). Successful wheat tissue cultures have also been established by using microspores (Hu and Kasha, 1997) and shoot tips and leaf bases (Viertel and Hess, 1996).

Immature wheat embryos, however, are the most frequently used explant in establishing tissue cultures (Sears and Deckard, 1982; He *et al.*, 1988; Weeks *et al.*, 1993; Özgen *et al*, 1996, 1998; Tiwari *et al.*, 1997). Immature embryos contain three meristematic areas that can give rise to embryogenic callus tissue. They are the scutellum, epiblast and apical meristem (He *et al.*, 1988). The scutellum is mostly utilised as source of embryogenic callus tissue (He *et al.*, 1988). Redway and his colleagues (1990) found that immature embryos between 1.0 and 1.5 mm in length responded robustly during callus initiation. Immature embryos, however, are available only during a limited time of the year (Özgen *et al.*, 1996) and appropriate developmental stages for callus initiation is also limited (Özgen *et al.*, 1998). Mature embryos, on the other hand, are available throughout the year. Although it has been found that some cultivars exhibit a high regeneration capacity, callus formation from mature embryos generally proceed with low efficiency (Özgen *et al.*, 1998). Thus, the high

efficiency of callus induction obtained from immature embryos render it ideal for tissue culture initiation.

2.2.3.1.2 Tissue culture initiation

The medium used during tissue culture initiation of wheat is mostly MS (Murashige and Skoog, 1962). Jähne and his colleagues (1991) also experimented with ML3 medium in order to initiate anther cultures. These initiation media are generally supplemented with the growth regulator 2,4-D (2,4 dichlorophenoxyacetic acid) (concentrations between 0.5 mg/L and 2 mg/L) and sucrose as carbon source (2% to 3%) (Sears and Deckard, 1982; Mohmand and Nabors, 1991; Ahloowalia, 1992; Altpeter *et al.*, 1996; Özgen *et al.*, 1996; Bommineni *et al.* 1997; Tiwari *et al.*, 1997). Casein hydrolysate (1 mg/L to 100 mg/L) is often added (Altpeter *et al.*, 1996; Bommeneni *et al.*, 1997; Tiwari *et al.*, 1997), as well as the sugar alcohol myo-Inositol (100 mg/L) (Bommeneni *et al.*, 1997; Tiwari *et al.*, 1997). Amino acids are supplied in the form of glutamic acid (Altpeter *et al.*, 1997) and aspartic acid (Sears and Deckard, 1982). Vitamins such as thiamine, nicotinic acid and pyridoxine are further added to enrich initiation media (Sears and Deckard, 1982; Jähne *et al.*, 1991; Mohmand and Nabors, 1991; Tiwari *et al.*, 1997). Explants placed on initiation media are kept in the dark at $\pm$ 25 to 27 °C (Altpeter *et al.*, 1996; Bommeneni *et al.*, 1997; Özgen *et al.*, 1998). The onset of callus formation from immature wheat embryos are generally obtained within 4 to 7 days after culture initiation (Ahloowalia, 1992). Further callus development is genotype dependent and callus tissue is obtained within 1 to 3 weeks after callus initiation (Sears and Deckard, 1982; Viertel *et al.*, 1998).

2.2.3.1.3 Callus maintenance

Calli obtained from immature wheat embryos are generally maintained on MS medium containing 2,4-D, but at a lower concentration than the initiation medium (Sears and Deckard, 1982). Indoleacetic acid (IAA) is occasionally added (Ahloowalia, 1992). Calli are then transferred to new media every 2 to 4 weeks (Sears and Deckard, 1982; Vasil *et al.*, 1993) and exposed to a 16-hour photoperiod (Weeks *et al.*, 1993).

2.2.3.1.4 Plantlet regeneration

Plant regeneration media are generally supplemented with IAA (Fennell *et al.*, 1996) and benzyladenine (BA) or zeatin (Redway *et al.*, 1990; Mohmand and Nabors, 1991). The formation of shoots are induced by transferring calli to sucrose-containing hormone-free MS (Altpeter *et al.*, 1996; Özgen *et al.*, 1996, 1998) or half-strength MS medium (Ahloowalia, 1992; Altpeter *et al.*, 1996). The material is exposed to a 16-hour photoperiod (Özgen *et al.*, 1996). Root development is stimulated by introducing calli to media without 2,4-D (Sears and Deckard, 1982; Özgen *et al.*, 1998), but containing naphthaleneacetic acid (NAA) (Ahloowalia, 1992) or BAP (benzylaminopurine) (Fennell *et al.*, 1996; Tiwari *et al.*, 1997). Plantlets reaching a height of 6 to 8 cm (Tiwari *et al.*, 1997), containing well-developed roots (Viertel *et al.*, 1997), are transferred to soil for further growth.

2.2.3.1.5 Post-bombardment selection

Calli that have been subjected to particle bombardment are either placed on osmotic medium (Vain *et al.*, 1993; Lonsdale *et al.*, 1998) or back on the initiation medium (Vasil *et al.*, 1992; Bommeneni *et al.*, 1997; Barro *et al.*, 1997; Witrzens *et al.*, 1998) immediately after bombardment. Bombarded material are

regenerated on media (generally MS) without growth regulators, but containing a relevant herbicide (Nehra *et al.*, 1994; Altpeter *et al.*, 1997). The specific marker gene that was inserted into the material during bombardment determines the herbicide used. This is important in ensuring that the offspring of the regenerated plants contain the genes conferring the desirable trait transferred to the plants (Eapen and Rao, 1986). The initial regeneration periods are then carried out in the dark or at low light intensities. Material are exposed to higher light intensities during later stages of development in order to enhance the frequency of regeneration (Nehra *et al.*, 1994; Altpeter *et al.*, 1997). Plantlets are obtained within 6 (Bommeneni *et al.*, 1997) to 12 weeks (Nehra *et al.*, 1994) after bombardment.

It is clear that the establishment of wheat embryo cultures requires media enriched with specific concentrations of growth regulators (Ahloowalia, 1992). The genotype (Özgen *et al.*, 1998) and age of the explant used (Viertel and Hess, 1996) are further considerations when establishing wheat tissue cultures.

2.2.3.2 Genetic manipulation

2.2.3.2.1 The method: particle bombardment

Two methods are mainly used for the transfer of foreign DNA into cereal crops. They are the direct transfer of genes to protoplasts and microprojectile bombardment of plant tissue (Brettell and Murray, 1995). Christou *et al.* (1991) suggested that an efficient transformation system should adhere to specific criteria. Firstly, transformation of any cultivar or genotype should be possible. Secondly, a large number of transgenic plants should be regenerated in order to assess the levels of gene expression. And thirdly, regeneration of the transformed material should take place quickly and efficiently to ensure lines without mutations or somaclonal variation. The ever developing and improving

particle bombardment process seems to be ideal in achieving such difficult prerequisites.

The most commonly used transformation system for cereals, however, has been the use of protoplasts (Chibbar *et al.*, 1991). Transformation was achieved by either electroporation (Callis *et al.*, 1987; Vasil *et al.*, 1989) or polyethylene glycol (PEG) (Shillito *et al.*, 1985). The major problem with these methods is the inability of the protoplasts to regenerate into plants (Chibbar *et al.*, 1991). Limited success has also been achieved by applying *Agrobacterium tumefaciens*-mediated transformation. This is due to the fact that monocotyledonous plants are not readily susceptible to this bacterium (Hooykaas, 1989; Rainieri *et al.*, 1990; Tingay *et al.*, 1997). It is thus thought that *Agrobacterium* has a limited host range (Schell, 1987) and the production of regenerable material still remains a problem (Park *et al.*, 1996).

Particle bombardment has several advantages over other transformation mechanisms. It is a very versatile method to obtain gene transfer (Brettell and Murray, 1995) for it can be applied to obtain transient gene expression (Ludwig *et al.*, 1990; Vain *et al.*, 1993), as well as stable transformation in plants (Klein *et al.*, 1988). The main advantage of particle bombardment is that intact plant tissue can serve as the target material (Finer *et al.*, 1992). DNA can be transferred to suspension culture cells, as well as to larger intact material such as callus tissue (Vasil *et al.*, 1991). The particle bombardment system rests on a basic concept that can be universally applied to all transformation procedures (Finer *et al.*, 1992). The method is simple, rapid (Sanford, 1988) and applicable to a wide range of host material (Hunold *et al.*, 1994). Further, this is the only method of direct gene transfer where the cell wall is not regarded as an obstacle (Hamilton *et al.*, 1992). Thus, it avoids the use of protoplasts (Vasil *et al.*, 1991). The particle gun method also allows better control over helium pressure, distributes the particles evenly over the target tissue and cause less tissue damage (Russell-Kikkert, 1993). Particle bombardment has been successfully

applied to transform sugarcane (Gambley *et al.*, 1993; Snyman *et al.*, 1996), oil palm (Parveez *et al.*, 1997), maize (Klein *et al.*, 1988; Vain *et al.*, 1993), rice (Christou *et al.*, 1991), barley (Ritala *et al.*, 1994), soybean (McCabe *et al.*, 1988) and white spruce (Ellis *et al.*, 1993).

Wheat has also been the subject of several transformation procedures. Fry and his colleagues (1998) transferred antifungal protein (AFP) genes into wheat via particle bombardment. Preliminary results indicated that these genes increased the level of resistance of the transformed plants to wheat head scab (*Fusarium graminearum*). Leckband and Lörz (1998), and Hain and his colleagues (1993) transformed wheat with the stilbene synthase gene (*Vst 1*) obtained from *Vitis vinifera*. This transformation was previously performed in barley and induced an increased level of resistance against the necrotrophic fungus *Botrytis cinerea*. It is, therefore, believed that wheat transformed with the *Vst 1* gene will also exhibit an increased level of resistance against certain pathogens (Leckband and Lörz, 1998). Wheat transformed with two coat proteins, namely BYMV-*cp* and WSMV-*cp*, (Hansen *et al.*, 1998) exhibited resistance to the barley yellow mosaic virus (BYMV) and wheat scab mosaic virus (WSMV) during greenhouse and field evaluations. Further results obtained after the transformation of wheat with the aim of improving the crop's resistance against pests and to improve quality traits, are summarised in Table 2.2.3.2.1.

Table 2.2.3.2.1 A summary of current wheat transformation studies using particle bombardment with the aim of improving the wheat plant.

Wheat cultivar	Gene/s transferred	Function of genes	Author/s
Hartog – soft white spring **Bobwhite** – soft white spring	HMW glutenin sub-unit gene, ***Sx2***	Improved dough and flour properties	Demeke *et al.*,1998
Bobwhite – soft white spring	HMW glutenin sub-unit genes, ***Dx5***, ***DY10***	Improved dough and flour properties	Blechl and Anderson, 1998
AC Karma – soft white spring **Fielder** – soft white spring **Columbus** – hard red spring	Starch-branching enzymes, ***Sbe1*** and ***Sbe2***	Improved starch content	Chibbar *et al.*, 1998
Florida – soft white winter	Snow drop lectin gene, **GNA**	Aphid resistance	De la Vinã *et al.*, 1998
Hartog – soft white spring **Bobwhite** – soft white spring	Glutenin gene, ***Sec2***	Improved dough qualities	Murray *et al.*, 1998
Triticum aestivum lines **L88-6** and **L88-31**	HMW glutenin sub-unit genes, **Ax1**, **Bx17**, **By18**, **Dx5** and **Dy10**	Improved dough and flour properties	Barro *et al.*, 1998

Several other studies are in progress concerning the transformation of wheat for increased levels of resistance against pathogens employing particle bombardment. Chitinase-, ß-1,3-glucanase- and ribosome inactivating protein (RIP)-genes are being transformed into wheat against *Septeria tritici* blotch and *Fusarium* head scab (Fennel *et al.*, 1998). Chen and his colleagues (1998) have inserted a rice thaumatin-like protein (*tlp*) gene into wheat plants and are currently screening these plants for resistance to fungal diseases.

2.2.3.2.2 Development of the particle bombardment system

The method of particle bombardment was developed during 1984 to 1987 (Sanford, 1988). E.D. Wolf, N.K. Allen and J.C. Sanford of Cornell University, USA, worked together to invent the process of biological ballistics or shortly, biolistics. Sanford and his co-workers experimented with several methods with the aim of obtaining stable transformation. The idea of literally shooting DNA into target tissue was raised. They proposed that DNA, because of its fragile nature, should be fired into the cells together with solid particles. Onion epidermis cells were used as the first target tissue since they are large, easily obtainable and the monolayer enabled them to assess the penetration easily. The cytoplasmic streaming of these cells also allowed direct evaluation of the cells' viability after bombardment. Sanford and his co-workers experimented with microprojectiles, mostly tungsten, of different sizes and found that several cell layers could be penetrated. These penetrated cells also remained viable and the microprojectiles could be transported within the cell's cytoplasm (Sanford, 1988).

Various methods of accelerating the microprojectiles were experimented with to ensure less tissue damage and to provide high velocity to the particles (Sanford, 1988). These methods included the use of a transferred pulse across a membrane that supplied energy to the particles, macro-projectile acceleration and the use of centripetal and electrostatic acceleration. They found that the application of macro-projectiles, together with the use of a vacuum chamber, delivered the micro-projectiles gently to smaller cells. Several other delivery systems have also been developed. They are the use of a high voltage electrical discharge through a droplet of water (Brettell and Murray, 1995), pneumatic discharge guns (Oard *et al.* 1990; Seki *et al.*, 1991; Russell-Kikkert, 1993), micro-targeting (Sautter *et al.*, 1991), gunpowder charges (Reggiardo *et al.*, 1991) and the use of nitrogen, carbon dioxide and helium to provide an efficient acceleration force (Birch and Franks, 1991; Sanford *et al.*, 1991; Finer *et al.*, 1992; Vain *et al.*, 1993; Brown *et al.*, 1994; Nabulsi *et al.*, 1994; Songstad *et al.*, 1995). All these

methods strived to provide a simple, safe, accurate and cost effective mechanism to deliver the microprojectiles (Brown *et al.*, 1994). Of all these methods, the use of helium as carrier-gas is preferred to accelerate the microprojectiles (Brettell and Murray, 1995).

Sanford (1988) further demonstrated that DNA, as well as RNA, could be bound to tungsten particles. The DNA and RNA remained biologically active in the bombarded cells and the marker genes could also be introduced and expressed in these cells. Consequently, they demonstrated the viability and efficiency of the biolistic process. Today, this process is widely used to study transient expression of foreign DNA in target tissue (Klein *et al.*, 1987).

2.2.3.2.3 The mechanism

The particle bombardment mechanism is quite simple. Microscopic gold or tungsten particles are covered with DNA and then shot into the target tissue (Sanford *et al.*, 1987). The particles are fired through a partial vacuum (Brown *et al.*, 1994) by a force that is provided by pressurised helium gas (Suatter *et al.*, 1991; Finer *et al.*, 1992). The newly introduced DNA is then transferred to the nuclei of the hit cells (Yamashita *et al.*, 1991; Hunold *et al.*, 1994) and subsequently integrated into the genome (Brettell and Murray, 1995). Here, the introduced DNA is expressed.

Several parameters have to be complied with in order to ensure maximal transformation (Russell-Kikkert, 1993), little tissue damage and a high cell viability (Hunold *et al.*, 1994). Parameters such as helium pressure, distance from the micro-carrier plate to the target tissue and vacuum applied should be optimised. Other factors, e.g. the type and age of the target tissue, osmotic pre-treatment of the tissue and the gene constructs used, should also be considered

in determining the transformation efficiency (Russell-Kikkert, 1993). It has been suggested that gentle bombardment conditions will result in high transformation efficiencies (Stiff *et al.*, 1995).

2.2.3.2.3.1 The particles

The particles or "DNA carriers" that are used during bombardment are accelerated in a stream of helium gas (Finer *et al.*, 1992) and carried downward until they hit and penetrate the target tissue (Russell-Kikkert, 1993). In order to retain the cells' viability and integrity, these particles have to be relatively small and uniformly spread over the target cells (Brettell and Murray, 1995). In order to reduce the tissue damage, the amount of particles used per bombardment should be reduced (Becker *et al.*, 1994).

Tungsten and gold particles are commonly used. Tungsten particles are favoured for their cost effectiveness and their heterogeneous size and shape (Russell-Kikkert, 1993). Tungsten does however, causes the degradation of DNA with time and may be toxic to specific cell types (Russell *et al.*, 1992). The advantages of gold particles are that they are rounder and more uniform in size than tungsten, they are biologically inert and cannot harm the bombarded tissue. Gold particles, however, tend to form irreversible agglomerates in aqueous solutions (Russell-Kikkert, 1993).

2.2.3.2.3.2 Vacuum

The vacuum within the vacuum chamber serves a triple function. First, the vacuum reduces the aerodynamic drag on the micro-carriers (Russell-Kikkert, 1993; Brown *et al.*, 1994). This results in an increase in the micro-carriers' velocity and, thus, penetration efficiency of the target cells (Sanford *et al.*, 1993; Parveez *et al.*, 1997). Secondly, the vacuum causes an even expansion of the helium gas that results in less tissue damage. Thirdly, the vacuum contributes to

increased particle acceleration in that it creates a pressure differential between the helium gas and vacuum chamber (Finer *et al.*, 1992).

2.2.3.2.3.3 Helium pressure

The expansion of compressed helium gas is preferred over other gasses such as nitrogen and carbon dioxide to accelerate the micro-carriers (Johnston *et al.*, 1991). Helium expands much faster than these gasses and can therefore accelerate the micro-carriers more efficiently (Russell-Kikkert, 1993). Furthermore, helium is an inert gas that leaves no residues after bombardment (Finer *et al.*, 1992).

The type of plant tissue being bombarded plays an important role in determining the optimum helium pressure to be used (Iida *et al.*, 1990). Furthermore, when the helium pressure is reduced, a decrease in the expression of the introduced DNA occurs. This may be due to a decrease in the velocity of the particles that results in low penetration efficiency. However, when the pressure is increased to increase the DNA expression, extensive tissue damage occurs (Parveez *et al.*, 1997).

The size of the particles used also determines the helium pressure that is applied. For example, the larger the particles, the higher the pressure should be in order to ensure sufficient acceleration and penetration of the plant cells. The highest transient expression is usually obtained with high helium pressure (Parveez *et al.*, 1997).

2.2.3.2.3.4 The solenoid

The helium gas is released into the vacuum chamber using a timer relay-driven solenoid (Finer *et al.*, 1992). The solenoid is easy to use for it is already

mounted within the vacuum chamber casing (Finer *et al.*, 1992; Brown *et al.*, 1994).

2.2.3.2.3.5 Distance between target tissue and micro-carrier plate

The distance between the target material in the vacuum chamber and the micro-carrier plate affects the transformation efficiency (Taylor and Vasil, 1991; Gambley *et al.*, 1993). Therefore, it is important to determine the optimal distance between the plate and target tissue to ensure an even distribution of the DNA-coated particles on the tissue (Parveez *et al.*, 1997). The amount of physical damage to the target tissue should also be minimised (Gambley *et al*, 1993). When the distance between the micro-carrier plate and the tissue increases, the velocity of the particles decreases. The particles are also spread more uniformly over the tissue and cause less tissue damage. More cells are thus transformed (Russell-Kikkert, 1993).

2.2.3.2.4 Shortcomings of the particle bombardment method

Hunold *et al.* (1994) has outlined a few limitations of the particle bombardment system. Firstly, some plant material could possibly withstand particle penetration due to strong cuticles, lignified cell walls or hair-covered surfaces. Secondly, only a few cells of the total amount of target material will be penetrated by the DNA and of these, only a few cells will express the introduced DNA (Klein *et al.*, 1989; Russell *et al.*, 1993). Thus, a large number of experiments are required to overcome this problem. A third limitation is the regeneration of the bombarded tissue. A very efficient selection and regeneration protocol has to be found in order to regenerate as many of the transformed cells as possible.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals

Hormones were purchased from Sigma Chemical Company. UltraMax DH5α-FT competent cells used during transformation procedures was purchased from Life Technologies. Pioneer Hibred, Inc supplied plasmid pHP 687 (Figure 3.1.1). The Cht B3 (Figure 3.1.2) and Glu B2 (Figure 3.1.3) plasmids were supplied by Labor Kombrink (Max Planck Institut, Köhl, Germany). Marcherey-Nagel NucleoSpin (GmbH & Co. KG, Germany) systems were used during purification of plasmid DNA. Tungsten (1 µm) and gold particles (1.0 µm) were purchased from Bio-Rad Laboratories, CA. Spherical gold particles (1.5 µm – 3.0 µm) were obtained from Aldrich Chemical Company. The QIAquick Gel Extraction Kit was purchased from QIAGEN Inc, USA. Other chemicals used were of analytical grade.

3.1.2 Biological material

The wheat tested was hard-red cultivars. The winter wheat cultivars included 'Betta', 'Betta' *Dn1* ('Betta' 5*/SA 1684), 'Betta' *Dn2* ('Betta' 5*/SA 2199), 'Tugela' and 'Tugela' *Dn1* ('Tugela' 5*/SA 1684). The spring wheat cultivars included 'Gamtoos', 'Gamtoos' *Dn2* ('Gamtoos' 5*/SA 2199), 'Gamtoos' *Dn5* ('Gamtoos' 5*/SA 463), 'Palmiet', 'Palmiet' *Lr29* ('Palmiet' 4*/RL6080 [*Lr29*]), 'Palmiet' *Lr34* ('Palmiet' 4*/RL6058 [*Lr34*]), 'Palmiet' *Dn1* ('Palmiet' 5*/SA 1684), 'Palmiet' *Dn2* ('Palmiet' 5*/SA 2199) and 'Palmiet' *Dn5* ('Palmiet' 5*/SA 463). Dr. H.A. van Niekerk, Small Grain Institute Bethlehem and Prof. Z.A. Pretorius, Department of Plant Pathology, University of the Orange Free State, Bloemfontein, South Africa supplied the wheat seed.

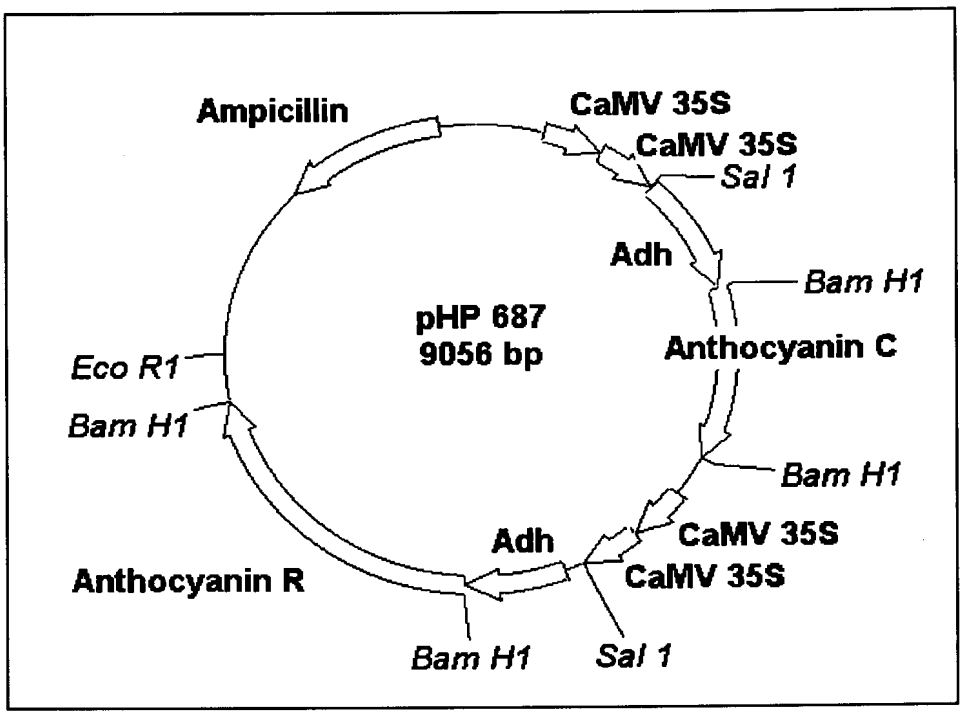


Figure 3.1.1 Plasmid pHP 687 containing two anthocyanin transcriptional activators R (1.9 kb) and C1 (1.1 kb), both under the control of a double CaMV 35S promoter. The plasmid contains an ampicillin resistance gene and an *Eco R1* restriction site.

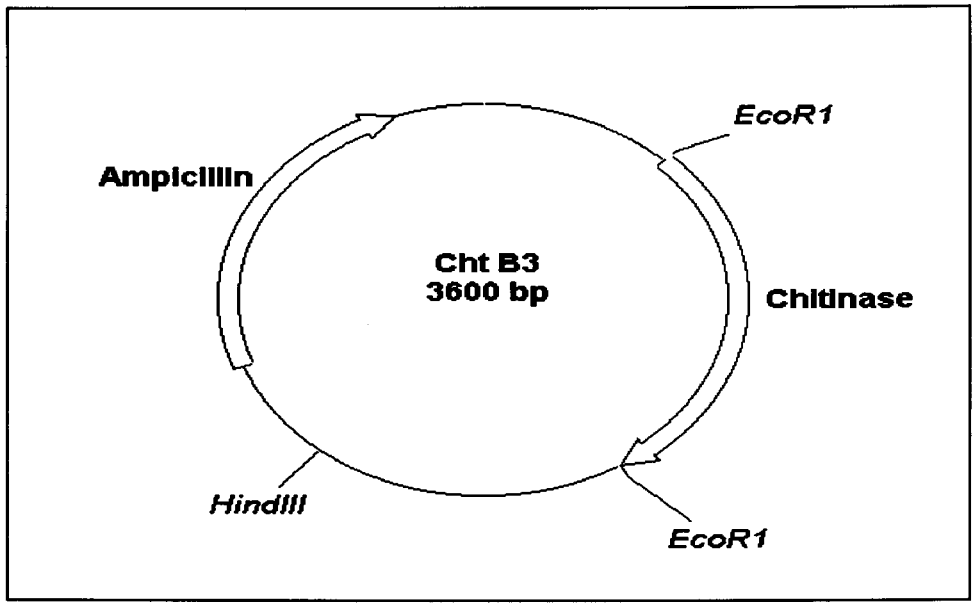


Figure 3.1.2 Plasmid Cht B3 contains the chitinase gene (0.877 kb) which is cloned into an *Eco R1* restriction site and contains an ampicillin resistance gene.

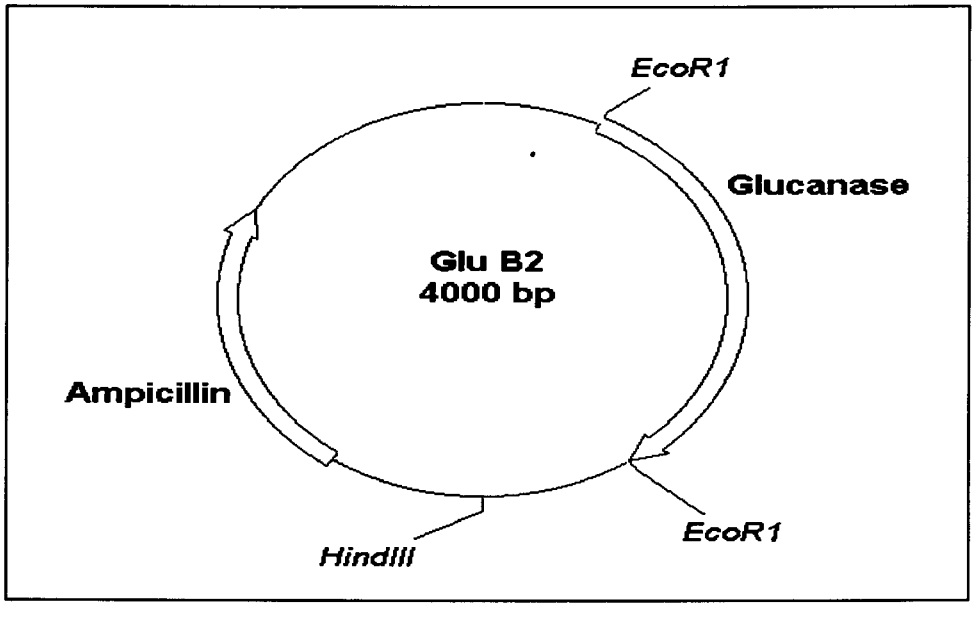


Figure 3.1.3 Plasmid Glu B2 containing the ß-1,3-glucanase gene (1.31 kb) cloned into an *Eco R1* restriction site and an ampicillin resistance gene.

3.2 Apparatus

The particle gun used was constructed similarly to the Particle Inflow Gun (PIG) of Finer and his colleagues (1992). It consisted of a thick-walled vacuum chamber enclosed in a metal casing and a single, large o-ring that ensured a tight vacuum within the chamber. The casing contained a solenoid and electronic control circuitry.

The vacuum chamber was connected to a vacuum pressure gauge that in turn was connected to a vacuum pump. A helium gas supply tank together with its pressure gauge was connected to the vacuum chamber. A third external connector consisted of a ventilation line that controlled the rate of air return in the vacuum chamber after bombardment.

A shelf carrying the target material could be positioned at four different levels within the chamber (7, 10, 13 and 16 cm). The micro-carrier launch assembly consisted of a micro-carrier plate onto which the micro-carrier/DNA preparation was loaded. The micro-carrier plate was placed inside the micro-carrier holder and screwed into position inside the vacuum chamber at the helium inlet. The vacuum chamber was placed in a laminar flow cabinet to ensure sterile handling of the plant tissue and to provide sterile air during ventilation.

3.3 Methods

3.3.1 Wheat growth conditions

Wheat was grown in a 500 x 100-mm pots containing a sand: peatmoss mixture (1:3 v/v). The plants were grown in a green house at a night temperature of 16 ± 1 °C and a day temperature of 25 ± 1 °C (Tiwari *et al.*, 1997).

3.3.2 Harvesting

The wheat plants were allowed to self-fertilise by covering the ears individually with the onset of anthesis. Four-week old ears were harvested and the seed stored at 4 °C.

3.3.3 Tissue culture initiation

Wheat seeds, collected shortly after fertilisation, were surface sterilised in 0.5% NaOCl for 5 min and imbibed overnight in sterilised water. The immature embryos were removed from the seeds under sterile conditions. The embryos were sterilised for 15 seconds in 70% ethanol, 10 minutes in 0.5% NaOCl and rinsed three times for 10 minutes each in sterilised water. The embryos were placed on either MS (Murashige and Skoog, 1962) or ML3 (Jähne *et al.*, 1991) growth medium solidified with 0.3% Gelrite (Saarchem) (Table 3.3.1). The embryos were placed in the dark at 26 ± 1 °C and subcultured every two weeks. The development of the embryos and the subsequent growth of plantlets on the two media were compared. The development of the plantlets was monitored by measuring the growth of the plantlets. The plants were regenerated on MS and ML3 media containing 5 mg/L benzylaminopurine (BAP) (Tiwari *et al.*, 1997).

Table 3.3.1 A comparison between the compositions of MS and ML3 media.

NUTRIENTS	NUTRIENT CONCENTRATIONS	
	MS Medium	ML3 Medium
Macro-elements	mg/L	mg/L
$CaCl_2 .2H_2O$	440	450
$FeSO_4 .7H_2O$	28	28
KH_2PO_4	170	200
KNO_3	1900	1 750
$MgSO_4 .7H_2o$	370	350
Na_2EDTA	37	37
NH_4NO_3	165	200
Micro-elements	mg/L	mg/L
$CoCl_2 . 6H_2O$	-	0.025
$CuSO_4 .5H_2O$	0.025	0.025
H_3BO_3	6.2	5.0
KI	0.83	0.75
$Na_2MoO_4 .2H_2O$	0.25	0.25
$MnSO_4 .4H_2O$	22.3	25
$ZnSO_4 .4H_2O$	8.6	7.5
Vitamins	mg/L	mg/L
Ascorbic acid	-	1.0
Biotin	-	0.005
Ca - pantothenate	-	0.5
Choline chloride	-	0.5
Folic acid	-	0.2
myo – Inositol	100.0	100.0
Nicotinic acid	1.0	1.0
p – Aminobenzoic acid	-	1.0
Pyridoxine – HCl	1.0	1.0
Riboflavin	-	0.1

Thiamine – HCl	10.0	10.0
Amino acids	**mg/L**	**mg/L**
Asparagine	100	100
Glutamine	750	750
Proline	150	150
Organic acids	**mg/L**	**mg/L**
Citric acid	-	10
Fumaric acid	-	10
Malic acid	-	10
Sodium pyruvate	-	5
Sugars	**g/L**	**g/L**
Cellobiose	-	0.125
Fructose	-	0.125
Maltose	60.0	30.0
Mannose	-	0.125
Rhamnose	-	0.125
Ribose	-	0.125
Xylose		0.125

3.3.4 Plasmid DNA preparation

3.3.4.1 Plasmid DNA transformation

Competent *Escherichia coli* cells (UltraMax DH5-FT) were used to transform all relevant DNA constructs (pHP 687, Cht B3 and Glu B2) used during this study. 100 µL competent cells were thawed on ice and 5 ng DNA added. The cells were incubated for 30 minutes on ice, given a heat-shock for 30 seconds at 42 °C and placed back on ice for 2 minutes. SOC (0.8 mL; 20 g/L bactotryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.19 g/L KCl, 1 mL 2 M Mg^{2+} solution, 2 mL 1 M glucose) at room temperature was added and shaken at 225 rpm for one hour at 37 °C. 10 µL and 100 µL of the suspension were spread on LB plates (Luria-Bertani-medium; 10 g/L NaCl, 10 g/L bactotryptone, 5 g/L yeast extract, 15 g/L agar) containing 100 µg/mL antibiotic and incubated overnight at 37 °C (Inoue *et al.*, 1990).

3.3.4.2 Plasmid purification

The transformed plasmid constructs were grown in LB medium containing 50 µg/mL ampicillin. The plasmid DNA was subsequently purified using NucleoSpin preparations (Marcherey-Nagel GmbH & Co. KG, Germany). The *E. coli* cells were pelleted in 1.5 mL eppendorf tubes at 10 000 rpm for 8 minutes at 4 °C. The pellet was resuspended in 250 µL RNase buffer to which 250 µL lysis buffer was added and incubated at room temperature for 5 minutes. 300 µL neutralisation buffer was added, incubated on ice for 5 minutes and centrifuged for 10 minutes at 14 000 rpm. The supernatant was loaded onto a NucleoSpin column and centrifuged for 1 minute at maximum speed. The column was washed with 500 µL buffer after which 700 µL ethanol buffer was added and centrifuged at maximum speed for 1 minute. Residual ethanol was removed by

centrifugation and the DNA was eluted with 50 µL elution buffer. The DNA was stored at -20 °C in sterilised water.

3.3.4.3 Plasmid restriction

Plasmid pHP 687 was restricted for 2 hours at 37 °C with *Bam H1* in order to recover the C1 and R fragments from the plasmid. Plasmid pHP 687 was further restricted with *Eco R1* in order to provide sticky ends for the ligation of the chitinase and β-1,3-glucanase genes into the vector. The Cht B3 and Glu B2 plasmids were restricted with *Eco R1* under similar conditions. The restricted DNA was run on a 1% agarose (Seakem, FMC BioProducts) gel for 30 minutes at 80 V with 1 x TAE buffer (4.84 g Tris-acetate, 1.142 mL acetic acid, 2 mL 0.5 M EDTA, pH 8.0) and visualised on UV light.

3.3.4.4 Fragment purification

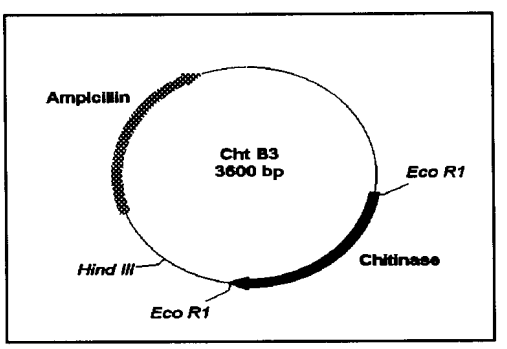
The R and C1 fragments, as well as the chitinase and β-1,3-glucanase genes, were run on a low-melt 1% agarose gel (Seakem). The fragments were excised from the gel and dissolved in a pH-indicating buffer for 10 minutes at 50 °C. Isopropanol was added to the samples and mixed. The samples were loaded on a QIAquick column placed on 2 mL collection tubes and centrifuged for 1 minute. The columns were washed with 750 µL ethanol-containing wash buffer and centrifuged for 1 minute. The flow-through was discarded and the columns centrifuged for an additional minute. The DNA was eluted with an elution buffer (10 mM Tris-HCl, pH 8.5) during a further centrifugation step.

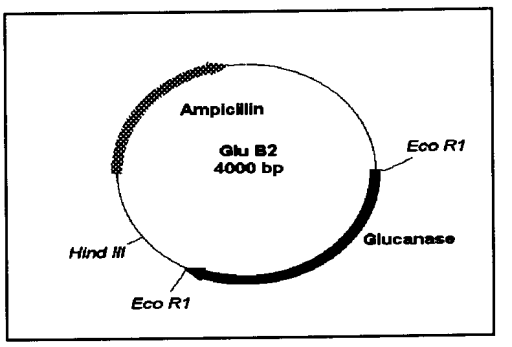
3.3.4.5 Plasmid dephosphorylation

The phosphorylated ends of plasmid pHP 687 that was restricted with *Eco R1*, were dephosphorylated for 20 minutes at 37 °C. The reaction consisted of 10 µL DNA, 4 µL 10 x alkaline phosphatase buffer, 0.5 µL alkaline phosphatase and 25.5 µL water.

3.3.4.6 Ligation

Plasmid pHP 687 was ligated with the chitinase and β-1,3-glucanase genes, respectively (Figure 3.3.1). These genes and the plasmid DNA were mixed in a 3:1 ratio and added to 1 µL 10 x ligase buffer, 1 µL T4 DNA ligase and 4 µL water.





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Restriction with *Eco R1*

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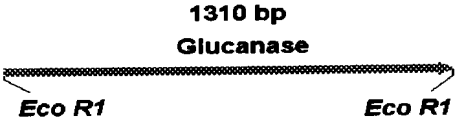

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Ligation into plasmid pHP 687 at *Eco R1* restriction sites

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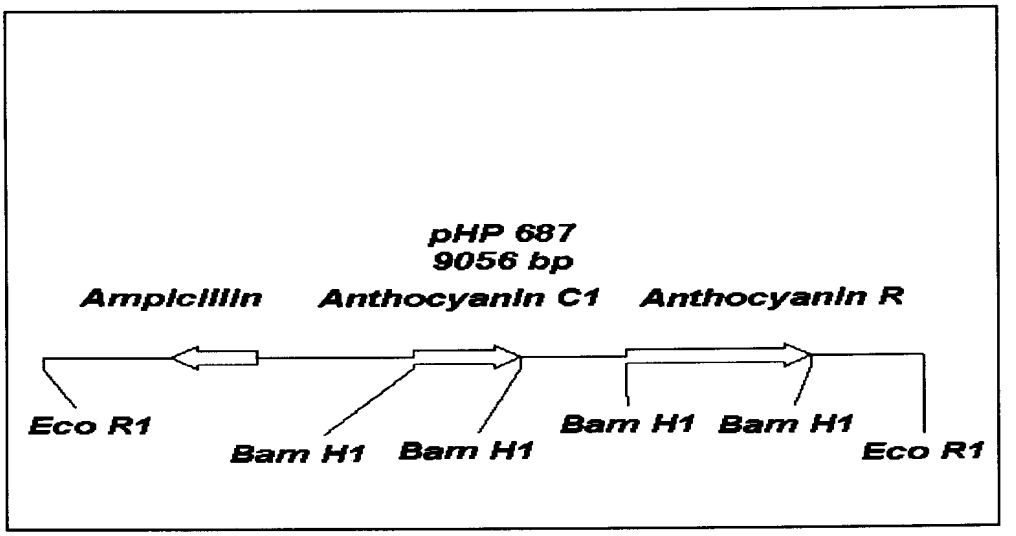
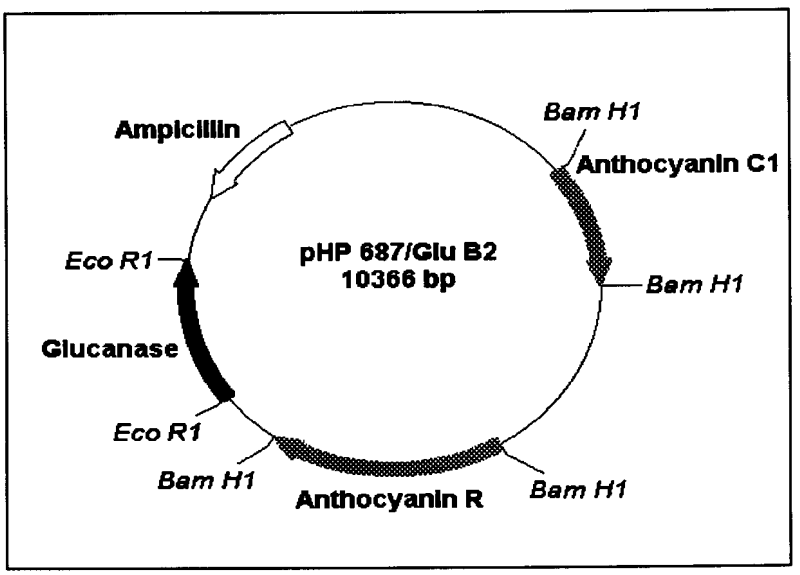


a



b

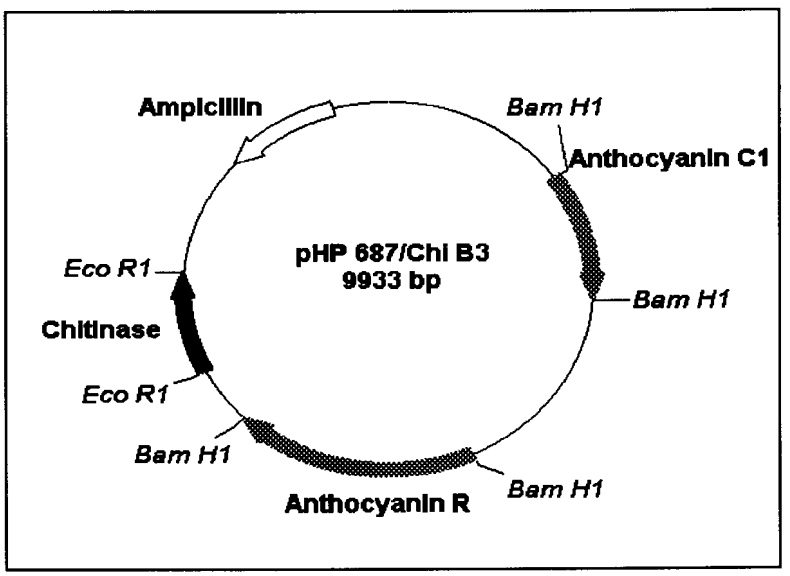


Figure 3.3.1 Schematic representation of the strategy to ligate β-1,3-glucanase and chitinase into pHP 687. **a**) Represents the pHP 687/Glu B2 construct and **b**) the pHP 687/Chi B3 construct.

3.3.5 Particle bombardment optimisation

The cultivars selected for use during particle bombardment optimisation are listed in paragraph 3.1.2, with the exclusion of 'Betta' *Dn2* and 'Gamtoos' *Dn2*.

3.3.5.1 Osmotic pre-treatment of target tissue

Tissue selected for bombardment included previously excised immature embryos and callus material ranging form 1 day- to 10 week-old material. The tissue to be bombarded, referred to as the target tissue, was placed on a 0.4 M osmotic medium 4 to 6 hours prior to bombardment. The osmotic medium consisted of 3.654% (m/v) mannitol, 3% (m/v) sucrose, 1 g/L casein hydrolysate and 0.6% (m/v) Gelrite. The tissue was placed in the dark at 26 ± 1 °C (Vain *et al.*, 1993).

3.3.5.2 DNA/micro-carrier preparation

The tungsten/gold particles were sterilised in 70% ethanol and rinsed three times in sterilised water. The particles were resuspended in sterilised water at a final concentration of 100 mg/mL. The bombardment mixture was prepared by mixing 25 µL of either the gold or tungsten suspension with 20 µL of the plasmid pHP 687 DNA (1 µg/µL) and 10 µL spermidine (0.2 M). The mixture was vortexed after the addition of each component and placed on ice for 5 min. The mixture was centrifuged briefly and the supernatant removed. The particles were resuspended in 30 µL 100% ethanol. The mixture was vortexed before each bombardment (Snyman *et al.*, 1996).

3.3.5.3 Bombardment procedure

The target tissue was placed on a grid inside the vacuum chamber at the various distances below the micro-carrier plate (Table 3.3.2). The bombardment mixture (3.5 µL per bombardment) was loaded in the centre of the micro-carrier plate. The micro-carrier assembly was screwed into position and the chamber closed. The chamber was evacuated to 70 kPa and one of the various helium pressures were applied (Table 3.3.2). The solenoid was triggered for 0.05 seconds to allow the acceleration of the particles towards the target tissue and the vacuum chamber was ventilated. These procedures were applied in order to optimise the particle bombardment system (Table 3.3.2). Each variable was tested individually in conjunction with every other variable. Each test consisted of 50 repetitions. The target tissue used during each repetition was subjected to similar preparatory procedures.

Table 3.3.2 The various bombardment parameters tested during optimisation.

Parameter	Parameter range	
Age of target tissue	1 day 3 days 4 days 5 days 1 week 2 weeks	3 weeks 6 weeks 7 weeks 8 weeks 9 weeks 10 weeks
Distance from micro-carrier plate to target tissue	10 cm 13 cm	16 cm
Helium pressure	1 200 kPa 1 400 kPa 1 600 kPa	1 800 kPa 2 000 kPa
Particles	Tungsten (1 µm) Gold (1 µm) Gold (1.5 – 3.0 µm)	

3.3.5.4 Post-bombardment treatment

The bombarded tissue was placed back on osmotic medium and placed in the dark for 24 to 48 hours. The expression of anthocyanin was assessed visually as red foci on the bombarded tissue. The proportion of bombarded tissue that expressed anthocyanin was determined as the number of embryos/calli expressing anthocyanin from the total number of embryos/calli that were bombarded. This will be referred to as the frequency of anthocyanin expression. The number of anthocyanin foci was determined as the number of cells per embryo/callus that expressed anthocyanin. The amount of tissue damage was assessed visually by estimating the percentage of dead, discoloured cells per embryo/callus.

Selection for transformed tissue was carried out on modified selection media as determined by Nehra and his colleagues (1994). MS basal medium was used and supplemented with the following concentrations of ampicillin: 50 mg/L and 75 mg/L (Table 3.3.3).

Table 3.3.3 The composition of the selection media on which the bombarded material was placed during selection.

Medium	Medium supplements	Antibiotic concentration (mg/L)	Conditions	Duration
MS basal medium	MS vitamins 2 mg/L 2,4-D 100 mg/L casamino acid 3% sucrose	0	± 26 °C, dark	2 weeks
MS basal medium	MS vitamins 2 mg/L 2,4-D 2% sucrose	50	± 26 °C, light	2-3 weeks
MS basal medium	MS vitamins 2% sucrose	75	± 26 °C, light	2-3 weeks

3.3.5.5 Plant regeneration

Wheat material surviving the strict selection process was transferred to a modified MS basal medium in order to stimulate further growth (Table 3.3.4).

Table 3.3.4 Composition of the regeneration media.

Time	MS medium supplements	Function
Week 8-10	2 mg/L 2,4-D 100 mg/L casein hydrolysate 1 mg/L nicotinic acid 100 mg/L myo-Inositol 1 mg/L pyridoxine HCl 10 mg/L thiamine HCl 500 mg/L glutamine 10 mg/L zeatin	Shoot production
Week 10-12	0.1 mg/L 2,4-D 100 mg/L casein hydrolysate 1 mg/L nicotinic acid 100 mg/L myo-Inositol 1 mg/L pyridoxine HCl 10 mg/L thiamine HCl 500 mg/L glutamine 5 mg/L BAP	Root induction

3.3.6 Bombardment of chitinase and β-1,3-glucanase

Target material was further bombarded with the chitinase and ß-1,3-glucanase gene constructs using similar preparations for the target material and implementing the optimum conditions as determined during optimisation.

Chapter 4

Results

4.1 Tissue culture initiation

Tissue cultures were initiated from immature wheat embryos (Figure 4.1.1). The scutellum cells of the immature embryos developed embryogenic callus tissue with somatic embryos. Non-embryogenic callus, consisting of soft, white, watery tissue formed from the dividing embryo cells. Embryogenic callus, consisting of yellow, nodular tissue, emerged from the side of the non-embryogenic callus (Figure 4.1.2).

The MS medium proved sufficient for the development of embryogenic callus tissue, as well as for the regeneration of plantlets from somatic embryos of all South African wheat cultivars (Figures 4.1.3 and 4.1.4). Very little growth was detected on the ML3 medium in comparison with the MS medium (Figure 4.1.5). All the cultivars tested exhibited a regeneration capacity of more than 80% on the MS medium (Figure 4.1.5).

The development of the calli into plantlets was monitored (Table 4.1.1). Onset of shoot development took place in the 'Palmiet' cultivars after the third successive subculture. The 'Palmiet' *Lr34* cultivar was the first to develop roots. 'Betta' *Dn2* was found to exhibit the most vigorous root growth and 'Palmiet' *Lr29* and 'Tugela' the most vigorous shoot growth. 'Gamtoos' and 'Tugela' *Dn1* exhibited vigorous growth in both organs. The above mentioned plantlets were transferred to soil and allowed to grow into mature plants.

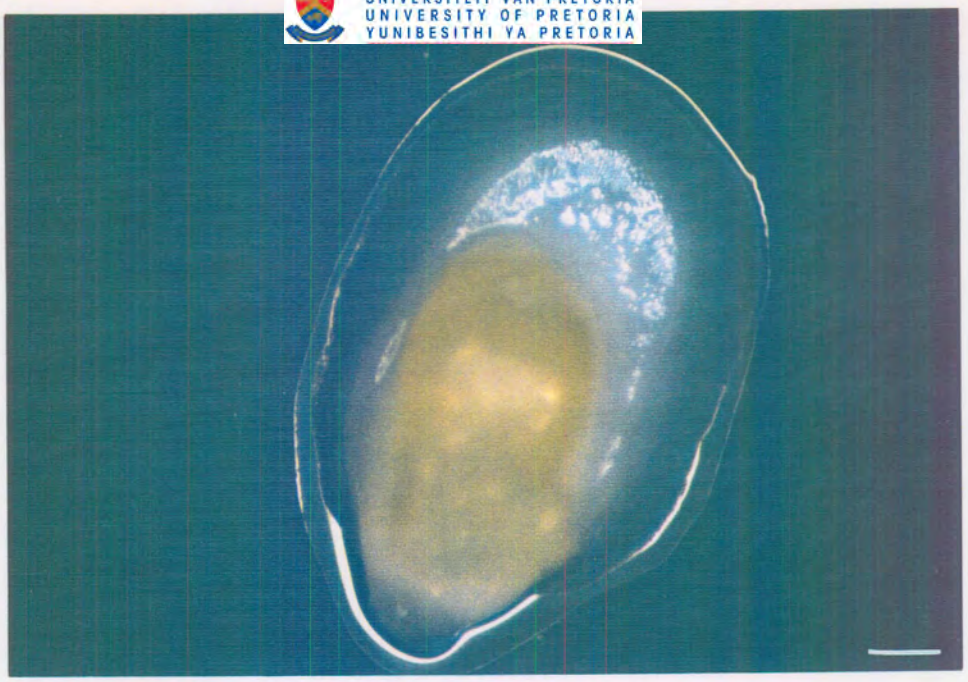


Figure 4.1.1 An immature wheat embryo excised from a 'Palmiet' *Dn5* seed used for tissue culture initiation. Bar represents 1 mm.

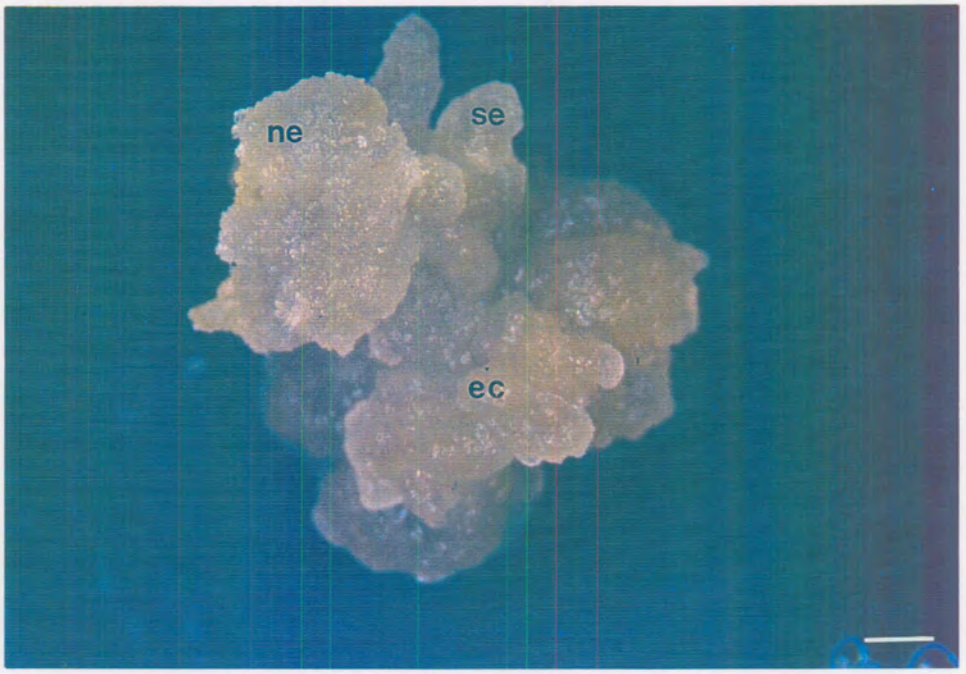


Figure 4.1.2 Embryogenic callus developing from an immature embryo of 'Tugela'*Dn1* after 4 weeks of culture initiation on MS medium. A somatic embryo is visible. Bar represent 5 mm. ec = embryogenic callus; ne = non-embryogenic callus; se = somatic embryo.

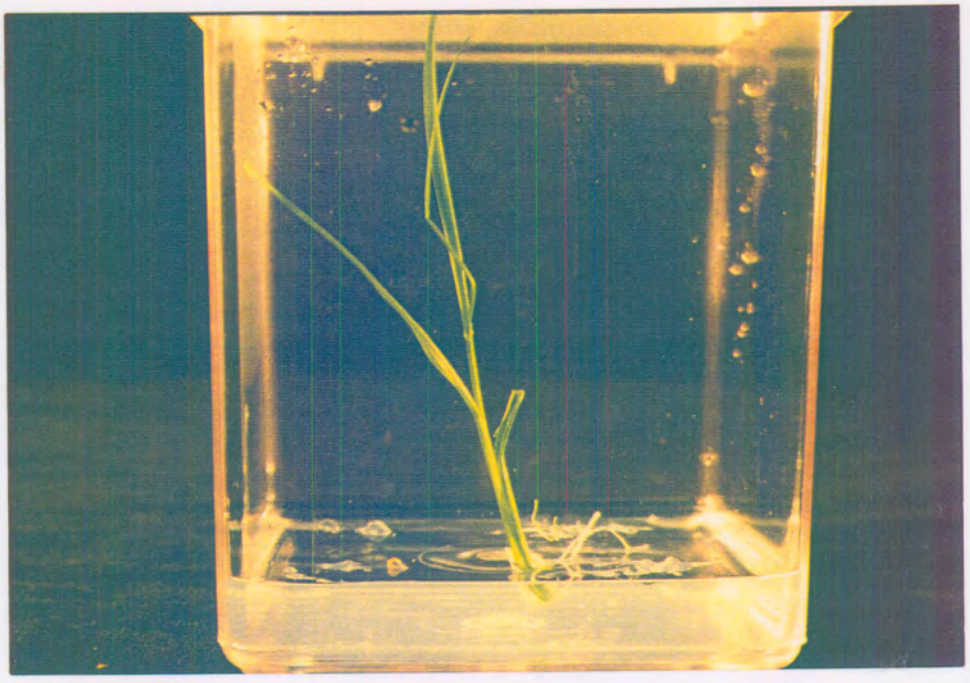


Figure 4.1.3 A 'Tugela' plantlet formed from a somatic embryo on MS medium after 8 weeks in culture.

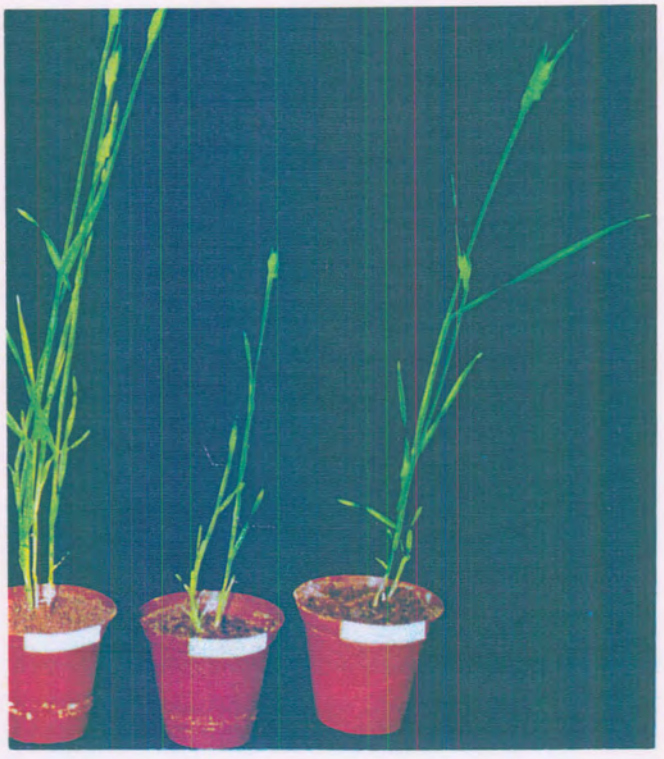


Figure 4.1.4 'Tugela' plants transferred to soil after 10 weeks in culture. Plants are 12 weeks old.

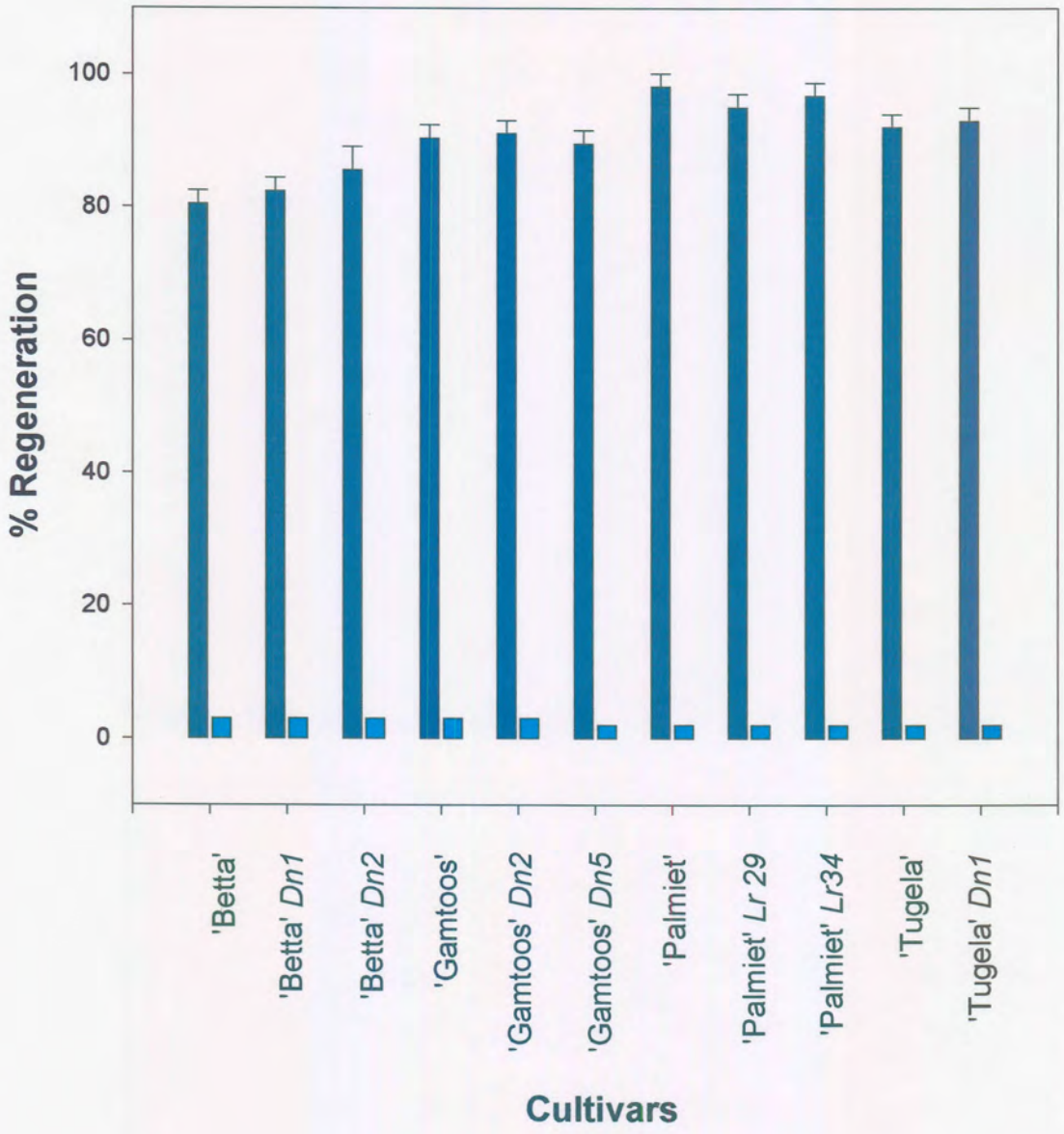


Figure 4.1.5 Influence of culture media on the regeneration capacity of different wheat cultivars. ■ = MS medium (Tiwari *et al.*, 1997); ■ = ML3 medium (Jähne *et al.*, 1991). Values are the mean of hundred replicas. SD was larger than 10% of the mean.

Table 4.1.1. The developmental progress of wheat plantlets on MS medium.

Cultivar	Age of plantlet	Growth medium	Shoot development	Root development
'Palmiet'	32 days	MS	2 leaves; 1-2 cm	0
		ML	0	0
'Betta' *Dn2*	34 days	MS	1-2 leaves, 0.5-2cm	0
		ML	0	0
'Palmiet'	35 days	MS	2 leaves; 2-3 cm	0
		ML	0	0
'Palmiet' *Lr34*		MS	1-3 leaves; 1-5 cm	2 roots;0.5-2cm
		ML	0	0
'Tugela'		MS	1-2 leaves; 0.5-1cm	1-2 roots;0.5-1.5cm
		ML	0	0
'Tugela' *Dn1*		MS	1-2 leaves; 0.5-2cm	1-2 roots;0.5-1.5 cm
		ML	0	0
'Betta' *Dn1*	39 days	MS	1 leaf; 0.5 cm	0
		ML	0	0
'Betta' *Dn2*		MS	1-3 leaves; 0.5-3cm	1-2 roots;0.5-2 cm
		ML	0	0
'Gamtoos'		MS	1-2 leaves; 0.5-3cm	1-2 roots;1-2 cm
		ML	0	0
'Gamtoos' *Dn2*		MS	1-3 leaves; 0.5-5cm	1-2 roots;1-2.5 cm
		ML	0	0
'Gamtoos' *Dn5*		MS	1 leaf; 1 cm	1-2 roots;0.5-1 cm
		ML	0	0
'Palmiet'	40 days	MS	2-3 leaves; 2.5-5cm	1-3 roots;1-2.5 cm
		ML	0	0
'Palmiet' *Lr29*		MS	1 leaf; 1.5 cm	1-2 roots;0.5 cm
		ML	0	0
'Palmiet' *Lr34*		MS	1-3 leaves; 2.5-6cm	1-3 roots;2-4 cm
		ML	0	0
'Tugela'		MS	1-3 leaves; 1-4 cm	1-3 roots;2-3.5 cm
		ML	0	0
'Tugela' *Dn1*		MS	2-3 leaves; 3-8 cm	1-2 roots;2.5-4.5 cm
		ML	0	
'Betta'	45days	MS	2 leaves; 6cm	1-2 roots;0.5-2 cm
		ML	0	0
'Betta' *Dn2*		MS	2-3 leaves; 2-15cm	4 roots; 2-6 cm
		ML	0	0
'Gamtoos'		MS	2-3 leaves; 4-10cm	3 roots;2-5 cm
		ML	0	0
'Gamtoos' *Dn2*		MS	1-3 leaves; 2-6 cm	0
		ML	0	0
'Palmiet' *Lr29*		MS	3-4 leaves; 1-6cm	1-2 roots;1-3 cm
		ML	0	0
'Palmiet' *Lr34*		MS	1-2 leaves; 1-8cm	1-2 roots; 1.5-4 cm
		ML	0	0
'Tugela'		MS	2-3 leaves; 1-10cm	4 roots; 2-4 cm
		ML	0	0
'Tugela' Dn1		MS	1-8 leaves; 1-10cm	4 roots; 3-8 cm
		ML	0	0

4.2 Plasmid DNA preparation

Plasmid pHP 687 was successfully transformed into and purified from the *E. coli* cells. The plasmid DNA was restricted with *Bam H1* restriction enzyme in order to obtain the C1 and R anthocyanin fragments (Figure 4.2.1). A restriction with *Sal 1* was performed as a control reaction.

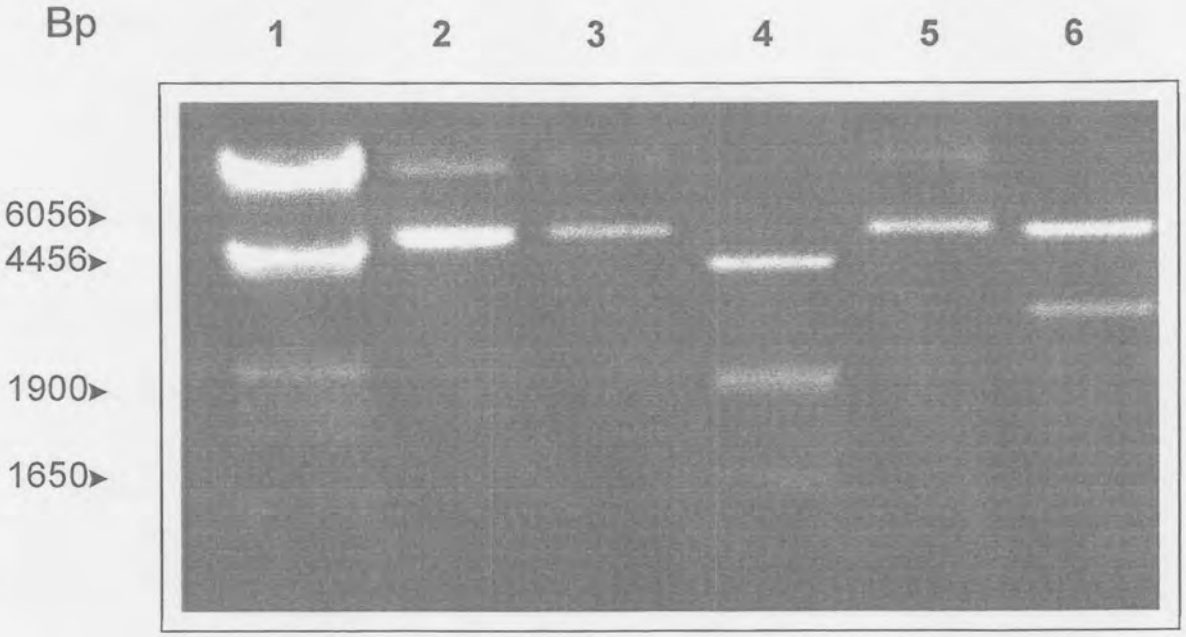


Figure 4.2.1 Plasmid pHP 687 restricted with *Bam H1* and *Sal 1* restriction enzymes. Lane 1 = molecular weight marker III; lane 2 = plasmid pHP 687 extracted from *E. coli* cells; lane 3 = control plasmid pHP 687 DNA; lane 4 = pHP 687 + *Bam H1*; lane 5 = control plasmid pHP 687 DNA; lane 6 = pHP 687 + *Sal 1*.

The Glu B2 and Cht B3 plasmids were restricted with *Eco R1* in order to purify the chitinase and ß-1,3-glucanase genes from the vectors (Figure 4.4.2).

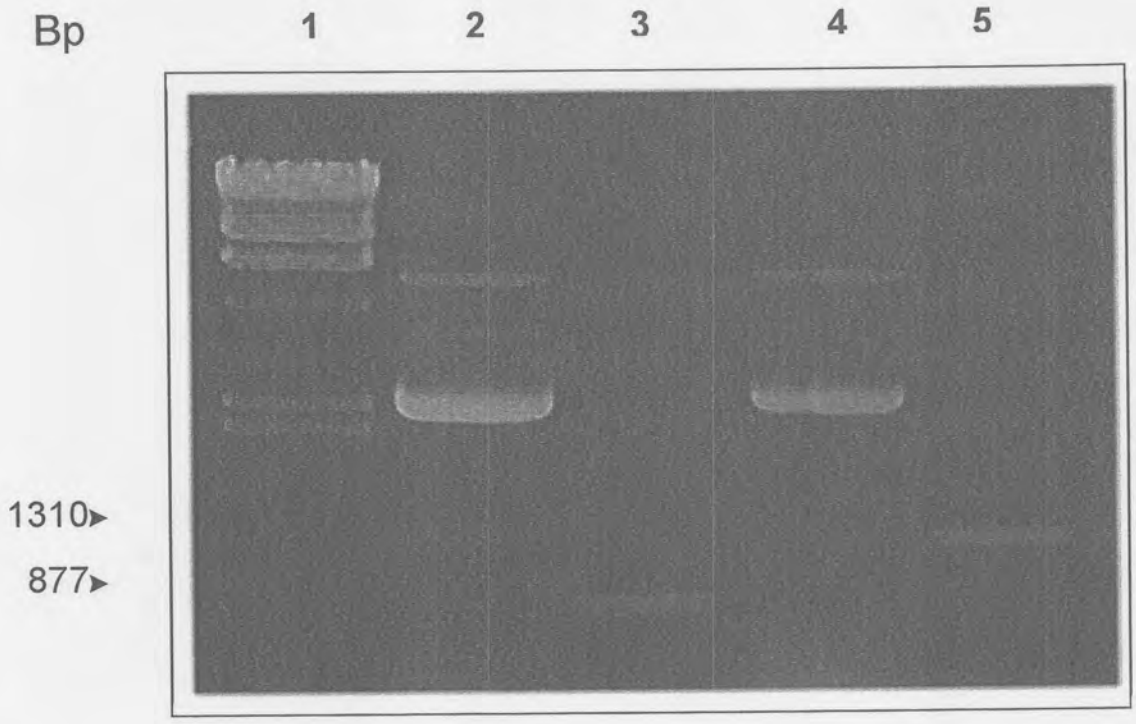


Figure 4.2.2 The restriction of plasmids Cht B3 and Glu B2 with *Eco R1*. Lane 1 = molecular weight marker III; lane 2 = plasmid Cht B3; lane 3 = chitinase gene; lane 4 = plasmid Glu B2; lane 5 = ß-1,3-glucanase gene.

The ligation of these two genes into the pHP 687 plasmid, however, was unsuccessful. Therefore, bombardment with the chitinase and ß-1,3-glucanase genes together with the marker gene was not carried out. The material bombarded with the Cht B3 and Glu B2 constructs was kept on culture media in order to regenerate plants from which DNA could be extracted for further analysis.

4.3 Particle bombardment optimisation

The particle bombardment system was optimised by determining the ideal conditions to be applied during bombardment. The tissue was bombarded with tungsten particles and the age ranged from 1 day-old embryos to 10 week-old calli. The frequency of transient anthocyanin expression was assessed by determining the amount of red foci produced in the bombarded material (Figures 4.3.1 and 4.3.2).

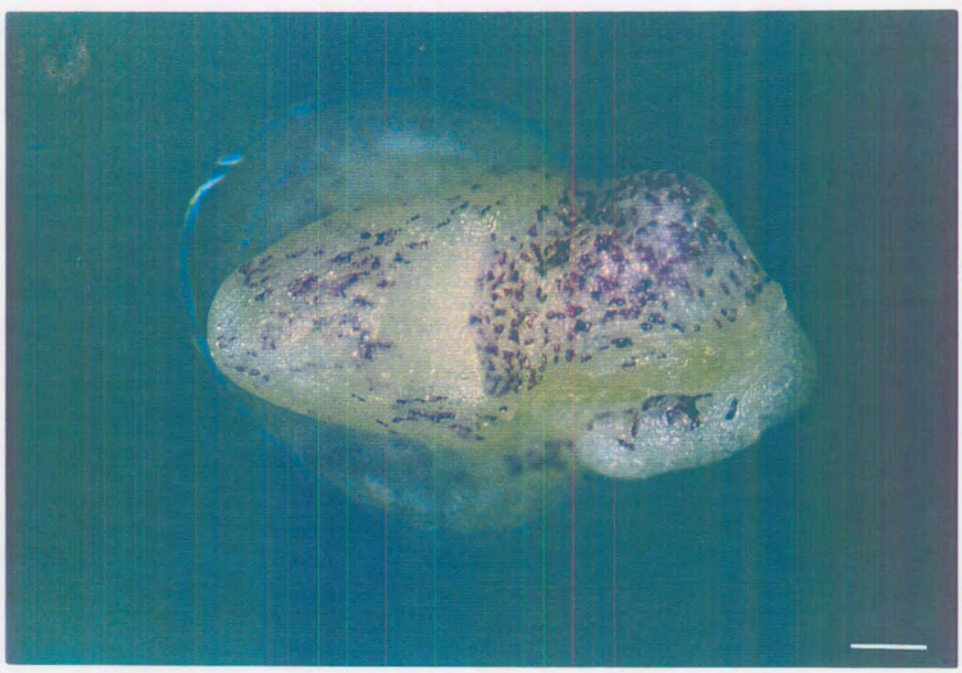


Figure 4.3.1 Transient anthocyanin expression in a 5-day old 'Palmiet' *Dn5* embryo 72 hours after bombardment with plasmid pHP 687. Bar represent 1 mm.

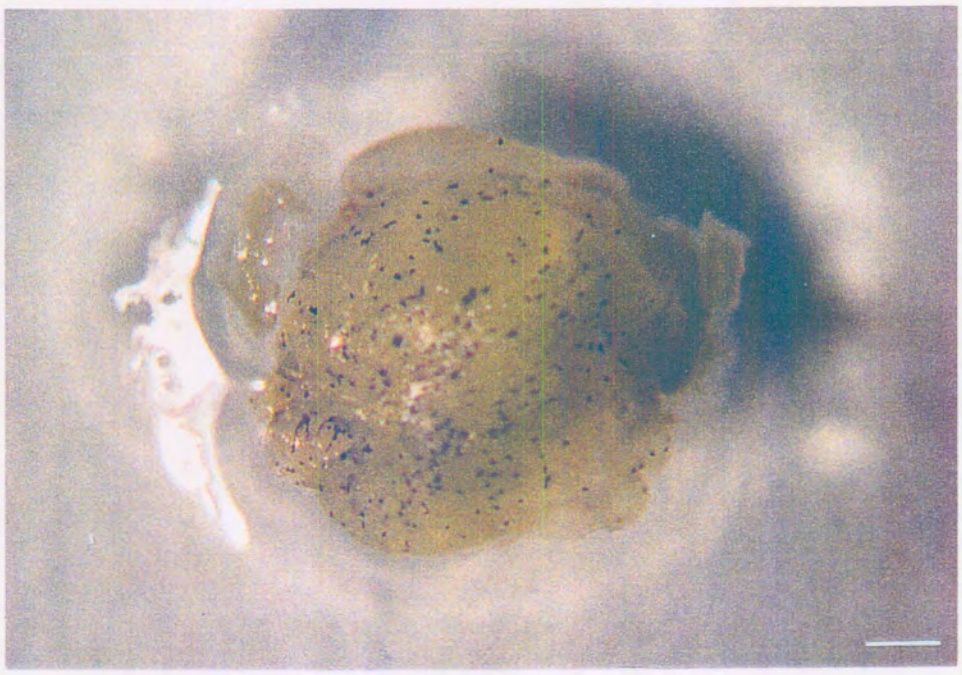


Figure 4.3.2 Transient anthocyanin expression in a developing, 10-day old 'Tugela' callus 48 hours after bombardment with pHP 687. Bar represents 5 mm.

The first bombardment parameter to be optimised was the distance between the target tissue and micro-carrier plate (Table 4.3.1). It was found that the closer the target tissue was placed to the DNA/micro-carrier assembly, the higher the average frequency of anthocyanin expression. In other words, the closer the tissue was placed to the DNA, the more the bombarded cells expressed anthocyanin. The highest average amount of foci per embryo/callus, however, was obtained at a distance of 16 cm and could be due to the low amount of tissue damage caused to the material. The amount of tissue damage caused at a distance of 10 cm was higher than at 16 cm, but this tissue could still be regenerated. Since tissue bombarded at 10 cm exhibited such a high frequency of anthocyanin expression, this distance was used during subsequent bombardments.

Table 4.3.1 Optimisation of the distance from the micro-carrier plate to target tissue.

Distance from micro-carrier plate to target tissue	Age of tissue	% Tissue expressing anthocyanin	Average foci per embryo/ callus	% Tissue damage
10 cm	1 day	65	60	10
	3 days	50	20	10
	4 days	66	10	15
	5 days	100	30	15
	1 week	44	30	15
	2 weeks	40	30	20
	3 weeks	42	30	30
	6 weeks	18	15	25
	7 weeks	6	10	20
	8 weeks	22	15	45
	9 weeks	6	10	15
	10 weeks	0	0	20
		38 ± 0.57	**22 ± 6.02**	**20 ± 1.29**
13 cm	1 day	66	20	10
	4 days	75	20	15
	1 week	40	30	15
	2 weeks	60	25	15
	3 weeks	28	35	30
	6 weeks	24	20	20
	7 weeks	10	20	20
	8 weeks	18	10	45
	9 weeks	17	10	15
	10 weeks	0	0	45
		34 ± 0.23	**20 ± 1.31**	**23 ± 3.33**
16 cm	1 day	20	20	5
	4 days	18	35	10
	1 week	25	18	15
	3 weeks	17	10	15
	6 weeks	20	20	22
	7 weeks	15	45	20
		19.2 ± 1.45	**24.6 ± 1.26**	**14.5 ± 2.34**

Another important bombardment parameter is the helium pressure used to accelerate the DNA-coated particles (Table 4.3.2). Pressures of 1 400 kPa and 2 000 kPa resulted in the highest frequency of anthocyanin expression. The amount of tissue damage caused at 2 000 kPa, however, was significantly more than the damage caused at 1 400 kPa.

Pressures of 1 200, 1 400 and 1 600 kPa resulted in a comparable amount of foci per embryo/callus. The low amount of tissue expressing anthocyanin at 1 200 kPa resulted from inefficient penetration of the target material by the micro-carriers. This was due to low velocity of the micro-carriers resulting from the lower helium pressure. The least amount of tissue damage was caused at pressures of 1 400 and 1 600 kPa. Since a pressure of 1 400 kPa resulted in the higher anthocyanin frequency when compared to 1 600 kPa, this pressure was applied during subsequent bombardment procedures.

Table 4.3.2 Optimisation of helium pressure used per bombardment.

Helium pressure	Age of tissue	% Tissue expressing anthocyanin	Average foci per embryo/ callus	% Tissue damage
1 200 kPA	1day	23	41	15
	4 days	27	29	15
	1week	24	30	20
	3 weeks	15	38	40
	8 weeks	21	28	23
		22 ± 1.83	**33.2 ± 2.36**	**22.6 ± 4.12**
1 400 kPa	1 day	55	100	5
	3 days	50	20	10
	1 week	34	20	10
	2 weeks	46	40	25
	7 weeks	33	15	20
	8 weeks	28	10	25
		41 ± 2.13	**34 ± 3.98**	**16 ± 1.19**
1 600 kPa	1 day	31	60	10
	4 days	42	48	10
	11 days	47	52	15
	3 weeks	12	20	15
	7 weeks	0	0	25
		26.4 ± 5.99	**36 ± 7.02**	**15 ± 2.45**
1 800 kPa	2 weeks	27	15	25
	3 weeks	27	10	25
	4 weeks	10	10	20
	5 weeks	10	10	15
	6 weeks	28	35	20
	7 weeks	25	70	18
	8 weeks	0	0	15
	9 weeks	15	10	15
	10 weeks	0	0	40
		16 ± 6.88	**18 ± 3.55**	**21 ± 6.52**
2 000 kPa	1day	67	20	15
	4 days	60	20	15
	5 days	100	20	15
	1 week	38	20	15
	2 weeks	65	30	20
	3 weeks	41	35	30
	6 weeks	32	10	22
	7 weeks	0	0	20
	8 weeks	10	10	33
	9 weeks	10	10	18
	10 weeks	0	0	50
		38.5 ± 9.01	**16 ± 2.49**	**23 ± 4.22**

The age of the material being bombarded was found to play a significant role in the transient expression of anthocyanin (Table 4.3.3). The older the bombarded tissue, the less anthocyanin expression was observed. Tissue ranging from 1 day-old embryos to 1 week-old calli exhibited the highest percentage of anthocyanin expression. Further, the maximum number of foci per embryo was exhibited in material between 2 and 3 weeks. The transient expression decreased rapidly as the age of the material increased. Calli exceeding 8 weeks exhibited the lowest percentage of anthocyanin expression. It can be clearly seen that as the age of the target material increased, the amount of injury caused during bombardment, increased accordingly.

Table 4.3.3 The effect of tissue age on the expression of anthocyanin and the amount of tissue damage.

Tissue age	% Anthocyanin expression[a]	Average foci per embryo/callus[a]	% Tissue damage[a]
1 day – 1 week	78.0	180	15.0
1 – 2 weeks	51.4	180	15.0
2 – 3 weeks	37.2	220	22.5
3 – 6 weeks	34.9	100	23
6 – 8 weeks	10.8	70	22.5
8 – 10 weeks	9.2	50	35.0

[a] Values presented are the mean of 10 replicas. SD was larger than 10% of the mean.

The percentage of anthocyanin expression between the different cultivars was tested. Eight wheat cultivars exhibited transient anthocyanin expression when bombarded with tungsten particles (Table 4.3.4). The 'Palmiet' *Dn1*, 'Palmiet' *Dn2* and 'Palmiet' *Dn5* cultivars exhibited the highest percentage of anthocyanin expression. The most foci per embryo, however, were found in the 'Palmiet' and 'Palmiet' *Dn2* cultivars. 'Palmiet' *Lr29*, 'Palmiet' *Lr34* and 'Palmiet' *Dn5* expressed lower numbers of anthocyanin foci per embryo/callus, whereas the other cultivars exhibited almost half the frequency of the above mentioned cultivars.

Table 4.3.4 Variation in anthocyanin transient expression of eight different cultivars.

Cultivar	% Bombarded calli expressing anthocyanin[a, b]	Average foci per embryo/callus
'Gamtoos'	20.0	90
'Palmiet'	56.3	200
'Palmiet' *Lr29*	41.2	170
'Palmiet' *Lr34*	39.5	160
'Palmiet' *Dn1*	80.0	80
'Palmiet' *Dn2*	75.0	200
'Palmiet' *Dn5*	75.4	160
'Tugela' *Dn1*	52.7	120

[a] Values presented are the mean of ten replicas. SD was larger than 10% of the mean.
[b] Callus tissue aged 2 to 3 weeks.

4.4 Tungsten versus Gold Particles

Target material was bombarded with tungsten (1 µm) and gold (1 µm and 1.5 - 3.0 µm) particles, respectively, in order to determine optimal anthocyanin expression and tissue viability (Figure 4.4.1). Material ranging from one day to four weeks was selected, since this was found to be the optimum age range for bombardment. A distance of 10 cm between the target material and micro-carrier assembly, and a helium pressure of 1 400 kPa, were applied.

For tissue ranging from 1 day to 1 week, bombardment with tungsten particles resulted in the highest frequency of anthocyanin expression. Bombardment with the tungsten and the small gold particles did not result in significant differences in anthocyanin expression for tissue between 1 week and 4 weeks. In all the age groups, the tissue expressed a low amount of anthocyanin when bombarded with the larger gold particles. This is specifically true for the older tissue (3 to 4 weeks). Figure 4.4.1 clearly indicates that the younger the bombarded material, the higher the frequency of anthocyanin expression.

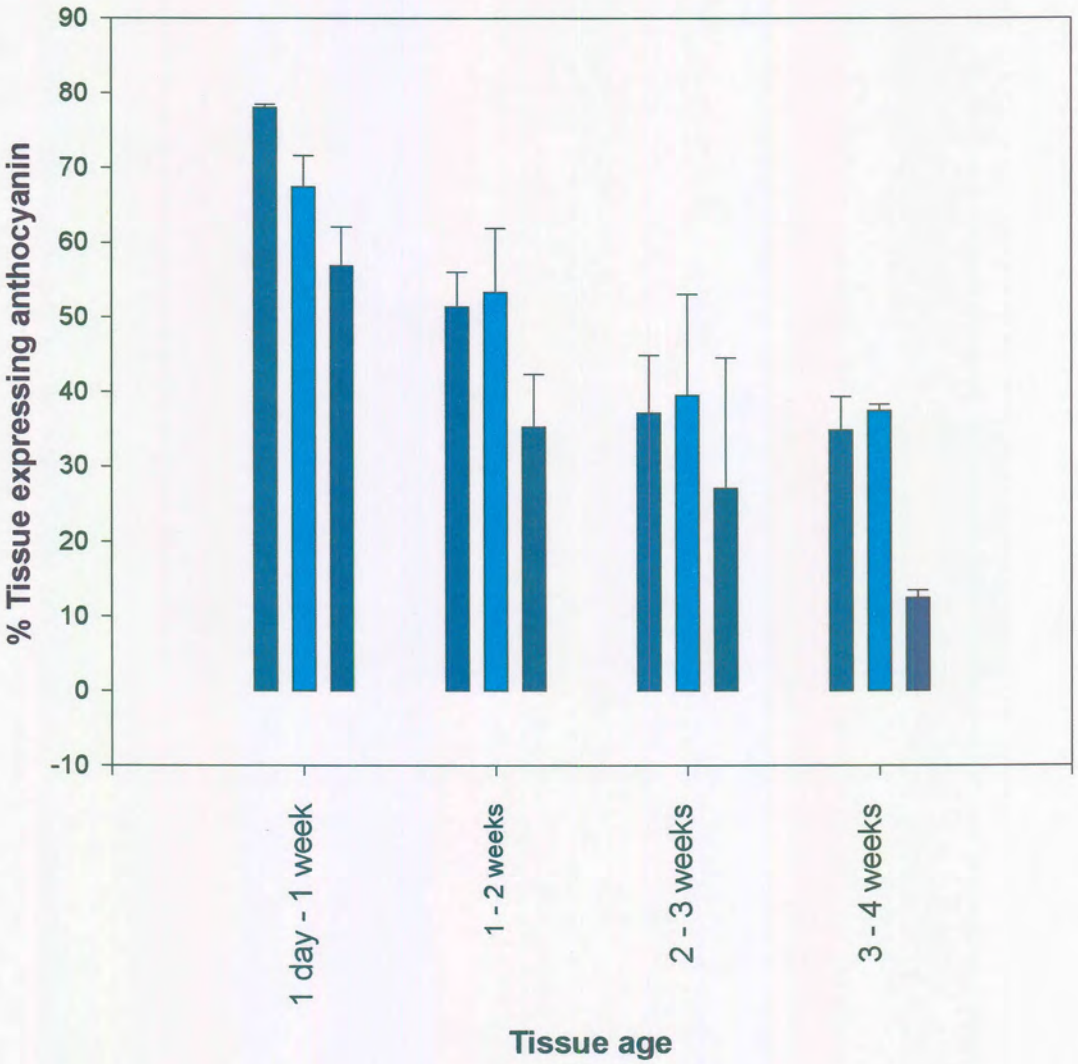


Figure 4.4.1 A comparison between the percentage of bombarded tissue expressing anthocyanin after bombardment with tungsten or gold particles. ■ = tungsten (1 µm); ■ = gold (1 µm); ■ = gold (1.5 - 3 µm).

The average amount of anthocyanin foci that were expressed by each embryo/callus was determined after bombardment with the different micro-carriers (Figure 4.4.2). For tissue up to 1 week, as well as for tissue between 3 and 4 weeks, bombardment with the small gold particles resulted in the highest amount of foci per embryo/callus. Tungsten seemed to be more efficient for tissue ranging from 1 to 3 weeks. Bombardment with the larger gold particles resulted in the least amount of foci for all the age groups.

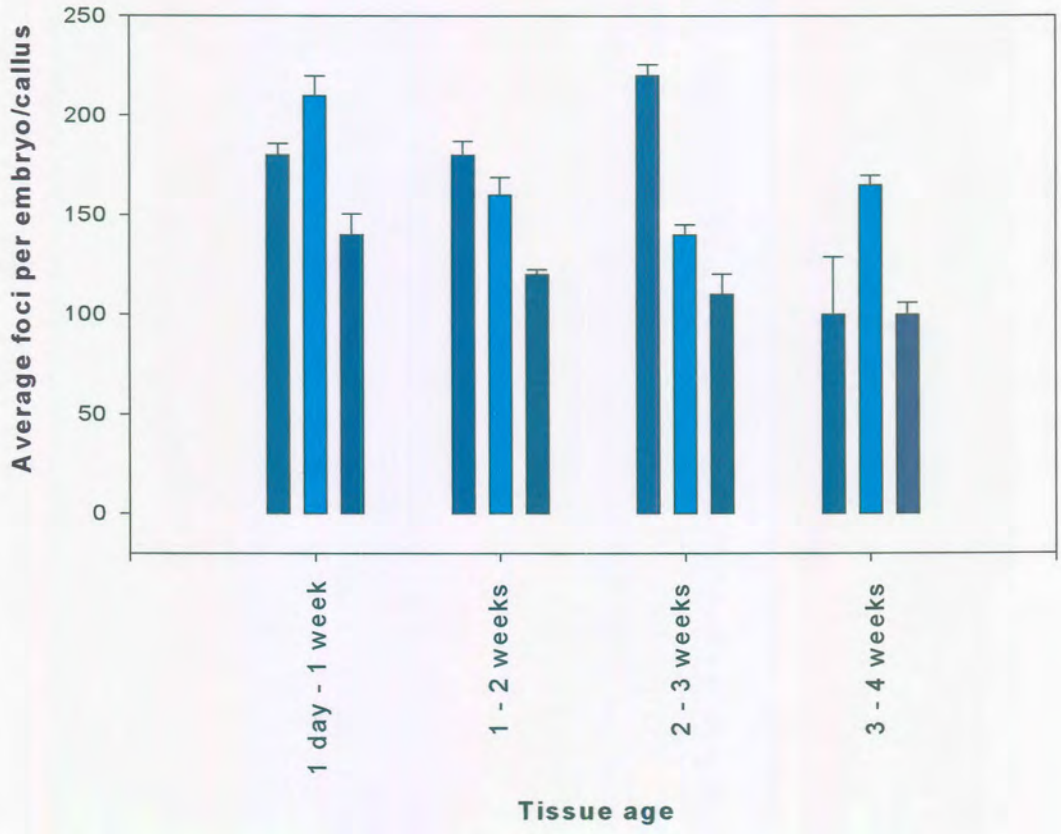


Figure 4.4.2 A comparison of the amount of anthocyanin foci in the embryo/callus tissue after bombardment with tungsten or gold micro-carriers at various ages. ■ = tungsten (1 µm); ■ = gold (1 µm); ■ = gold (1.5 - 3 µm).

Figure 4.4.3 indicates that as the age of the target material increases, the amount of tissue damage increases accordingly. In tissue ranging from 2 to 4 weeks, the larger gold particles resulted in the highest percentage of injured cells. In tissue ranging from 1 day to 1 week-old, bombardment with tungsten had the most destructive consequences. The effect of the smaller gold particles on the tissue of each age category, except for 1 – 2 weeks, was not as damaging as the other particles.

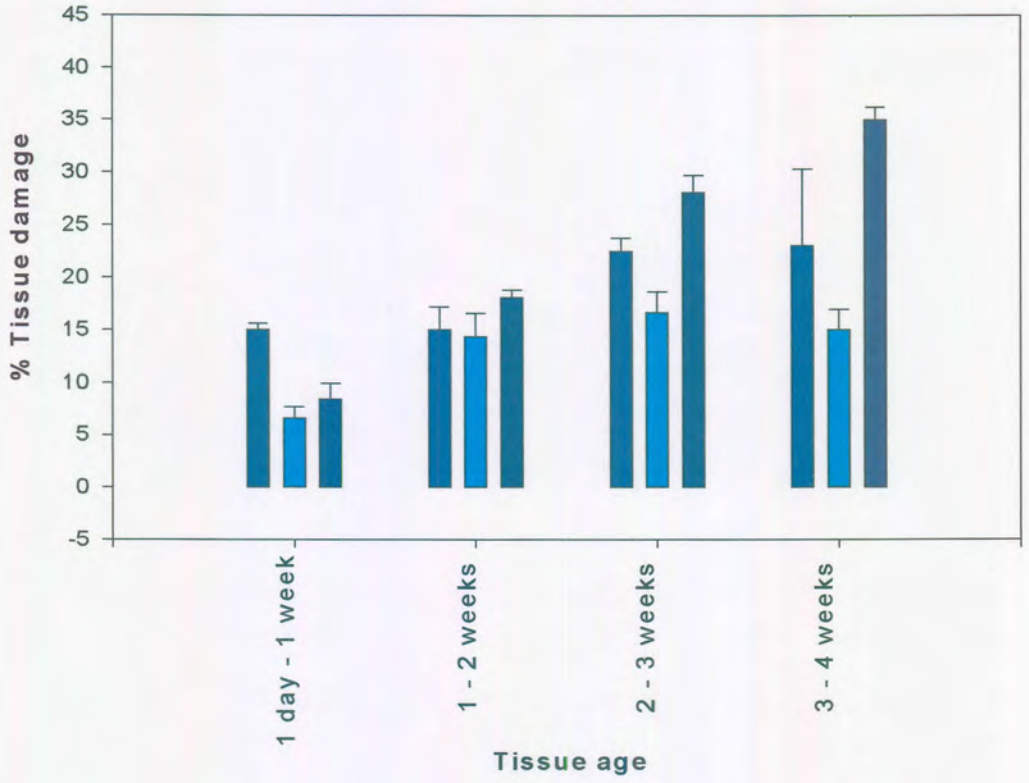


Figure 4.4.3 A comparison between the amount of tissue damage caused to wheat embryos/calli bombarded with tungsten or gold particles, respectively. ■ = tungsten (1 µm); ■ = gold (1 µm); ■ = gold (1.5 - 3 µm).

The frequency of anthocyanin expression between the various cultivars bombarded with tungsten particles, was monitored and compared (Figure 4.4.4). When bombarded with tungsten, the 'Palmiet' *Dn1*, *Dn2* and *Dn5* cultivars exhibited a frequency of anthocyanin expression of more than 75% each. The frequency anthocyanin expression in the other cultivars was less.

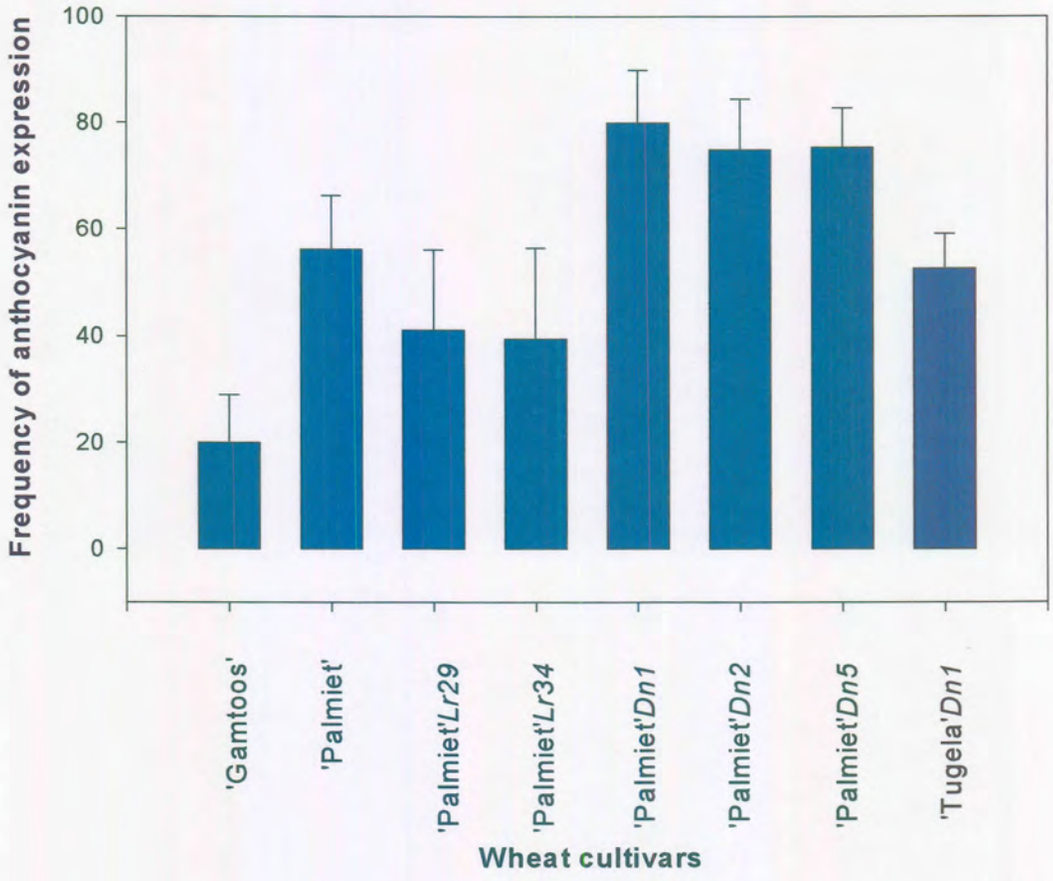


Figure 4.4.4 A comparison between the percentage of tissue bombarded with tungsten particles exhibiting anthocyanin expression as expressed by different wheat cultivars.

The cultivars bombarded with the smaller gold particles (1 µm), generally exhibited a higher frequency of anthocyanin expression than when these cultivars were bombarded with the larger particles (1.5 – 3 µm). 'Betta', 'Betta' *Dn1* and 'Palmiet' *Dn1* exhibited the highest percentage of anthocyanin expression: 91.7%, 80% and 71.1%, respectively. The 'Gamtoos', 'Gamtoos' *Dn5*, 'Palmiet' *Dn5* and 'Tugela' cultivars exhibited an average of 60% anthocyanin expression.

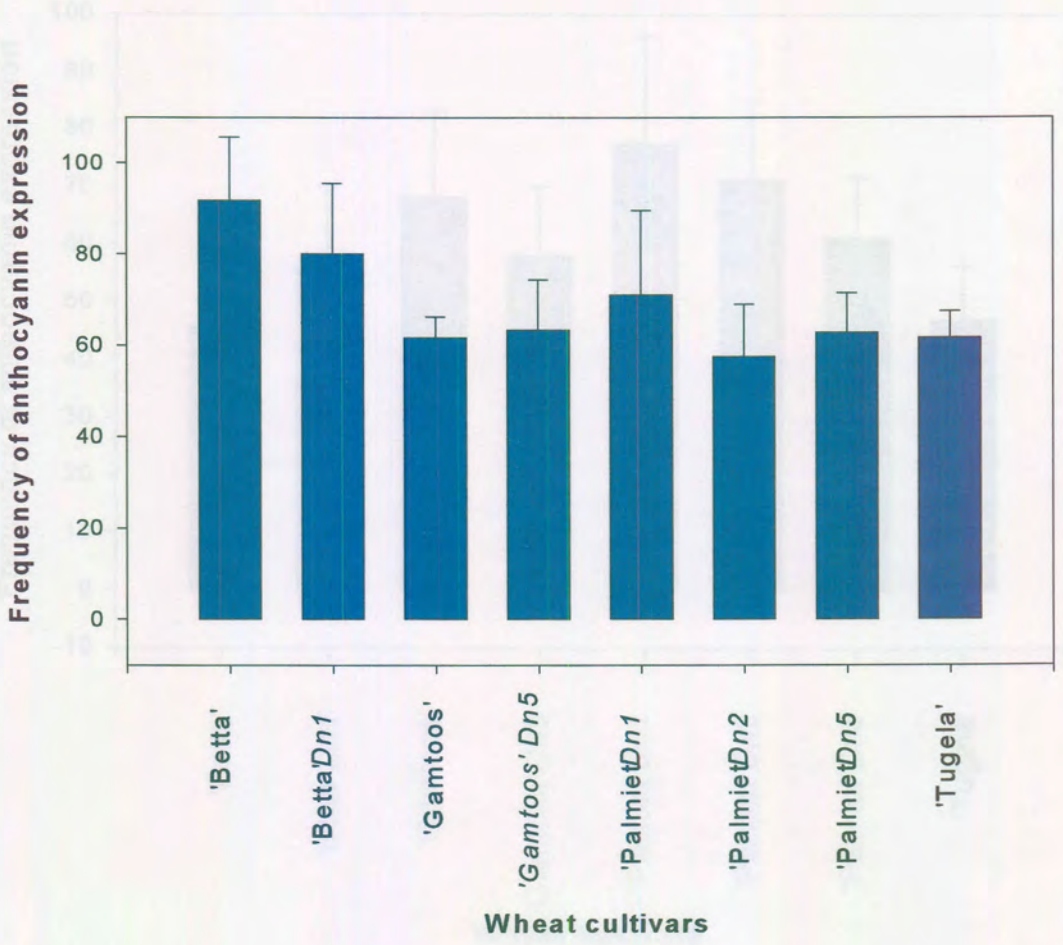


Figure 4.4.5 A comparison between the percentage of tissue exhibiting anthocyanin expression when bombarded with 1 µm gold particles as expressed by different wheat cultivars.

The cultivars displaying the highest percentage of anthocyanin expression when bombarded with the larger gold particles, were 'Palmiet' *Dn1* (77.8%), 'Palmiet' *Dn2* (71.3%) and 'Gamtoos' (68.3%). All other cultivars exhibited a frequency of 60% and less.

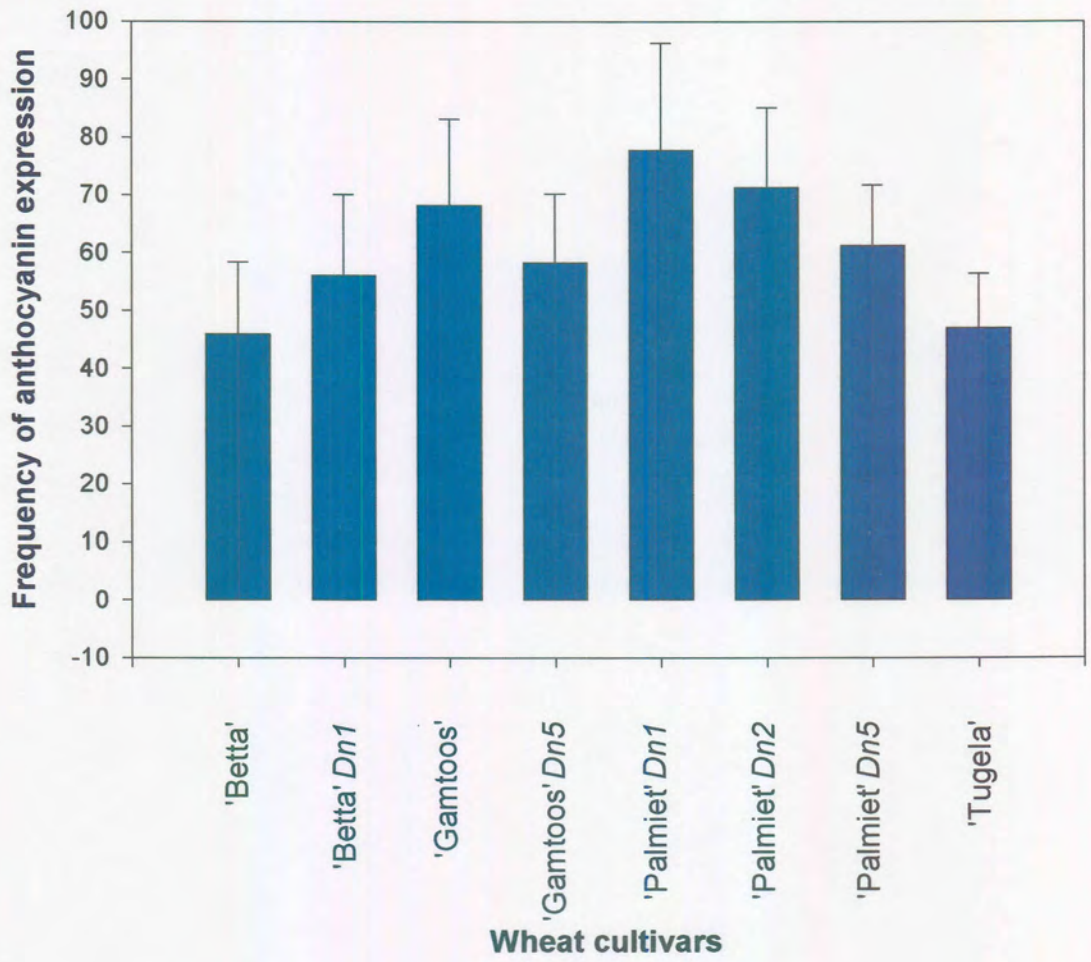


Figure 4.4.6 A comparison between the percentage of tissue bombarded with 1.5 – 3 µm gold particles exhibiting anthocyanin expression as expressed by different cultivars.

A comparison between the frequencies of anthocyanin expression between the cultivars bombarded with the smaller and larger gold particles revealed that most cultivars are more receptive towards the smaller particles. 'Betta' and 'Betta' *Dn1* are definitely more responsive towards the small gold particles. No significant differences were detected between the anthocyanin expression of the other cultivars.

Anthocyanin expression of the 'Gamtoos' and 'Palmiet' *Dn* cultivars were compared after bombardment with tungsten and gold particles. No significant differences in expression were observed between the 'Palmiet' *Dn* tissues after bombardment with the respective particles. 'Gamtoos', however, is much more responsive towards bombardment with gold than tungsten particles.

4.5 Plant regeneration

Bombarded material displaying anthocyanin expression of more than 150 foci per embryo/callus was subjected to various selection steps. The number of calli that were able to survive on the various selection media decreased dramatically from the initial amount of bombarded calli. These selection steps indicated that the 'Palmiet' *Dn1* and *Dn2* cultivars could withstand more selection pressure than the other cultivars, since 7.1% and 4.1% of the calli survived 6 weeks of selection, respectively. None of the material, however, was able to withstand the entire selection process. Thus, none of the calli could be regenerated into plantlets that could be transferred to soil.

Table 4.5.1 A comparison between the regeneration efficiency of bombarded material on selection medium.

Cultivar	Number of bombarded embryos/ calli	Ampicillin (50 mg/L) resistant calli after 3 weeks[a]	% Survival after 3 weeks	Ampicillin (75 mg/L) resistant calli after 6 weeks[a]	% Survival after 6 weeks
'Betta'	250	26	10.4	6	2.4
'Betta' *Dn1*	275	20	7.3	7	2.5
'Gamtoos'	161	17	10.6	3	1.9
'Gamtoos' *Dn5*	173	10	5.8	3	1.7
'Palmiet' *Dn1*	268	48	17.9	19	7.1
'Palmiet' *Dn2*	267	39	14.6	11	4.1
'Palmiet' *Dn5*	182	29	15.9	5	2.7
'Tugela'	288	31	10.8	8	2.8

[a] Values are presented as the mean of 10 replicas. SD was larger than 10% of the mean.

Chapter 5

Discussion

Wheat, as one of the most important cereal crops in the world, is increasingly becoming a target for genetic manipulation (Brettell and Murray, 1995). In order to regenerate transformed plants, a successful tissue culture system should be developed. Immature wheat embryos (Figure 4.1.1) were found to be very suitable material for plant regeneration (Vasil, 1990). The scutellum of the wheat embryo was also suitable for development of embryogenic callus tissue (Figure 4.1.2).

This study proved that a modified MS medium is sufficient for the development of callus tissue, as well as for plant regeneration from a wide range of cultivars (Figure 4.1.3). Although Jähne and his colleagues (1991) detected the development of soft callus tissue from anther cultures on the ML3 medium, this medium could not sustain the development of callus tissue or plantlets from the explant material used during this study (Figure 4.1.5). Therefore, the little development that was detected on this medium could be ascribed to the fact that specific tissue types require specific media for tissue culture establishment. Fennell *et al.* (1996), as well as Viertel *et al.* (1998), also found that different cultivars exhibited varying degrees of callus development and plant regeneration efficiencies on different initiation media. The regeneration frequencies obtained during this study (80.5 - 98.2%) are more consistent in comparison to frequencies obtained by, e.g., Ahloowalia (1982; 28 – 100%), Sears and Deckard (1982; 0 – 97%) and Fennell *et al* (1996; 0 – 67.7%).

Regular subculturing of the callus material on MS medium supplemented with 5 mg/L BAP resulted in the formation of more somatic embryos and improved germination thereof (Tiwari *et al.*, 1997). Plantlets were generated 10 weeks after callus initiation. It was, further, possible to transfer these plantlets to soil and regenerate plants from them (Figure 4.1.4).

The growth of the various hard-red spring and winter wheat cultivars indicated which cultivars are suitable for tissue culture initiation and regeneration (Table 4.1.1), and, therefore, subsequent transformation. 'Gamtoos' (spring wheat) and 'Tugela' (winter wheat) exhibited vigorous growth of the roots and shoots. 'Betta' *Dn2* (spring wheat), and 'Palmiet' *Lr29* and 'Tugela' (winter wheat) are also suitable for tissue culture establishment due to the development of viable roots and shoots. Thus, these hard-red South African cultivars are very suitable for tissue culture initiation.

Plasmid pHP 687 proved to be an efficient vector to use during particle bombardment optimisation. The plasmid acted as a non-destructive tool in the process of determining the frequency of anthocyanin expression in the bombarded tissue. Thus, the tissue could be submitted to the selection conditions directly after bombardment in order to regenerate transformed material. The double CaMV 35S promoters of both the C1 and R anthocyanin genes were efficient in expressing these genes. The ligation of pHP 687 with the ß-1,3-glucanase and chitinase genes, however, was unsuccessful. This could be due to unsuccessful dephosphorylation of the phosphorylated ends of plasmid pHP 687 acquired after plasmid restriction. Therefore, bombardment with the proposed vectors (Figure 3.3.1) could not be carried out. The analysis of tissue that was bombarded with the Glu B2 and Chi B3 plasmids is still in progress. Since these two genes were not bombarded together with anthocyanin, visual assessment of expression was not possible. The bombarded material was placed on selection media.

The particle bombardment system is a very simple transformation method since a basic protocol can be applied universally (Sanford, 1988). Optimisation of each individual system, however, is still necessary since the bombardment apparatus, type of material and micro-carriers used, vary. This is evident when various methods are compared (McCabe *et al.*, 1988; Morikawa *et al.*, 1989; Lonsdale *et al.*, 1990; Witrzens *et al.*, 1998).

The particle inflow gun system used was optimised for the use of small micro-carriers (1 µm to 3.0 µm) and wheat as the target material. Optimisation was based on the visual assessment of anthocyanin expression in the target material (Figures 4.3.1 and 4.3.2). It was found that the nearer the target material was placed to the micro-carrier assembly, the higher the anthocyanin expression frequency (Table 4.3.1). The short distance that the DNA-coated micro-carriers had to travel resulted in a higher penetration efficiency of the target cells. The aerodynamic drag on the particles was also decreased and the particles were distributed more evenly over the tissue (Klein *et al.*, 1988). The amount of tissue damage caused at a distance of 16 cm was less than the damage at 10 and 13 cm. This is in accordance with what would be expected, since the closer the material is placed to the DNA/micro-carrier assembly, the higher the velocity of the particles. This would result in more cells being injured. The average amount of foci exhibited by the material at all three distances did not differ significantly.

The frequency of transient expression, as well as the amount of foci on the bombarded material, varied considerable between the different helium pressures (Table 4.3.2). Pressures of 1 400 and 2 000 kPa resulted in the highest percentage of expression. Brown and his colleagues (1994) found that, as the helium pressure decreases, the frequency of expression decreases accordingly. During this study, however, it was found that an intermediate pressure, namely 1 400 kPa, resulted in the highest expression frequency, the highest amount of foci per embryo/callus and the least amount of tissue damage. As expected, the amount of tissue damage increased, with the pressure increase, since more cells were dislodged by the high force at which the particles hit the cells (Parveez *et al.*, 1997). These findings clearly indicate that the wheat material only responded favourable to a specific helium pressure.

The optimum age of target material was an important determining factor during bombardment optimisation (Russell *et al.*, 1992) (Table 4.3.3). It appeared that the youngest tissue (1 day to 1 week) was more viable than the older tissue since 78% of the younger tissue expressed anthocyanin. The older tissue was softer and more fragile than the younger tissue. Therefore, the micro-carriers could penetrate the older cells more easily, but in effect resulted in more tissue damage. Thus, the observation that tissue up to 3 weeks still displayed high amounts of foci may be due to the fact that these cells were able to recover more easily form the damage than the older cells. The advantage of bombarding young tissue is that it will regenerate more efficiently than older cells.

Hunold *et al.*, (1994) and Russell *et al.* (1992) found that the type of material used as target during bombardment, is an important determinant of transient expression. The three 'Palmiet' *Dn* cultivars are definitely the most susceptible to transfer of foreign DNA when considering the percentage material that expresses anthocyanin (Table 4.3.4). The 'Palmiet' and 'Palmiet' *Dn2* cultivars, however, exhibited a large amount of foci per embryo/callus. These cultivars are the most suitable for use during transformation studies.

Brettell and Murray (1995) suggested that the particles used during bombardment should be relatively small to ensure efficient penetration of the cell walls and membranes of the target cells. The results of figure 4.4.1 support this theory. The small gold and tungsten particles (1 µm in diameter) resulted in the highest percentage tissue to express anthocyanin for all the age groups tested. The tungsten particles, however, resulted in the most efficient penetration of target cells aged 1 day to 1 week. The large gold particles (1.5 – 3 µm) probably resulted in too much tissue damage and exhibited low expression frequencies. Brown and his colleagues (1994) reported that particles of 1.67 µm in diameter resulted in the highest transient expression frequency. Klein *et al.* (1988) demonstrated that 1.2 µm particles delivered the DNA efficiently to the target

cells. The conclusion can be drawn form figure 4.4.1 that 1 µm particles can also serve as efficient DNA carriers.

Bombardment with the small gold and tungsten particles further resulted in a high number of foci per embryo/callus (Figure 4.4.2) for tissue from 1 day – 1 week and 3 – 4 weeks, and 1 –3 weeks, respectively. Once again this can be attributed to efficient penetration of the target cells and little tissue damage caused by the small particles. Hunold and his co-workers (1994) demonstrated that a low amount of foci were found when target tissue was bombarded with 1.5 – 3 µm gold particles. Figure 4.4.2 supports these findings.

The large gold particles resulted in a high percentage of damage to the cell walls when they hit and penetrated the target cells. Bombardment of the older, softer tissue (2 – 4 weeks) with these particles, therefore, resulted in extensive tissue damage (Figure 4.4.3). The high penetration efficiency of the small gold particles caused little tissue damage to all age groups. Although the tungsten particles were small, the toxicity of these particles could account for the higher percentage of tissue damage. Parveez *et al.* (1997) also preferred to use 1 µm gold particles during bombardment since these particles are biologically inert and cause little tissue damage. Comparing the anthocyanin expression frequency between cultivars bombarded with the various particles revealed some genotype specificity regarding particle size and type, e.g. only gold particles are recommended for the bombardment of 'Gamtoos' and small gold particles for 'Betta'.

The regeneration of transformed material into mature plants plays and important role in genetic manipulation of plants. Barcelo and his colleagues (1998) stated that an efficient regeneration system should be strict by applying longer periods of selection and high concentrations of the selection agent. This argument was taken into consideration when selecting for transformed tissue during this study. The number of bombarded embryos/calli expressing

anthocyanin that were subjected to selection, exceeded 150 foci for all the cultivars tested (Table 4.5.1). The number of calli, however, surviving the strict selection conditions, declined drastically during the first three weeks. The amount of cells containing the anthocyanin vector were, therefore, not enough to allow growth on the ampicillin-containing media. The amount of tissue that was able to survive the first three weeks did not decrease so drastically during the next selection process. This could be an indication that the vector-containing cells were viable enough to withstand further selection pressure. Some of the cells that did not survive the last selection step could be regarded as escapes from the previous step.

When the material was placed back on regeneration media after the six-week selection period, however, none of the calli was able to regenerate into plantlets. The selection process was, therefore, too severe. Material that is placed on selection medium should contain a higher percentage cells that transiently express anthocyanin. This would enable the material to withstand the ampicillin-containing media. Further, the material that would be subjected to regeneration would consist of an agglomerate of cells containing the inserted DNA and this would facilitate the regeneration of the material greatly. Vasil *et al.* (1993) suggested that selection should be carried out on media supplemented with a gradually increasing concentration of the selective agent.

Although the percentage of bombarded calli regenerated after a 6-week selection period was low (1.7% to 7.1%), it is comparable to results that were obtained by other researchers. Results obtained during the transformation of soft white wheat cultivars exhibited regeneration efficiencies of 0.01 – 2.0% (Altpeter *et al.*, 1996), 0.75% (Chibbar *et al.*, 1998) and 0.6 – 1.2% (Hansen *et al.*, 1998). Chen and his colleagues (1998) obtained only 10 regenerated plantlets after selection from 155 bombarded Bobwhite embryos (i.e. a regeneration efficiency of 6.45%). This study was the first to be conducted on South African hard-red

cultivars and, when comparing my findings to that of other researchers, regeneration of transformed plants from these cultivars are within reach.

The embryo/calli that were bombarded with the Glu B2 and Chi B3 constructs, respectively, are currently placed on selection media (50 mg/L ampicillin). The embryo/calli cells are differentiating and it appears that the effect of the antibiotic on this tissue is less severe than on tissue bombarded with the pHP 687 construct. Thus, it would appear that the regeneration of this tissue would be successful.

By definition, transient expression in the bombarded material is not necessarily permanent, but it can however be regarded as a good indication of expected stable transformation of the material. The data obtained during this study indicated that particle bombardment could be used efficiently in order to obtain transient expression of marker genes in wheat material. The severe selection procedures applied, however, restricted the regeneration of the bombarded material into plants. A decrease in cell viability due to tissue damage after bombardment further played a role in the low regeneration efficiency. The regeneration of embryos, in stead of callus material, into plantlets would also proceed with more efficiency, since embryo differentiation and development proceed much faster. Low transformation efficiencies have been reported (Weeks *et al.*, 1993; Altpeter *et al.*, 1996; Takumi and Shimada, 1996; Hansen *et al.*, 1998) and is a common problem in the production of transgenic wheat plants. Thus, an optimum transformation system, as well as an efficient tissue culture system, are prerequisites in the process of obtaining transformed wheat plants. The South African wheat cultivars tested were shown to be suitable material to be used during tissue culture establishment and transformation procedures.

Chapter 6

Summary / Opsomming

The battle of wheat against pests and pathogens can be strengthened by genetically engineering the wheat plant for disease tolerance, e.g. by enhancing the expression of chitinase and β-1,3-glucanase genes. In order to obtain this long-term goal, an efficient tissue culture system, as well as an optimal transformation procedure, was produced.

Fourteen spring and winter hard-red South African wheat (*Triticum aestivum* L.) cultivars were compared for their regeneration and transient anthocyanin expression efficiencies. Embryonic and non-embryonic callus, as well as plantlets were obtained from all the cultivars using a modified MS basal medium supplied with 5 mg/L BAP. The modified ML3 medium could not sustain callus or plantlet development from any of the cultivars. The cultivars exhibiting the most vigorous growth were 'Betta' *Dn2* and 'Gamtoos' (spring wheat), and 'Palmiet' *Lr29* and 'Tugela' (winter wheat). These cultivars are, therefore, the most suitable for tissue culture establishment.

The particle bombardment system was efficiently optimised using the anthocyanin reporter gene cloned into the pHP 687 vector. It was found that the optimal distance between the micro-carrier assembly and target material should not exceed 13 cm. A helium pressure of 1 400 kPa produced the highest percentage of anthocyanin expression, the most foci per embryo/callus and the least amount of tissue damage. The age of the target material was found to be an important determining factor during bombardment and, thus, the age of target material should not exceed 3 weeks. The cultivars most suitable for transformation were 'Palmiet', 'Palmiet' *Dn1*, 'Palmiet' *Dn2* and 'Palmiet' *Dn5*. Bombardment with small particles, namely 1 µm tungsten and 1 µm gold, resulted in efficient penetration of the target cells and relatively little tissue damage. This, in turn, enabled the bombarded tissue to express a high percentage of anthocyanin. If was further found that the cultivar bombarded is receptive towards the particles used.

The material subjected to selection after bombardment should contain a large amount of cells transiently expressing anthocyanin. Plantlets could not be recovered from the material bombarded with anthocyanin since the applied selection procedure was too strict. Material bombarded with chitinase and β-1,3-glucanase are differentiating more efficiently and appears to survive the strict selection pressure.

OPSOMMING

Koringplante se oorlewingstryd teen peste en patogene kan grootliks verbeter word deur die plant geneties te manipuleer vir verhoogde siekteweerstand. Dit kan bewerkstellig word deur die verhoging van chitinase- en ß-1,3-glukanase-geenuitting. Hierdie langtermyn mikpunt kan slegs bereik word indien 'n effektiewe weefselkultuur- en geentransformeringsisteem daar gestel is.

Veertien lente en winter, harde-rooi Suid-Afrikaanse koringkultivars (*Triticum aestivum* L.) is vergelyk ten opsigte van hul regenerasievermoë en uitdrukking van antosianien-aktiwiteit. Embriogeniese en nie-embriogeniese kallus, asook plante, is verkry van al die kultivars deur van 'n gewysigde MS - basale medium, wat met 5 mg/L BAP verryk is, gebruik te maak. Die gewysigde ML3-medium kon nie die ontwikkeling van kallusweefsel of plante stimuleer nie. Die kultivars wat die mees weerbarstige groei getoon het, was 'Betta' *Dn2* en 'Gamtoos', as voorbeelde van lentekoring, en 'Palmiet' *Lr29* en 'Tugela', as voorbeelde van winterkoring. Hierdie kultivars is dus die geskikste vir die vestiging van weefselkulture.

Die bombarderingsisteem is suksesvol geoptimiseer deur van die antosianienmerkergeen gebruik te maak. Die geen is in die pHP 687-vektor gekloneer. Die optimale afstand tussen die mikro-draer-eenheid en die teikenweefsel behoort nie 13 cm te oorskry nie. 'n Heliumdruk van 1 400 kPa het die hoogste persentasie antosianien-uitting tot gevolg gehad, asook die meeste foki per embrio/kallus. Hierdie druk het ook die kleinste hoeveelheid weefselbeskadiging veroorsaak. Daar is gevind dat die ouderdom van die teikenmateriaal 'n bepalende faktor tydens bombardering is. Die teikenmateriaal se ouderdom behoort nie 3 weke te oorskry nie. Die beste kultivars om tydens transformasie te gebruik was 'Palmiet', 'Palmiet' *Dn1*, 'Palmiet' *Dn2* en 'Palmiet' *Dn5*. Bombardering met klein partikels, naamlik 1 µm tungsten en 1 µm goud, het tot effektiewe penetrasie van die teikenselle gelei. Min weefselbeskadiging is waargeneem. Hierdie faktore het weer daartoe bygedra dat die teikenweefsel 'n hoë antosianien-uitting getoon het. Daar is ook gevind dat die kultivar wat as teikenmateriaal dien, varieer met betrekking tot die ontvanklikheid vir die partikels wat gebruik word.

Materiaal wat na bombardering aan seleksie blootgestel word behoort 'n groot hoeveelheid selle wat antosianien-uitting toon, te bevat. Plante kon egter nie van die gebombardeerde weefsel verkry word nie, aangesien die seleksiedruk wat toegepas is, te streng was. Die materiaal wat egter met die chitinase- en glukanase-konstrukte gebombardeer is, differensieer meer effektief en dit blyk of hierdie materiaal die streng seleksietoestande sal oorleef.

Chapter 7

Literature Cited

Ahloowalia BS. 1992. Plant regeneration from callus culture in wheat. Crop Science 22: 405 - 410.

Altpeter F, Vasil V, Srivastava V, Stöger E and Vasil IK. 1996. Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Reports 16: 12 – 17.

Arlorio M, Ludwig A, Boller T and Bonfante P. 1992. Inhibition of fungal growth by plant chitinases and ß-1,3-glucanases. A morphological study. Protoplasma 171: 34 – 43.

Aung T and Kerber E. 1998. Incorporation of stem rust and leaf rust resistance from *Aegilops triuncialis* into common wheat. Proceedings of the 9th International Wheat Genetics Symposium 2: 7 - 9.

Badur R, Herbers K, Mönke G, Ludewig F and Sonnewald U. 1994. Induction of pathogenesis-related proteins in sugar accumulating tobacco leaves. Photosynthetica 30 (4): 575-582.

Balyan HS, Sharma PC, Ramesh B, Kumar A, Varshney RK Roy JK, Dhaliwal HS Singh H and Kupta PK. 1998. Towards development of molecular markers for tagging genes for qaulity traits in bread wheat. Proceedings of the 9th International Wheat Genetics Symposium 3: 84 - 88

Barcelo P, Rasco-Gaunt S, Sparks C, Cannell M, Salgueiro S, Rooke L, He GY, Lamacchia C, De la Viña G, Shewry PR and Lazzeri PA. 1998. Transformation of wheat: State of the technology and examples of application. Proceedings of the 9th International Wheat Genetics Symposium 1: 143 – 147.

Barro F, Rooke L, Békés F, Gras P, Tatham AS, Fido R, Lazzeri PA, Shewry PR and Barceló P. 1997. Transformation of wheat with high molecular weight sub-unit genes results in improved functional properties. Nature Biotechnology 15:1295 – 1299

Becker D, Brettschneider R and Lörz H. 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. The Plant Journal 5(2): 299 - 307.

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J and Staskawicz BJ. 1994. RPS2 of *Arabidopsis thaliana*: A leucine-rich repeat class of plant disease resistance genes. Science 265: 1856 – 1860.

Bhojwani SS and Razdan MK. 1983. *Plant tissue culture: theory and practice*, Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 1 – 112.

Birch RG and Franks T. 1991. Development and optimization of microprojectile systems for plant genetic transformation. Australian Journal of Plant Physiology 18: 453 – 469.

Blechl AE and Anderson OD. 1998. Engineering qualitative and quantitative changes in wheat high-molecular-weight glutenin sub-unit composition via genetic transformation. Proceedings of the 9th International Wheat Genetics Symposium 1: 163 – 164.

Blechl AE, Le HQ and Anderson OD. 1998. Engineering changes in wheat flour by genetic transformation. Journal of Plant Physiology 152: 703 – 707

Bol JF, Linthorst HJM and Cornelissen BJC. 1990. Plant pathogenesis-related proteins induced by virus infection. Annual Review of Phytopathology 28: 113-138.

Bommineni VR, Jauhar PP and Peterson TS. 1997. Transgenic durum wheat by microprojectile bombardment of isolated scutella. The Journal of Heredity 88(6): 475 - 481.

Botha A-M, Nagel MAC, Van der Westhuizen AJ and Botha FC. 1998. Chitinase isoenzymes in near-isogenic wheat lines challenged with Russian wheat aphid, exogenous ethylene, and mechanical wounding. Botanical Bulletin of Academia Sinica 39: 99 – 106.

Braun H-J, Payne TS, Morgounov AI, Van Ginkel M and Pajaram S. 1998. The challenge: one billion tons of wheat by 2020. Proceedings of the 9th International Wheat Genetics Symposium 1: 33 – 40.

Brettell RIS and Murray F. 1995. DNA transfer and gene expression in transgenic cereals. Biotechnology and Genetic Engineering Reviews 13: 315 – 334.

Broekaert WF, Van Parijs J, Allen AK and Peumans WJ. 1988. Comparison of some molecular, enzymatic and antifungal properties from chitinases from thornapple, tobacco and wheat. Physiological and Molecular Plant Pathology 33: 319 – 331.

Broekaert WF, Van Parijs J, Leyns F, Joos H and Peumans WJ. 1989. A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science 245: 1100 – 1102.

Broglie R and Broglie K. 1993. Chitinases and plant protection. In: *Mechanisms of plant defense responses*, Fritig B and Legrand M (eds.), Kluwer Academic Publishers, Dordrecht, pp. 411 – 421.

Brown DCW, Tian L, Buckley DJ, Lefebvre M, McGrath A and Webb J. 1994. Development of a simple particle bombardment device for gene transfer into plant cells. Plant Cell, Organ and Tissue Culture 37: 47 – 53.

Bushnell WR and Rowell JB. 1981. Suppressers of defense reactions: a model for roles in specificity. Phytopathology 71: 1012-1014.

Callis J, Fromm ME and Walbot V. 1987. Introns increase gene expression in culture maize cells. Genes and Development. 1: 1183 - 1200.

Chan M-T, Lee T-M and Chang H-H. 1992. Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. Plant Cell Physiology 33: 577 – 583.

Chen WP, Chen PD, Liu DJ, Muthukrishnan S and Gill BS. 1998. Constitutive expression of rice thaumatin-like protein gene in T0, T1 and T2 transgenic wheat plants. Proceedings of the 9th International Wheat Genetics Symposium 3: 169 – 171.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW and Wan Y. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens.* Plant Physiology 115: 971 – 980

Chibbar RN, Kartha KK, Leung N, Qureshi J and Caswell K. 1991. Transient expression of marker genes in immature zygotic embryos of spring wheat (*Triticum aestivum* L.) through microprojectile bombardment. Genome 34: 453 – 460.

Chibbar RN, Båga M, Caswell K, Repellin A, Leung N, Abdel AAL and Hucl P. 1998. Genetic transformation strategies to alter starch structure in wheat. Proceedings of the 9th International Wheat Genetics Symposium 1: 167 – 170.

Christou P, Ford TL and Kofron M. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electrical discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/technology 9: 957 – 962.

Clarke BC, Taylor W, Morell M, Li Z, Rahman S, Ali S and Appels R. 1998. Gene expression in wheat: the quality genes in grain endosperm. Proceedings of the 9th International Wheat Genetics Symposium 1: 65 - 80.

Danhash N, Wagemakers CAM, Van Kan JAL and De Wit PJGM. 1993. Molecular characterization of four chitinase cDNAs obtained from *Cladosporium fulvum*-infected tomato. Plant Molecular Biology 22: 1017-1029.

Daniel D. 1994. *Aspects of durable resistance in wheat to yellow rust.* CIP-DATA Koninklijke Bibliothheek, Den Haag, pp. 1 – 3.

Delaney TP. 1997. Genetic dissection of acquired resistance to disease. Plant Physiology 113: 5 – 12.

De la Viñã G, Smart L, Pickett J, Lazzeri PA and Barceló P. 1998. Production of transgenic wheat containing the snow drop lectin (GNA) gene to engineer resistance to aphids. Proceedings of the 9th International Wheat Genetics Symposium 3: 172 – 173.

Demeke T, Hucl P, Båga M, Caswell K, Leung N and Chibbar RN. 1998. Transgene stability and inheritance in spring wheat. Proceedings of the 9th International Wheat Genetics Symposium 1: 103 – 106.

De Wit PJGM. 1995. Fungal avirulence genes and plant resistance genes: Unraveling the molecular basis of gene-for-gene interactions. Advances in Botanical Research 21: 147-185

Dixon RA and Lamb CJ 1990 Molecular communication in interactions between plants and microbial pathogens. Annual Review of Plant Physiology and Plant Molecular Biology 41: 339 – 367.

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K and Jones JDG. 1996. The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84: 451 – 459.

Dodds JH And Roberts LW. 1985. *Experiments in plant tissue culture*, 2nd edition, Cambridge University Press, USA, pp. 1 – 15.

Eapen S and Rao PS. 1986. Spontaneous and induced variation in tissue cultures and regenerated plants of breadwheat. In: *Plant Tissue Culture and its Agricultural Applications.* Withers LA and Alderson PG (eds.), Butterworths, London, pp. 461 - 467.

Ellis DD, McCabe DE, McInnis S, Ramachandran R, Russell DR, Wallace KM, Martinell BJ, Roberts DR, Raffa KF and McCown BH. 1993. Stable transformation of *Picea glauca* by particle acceleration. Bio/technology 11: 84 – 89.

Fedak G. 1998. Procedures for transferring agronomic traits from alien species to crop plants. Proceedings of the 9th International Wheat Genetics Symposium 1: 1 - 7.

Fennell S, Bohorova N, Van Ginkel M, Crossa J and Hoisington D. 1996. Plant regeneration from immature embryos of 48 elite CIMMYT bread wheats. Theoretical and Applied Genetics 92: 163 - 169.

Fennel S, Bohorova N, McLean S, Olivares-Villegas JJ, Hernandez-Reyes R, Sakgado-Siclan MM, Pacheco M, Diaz L and Hoisington D. 1998. Insertion of chitinase, glucanase and RIP transgenes in CIMMYT bread wheat varieties. Proceedings of the 9th International Wheat Genetics Symposium 3: 178 – 180.

Finer JJ, Vain P, Jones MW and McMullen MD. 1992. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports 11: 323 – 328.

Flor HH. 1956. The complementary gene systems in flax and flax rust. Advances in Genetics 8: 29 – 54.

Frick MM, Hucl R, Nykiforuk CL, Conner RL, Kuzyk A and Laroche A. 1998. Molecular characterization of a wheat stripe rust resistance gene in Moro wheat. Proceedings of the 9th International Wheat Genetics Symposium 2: 181 –182.

Fry JE, Cheng M, Hu T, Layton J, Wan Y, Zhou H, Duncan DR, Hironaka L, Pang S, Lianag J and Conner T. 1998. Advances in genetic engineering in wheat. Proceedings of the 9th International Wheat Genetics Symposium 1: 156 - 158.

Gambley RL, Ford R and Smith GR. 1993. Microprojectile transformation of sugarcane meristems and regeneration of shoots expressing ß-glucuronidase. Plant Cell Reports 12: 343 – 346.

Gama MICS, Leite RP, Cordeiro AR and Cantliffe DJ. 1996. Transgenic sweet potato plants obtained by *Agrobacterium tumefaciens* – mediated transformation. Plant Cell, Organ and Tissue Culture 46: 237 – 244.

Gasser CS and Fraley RT. 1989. Genetically engineering plants for crop improvement. Science 244: 1293 – 1299.

Greenberg TJ, Guo A, Klessig DF and Ausubel FM. 1994. Programmed cell death in plants: A pathogen triggered response coordinately with multiple defense functions. Cell 77: 551 – 563.

Grumet R and Lanina-Zlatkina T. 1996. Molecular approaches to biological control of virus diseases in plants. In: *Molecular Biology of the Biological control of Pests and Diseases of Plants* (Gunasekaran M, Weber DJ, eds.) CRC Press, Boca Raton Fla., pp. 15-37.

Hain R, Rei H-J, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stöcker RH and Stenzel K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361: 153 - 156.

Hamilton DA, Roy M, Rueda J, Sindhu RK, Sanford J and Mascarenhas JP. 1992. Dissection of a pollen-specific promoter form maize by transient transformation assays. Plant Molecular Biology 18: 211 – 218.

Hansen J, Shiel PJ, McCarthy P, Bergar PH and Zemetra RS. 1998. Transformation of soft white winter wheat (*Triticum aestivum*) for virus resistance. Proceeding of the 9th International Wheat Genetics Symposium 3: 186 - 188.

He DG, Yang YM and Scott KJ. 1988. A comparison of scutellum callus and epiblast callus induction in wheat: the effect of genotype, embryo and medium. Plant Science 57: 225 - 233.

Heath MC. 1981. A generalized concept of host-parasite specificity. Phytopathology 71: 1121-1123.

Hiei Y, Ohta S, Komari T and Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant Journal 6: 271 – 282.

Hooykaas PJJ. 1989. Transformation of plant cells via *Agrobacterium*. Plant Molecular Biology 13: 327 – 336.

Hu T and Kasha KJ. 1997. Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.) through ovary culture. Plant Cell Reports 16: 520 - 525.

Hunold R, Bronner R and Hahne G. 1994. Early events in microprojectile bombardment: cell viability and particle location. The Plant Journal 5(4): 593 – 604.

Ignatius SMJ, Chopra RK and Muthukrishnan S. 1994. Effects of fungal infection and wounding on the expression of chitinases and ß-1,3-glucanases in near-isogenic lines of barley. Physiologia Plantarum 90: 584-592.

Iida A, Seki M, Kamada M, Yamada and Morikawa H. 1990. Gene delivery into cultured plant cells by DNA-coated gold particles accelerated by a pneumatic particle gun. Theoretical and Applied Genetics 80: 813 - 816.

Inoue H, Nojima H and Okayama H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene: 96: 23 – 28.

Ishida Y, Saito, H, Ohta S, Hiei Y, Komari T and Kumashiro T. 1996. High efficiency transformation of maize (*Zea maize* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology 14: 745 – 750.

Jähne A, Lazzeri PA, Jäger-Gussen M and Lörz H. 1991. Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics 82: 74 – 80.

Jähne A, Becker D and Lörz H. 1995. Genetic engineering of cereal crop plants: a review. Euphytica 85: 35 - 44.

Johnson R. 1992. Past, present and future opportunities in breeding for resistance, with examples form wheat. Euphytica 63: 3 - 22.

Johnston SA, Riedy M, DeVit MJ, Sanford JC, McElligott S and Williams RS. 1991. Biolistic transformation of animal tissue. In Vitro Cellular and Developmental Biology 27: 11 – 14.

Jones DA and Jones JDG. 1997. The role of leucine-rich repeat proteins in plant defense. Advances in Botanical Research 24:89 – 167.

Kajita S, Osakabe K, Katayama Y, Kawai S, Matsumoto Y, Hata K and Morohoshi N. 1994. *Agrobacterium* – mediated transformation of poplar using a disarmed binary vector and the over-expression of a specific member of a family of poplar peroxidase genes in transgenic poplar cells. Plant Science 103: 231 – 239.

Kauffmann S, Legrand M and Fritig B. 1990. Isolation and characterization of six pathogenesis-related (PR) proteins of Samsun NN tobacco. Plant Molecular Biology 14: 381-390.

Keen NT. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annual Review of Genetics 24: 447 – 463.

Kemp G. 1996. Effect of *Puccinia recondita* f. sp. *tritici* on pathogenesis-related gene expression in wheat. M.Sc thesis, University of the Orange Free State, Bloemfontein, pp. 1 - 94.

Kemp G, Botha A-M, Kloppers FJ and Pretorius ZA. 1998. Disease development and ß-1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings. Physiological and Molecular Plant Pathology. (In press).

Klein TM, Wolf ED and Sanford JC. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70 – 73.

Klein TM, Gradziel T, Fromm ME and Sanford JC. 1988. Factors influencing gene delivery into *Zea maize* cells by high-velocity microprojectiles. Bio/technology 6: 559 – 564

Klein TM, Kornstein L, Sanford JC and Fromm ME. 1989. Genetic transformation of maize cells by particle bombardment. Plant Physiology 91: 440 – 444.

Kombrink E and Somssich IE. 1995. Defense responses of plants to pathogens. Advances in Botanical Research 21: 1-34

Kragh KM, Jacobsen S, Mikkelsen JD and Nielsen KA. 1993. Tissue specificity and induction of class I, II and III chitinases in barley (*Hordeum vulgare*). Physiologia Plantarum 89: 490-498.

Krebs SL and Grumet R. 1993. Affinity purification and characterization of a ß-1,3-glucanase from celery. Plant Science 93: 31-39.

Lagudah ES, Moullet O and Appels R. 1997. Map-based cloning of a gene sequence encoding a nucleotide-binding domain and a leucine-rich region at the *Cre3* nematode resistance locus of wheat. Genome 40: 659 – 665.

Lamb CJ, Ryals JA, Ward ER and Dixon RA. 1992. Emerging strategies for enhancing crop resistance to microbial pathogens. Bio/technology 10: 1436-1445.

Langridge P and Chalmers K. 1998. Techniques for marker development. Proceedings of the 9th International Wheat Genetics Symposium 1: 107 - 117.

Leckband G and Lörz H. 1998. Transformation and expression of a stilbene synthase gene on *Vitis vinifera* L. in barley and wheat for increased fungal resistance. Theoretical and Applied Genetics 96: 1004 – 1012.

Linthorst HJM. 1991. Pathogenesis-related proteins of plants. Critical Review of Plant Science 10: 123 – 150.

Lonsdale D, Önde S and Cuming A. 1990. Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. Journal of Experimental Botany 41: 1161 - 1165.

Lörz H, Becker D and Lütticke S. 1998. Molecular wheat breeding by direct gene transfer. Euphytica 100: 291 - 223.

Lu W, Cheng S, Shen X, Zhou M, Wang Y and Yao G. 1998. Utilization of biotechnology of wheat for scab resistance. Proceedings of the 9th International Wheat Genetics Symposium 3: 135 - 137.

Ludwig SR, Bowen B, Beach L and Wessler SR. 1990. A regulatory gene as a novel visible marker for maize transformation. Science 247: 449 – 450.

Maher EA, Lamb CJ and Dixon RA. 1993. Stress response in alfalfa (*Medicago sativa* L) XVII. Identification of multiple hydrolases and molecular characterization of an acidic glucanase. Physiological and Molecular Plant Pathology 43: 329-342.

Malany J and Klessig DF. 1992. Salicylic acid and plant disease resistance. The Plant Journal 2: 643 – 654.

Mauch F, Mauch-Mani B and Boller T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and ß-1,3-glucanase. Plant Physiology 88: 936-942.

McCabe DE, Swain WF, Martinell BJ and Christou P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/technology 6: 923 - 926.

Melchinger AE. 1990. Use of molecular markers in breeding for oligogenic resistance. Plant Breeding 104: 1 - 19.

Memelink J, Linthorst HJM, Schilperoort RA and Hoge JHC. 1990. Tobacco proteins encoding acidic and basic pathogenesis-related proteins display different expression patterns. Plant Molecular Biology 14: 119-126.

Mohmand AS and Nabors MW. 1991. Comparison of two methods for callus culture and plant regeneration in wheat (*triticum aestivum*). Plant Cell, Organ and Tissue Culture 26: 185 - 187.

Morikawa H, Iida A and Yamada Y. 1989. Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle gun). Applied and Microbial Biotechnology 31: 320 - 322.

Murashige T and Skoog F 1962 A revised medium for rapid growth and bioassays with tobacco cultures. Physiologia Plantarum 15: 473 – 479.

Murray FR, Hill A, Appels R and Brettell RIS. 1998. Transformation of Australian wheat cultivars. Proceedings of the 9th International Wheat Genetics Symposium 1: 160

Nabulsi SM, Page NW, Duval AL, Seabrook YA and Scott KJ. 1994. A gas-driven gene gun for microprojectile methods of genetic engineering. Measurement Science and Technology 5: 267 – 274.

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M and Kartha KK. 1994. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. The Plant Journal 5(2): 285 – 297.

Nielsen KK, Bojsen K, Collinge DB and Mikkelsen JD. 1994. Induced resistance in sugar-beet against *Cercospora beticola*: induction by dichloro-isonicotinic acid is independent of chitinase and ß-1,3-glucanase transcript accumulation. Physiological and Molecular Plant Pathology 45: 89-99.

Oard JH, Paige DF, Simmonds JA and Gradziel TM. 1990. Transient gene expression in maize, rice and wheat cells using an airgun apparatus. Plant Physiology 92: 334 – 339.

Özgen M, Türet M, Özcan S and Sancak C. 1996. Callus induction and plant regeneration from immature and mature wheat embryos of winter durum wheat genotypes. Plant Breeding 115: 355 - 458.

Özgen M, Türet M, Altinok S and Sancak C. 1998. Efficient callus induction and plant regeneration from immature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. Plant Cell Reports 18: 331 - 335.

Park SH, Pinson SRM and Smith RH. 1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Molecular Biology 32:1135 – 1148.

Parveez GKA, Chowdhury MKU and Saleh NM. 1997. Physical parameters affecting transient GUS gene expression in oil palm (*Elaeis guineensis* Jacq.) using the biolistic device. Industrial Crops and Products 6: 41 – 50.

Peña L, Cervera M, Juúrez J, Ortega C, Piña JA, Durán-Vila N and Navarro L. 1995. High efficiency *Agrobacterium* – mediated transformation and regeneration of citrus. Plant Science 104: 183 – 191.

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

Punja ZK and Zhang YY. 1993. Plant chitinases and their role in resistance to fungal diseases. Journal of Nematology 25 (4): 526-540.

Rainieri DM, Bottino P, Gordon MP and Nester EW. 1990. *Agrobacterium*-mediated transformation of rice *Oryza sativa* L. Bio/technology 8: 33 – 38.

Rashid H, Yokoi S, Toriyama K and Hinata K. 1996. Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. Plant Cell reports 15: 727 – 730.

Razdan MK. 1993. *An introduction to plant tissue culture*, Intercept, Andover, Hampshire, U.K., pp. 1- 100.

Redway FA, Vasil V, Lu D and Vasil IK. 1990. Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics 79: 609 - 617.

Reggiardo MI, Arana JL, Orsaria LM, Permingeat HR, Spittele MA and Vallejos RH. 1991. Transient transformation of maize by microprojectile bombardment. Plant Science 75: 237 – 243.

Riley R, Chapman V and Johnson R. 1968. Introduction of yellow rust resistance of *Aegilops comosa* into wheat by genetically induced homoeologous recombination. Nature 217: 383 - 384.

Ritala A, Aspegren K, Kurtén U, Salmenkallio-Marttila M, Mannonen L, Hannus R, Kauooinen V, Teeri TH and Enari TM. 1994. Fertile transgenic barley by particle bombardment of immature embryos. Plant Molecular Biology 24: 317 – 325.

Roberts WK and Selitrennikoff CP. 1990. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. Journal of General Microbiology 136: 1771-1778.

Ross AF. 1961. Systemic acquired resistance induced by localized virus infections in plants. Virology 14: 340 – 358.

Russell JA, Roy MK and Sanford JC. 1992. Major improvements in biolistic transformation of suspension-cultures tobacco cells. In Vitro Cellular and Developmental Biology 28: 97 – 105.

Russell DR, Wallace KM, Bathe JH, Martinell BJ and McCabe DE. 1993. Stable transformation of *Vaseolus vulgaris* via electric-discharge mediated particle acceleration. Plant Cell Reports 12: 165 – 169.

Russell-Kikkert J. 1993. The Biolistics PDS-1000/He Device. Plant Cell, Tissue and Organ Culture 33: 221 – 226.

Ryals J, Ukness S and Ward E. 1994. Systemic acquired resistance. Plant Physiology 104: 1109 – 1112.

Salzer T, Hübner B, Sirrenberg A and Hager A. 1997. Differential effect of spruce chitinases on the activity of elicitors from Ectomycorrhizal fungi. Plant Physiology 114: 957 – 968.

Sanford JC. 1988. The biolistic process. Techniques in Biotechnology 6: 299 – 302.

Sanford JC, Klein TM, Wolf ED and Allen N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. Particulate Science and Technology 5: 27 – 37.

Sanford JC, DeVit MJ, Russell JA, Smith FD, Harpending PR, Roy MK and Johnston SA. 1991. An improved helium-driver biolistic device. Technique – A Journal of Methods in Cell and Molecular Biology 3: 3 – 16.

Sanford JC, DeVit MJ, Russell JA, Smith FD, Harpending PR, Roy MK and Johnston SA. 1993. Optimizing the biolistic process for different biological applications. Methods in Enzymology 217: 483 – 509.

Sautter C, Waldner H, Neuhaus-Url G, Galli A, Neuhaus G and Potrykus I. 1991. Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. Bio/technology 9: 1080 – 1085.

Schell JS. 1987. Transgenic plants as tools to study the molecular organization of plant genes. Science 237: 1176 – 1182.

Schlumbaum A, Mauch F, Vögeli U and Boller T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367.

Sears RG and Deckard EL. 1982. Tissue culture variability in wheat: callus induction and plant regeneration. Crop Science 22: 546 - 550.

Seki M, Komeda Y, Iida A, Yamada Y and Morikawa H. 1991. Transient expression of ß-glucuronidase in *Arabidopsis thaliana* leaves and roots and *Brassica napus* stems using a pneumatic particle gun. Plant Molecular Biology 17: 259 – 263.

Shillito RD, Saul MW, Paszkowski J, Muller M and Potrykus I. 1985. High efficiency direct gene transfer to plants. Bio/technology 3: 1099 – 1103.

Snape JW. 1998. Golden calves or white elephants? Biotechnologies for wheat improvement. Euphytica 100: 207 – 217.

Snyman SJ, Meyer GM, Carson DL and Botha FC. 1996. Establishment embryonic callus and transient gene expression in selected sugarcane varieties. South African Journal of Botany 62(3): 151 – 154.

Songstad DD, Somers DA and Griesbach RJ. 1995. Advances in alternative DNA delivery techniques. Plant Cell, Organ and Tissue Culture 40: 1 – 15.

Stiff CM, Kilian A, Huaping Z, Kudrna DA and Kleinhofs A. 1995. Stable transformation of barley using biolistic particle bombardment and the phosphinotricin acetyltransferase (*bar*) gene. Plant Cell, Tissue and Organ Culture 40: 243 – 248.

Stinzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M and Fritig B. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie 75: 687-706.

Strauss SH, Knowe SA and Jenkins J. 1997. Benefits and risks of transgenic plants. Journal of Forestry (May 1997): 12 – 19.

Susi A, Mikkelsen JD, Von Weissenberg K and Nielsen KK. 1995. Sugar-beet chitinase inhibits the growth of a spruce pathogen. European Journal of Forest Pathology 25: 61-64.

Takumi S and Shimada T. 1996. Production of transgenic wheat through particle bombardment of scutellar tissue: Frequency is influenced by culture duration. Journal of Plant Physiology 149: 418 - 423.

Tanksley Sd, Young ND, Paterson AH and Boniebale MW. 1989. RFPL mapping in plant breeding: new tool for an old science. Bio/technology 7: 257 - 264.

Taylor MG and Vasil IK. 1991. Histology of, and physical factors affecting, transient GUS expression in pearl millet (*Pennisetum glaucum* (L.) P.Br) embryos following microprojectile bombardment. Plant Cell Reports 10: 120 – 125.

Tepper CS and Anderson AJ. 1984. The genetic basis of plant-pathogen interaction. Phytopathology 74 (10): 1143-1144

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettell R. 1997. *Agrobacterium tumefaciens* - mediated transformation. The Plant journal 11: 101 – 108.

Tiwari VK, Botha A-M and Van der Westhuizen AJ. 1997. A new technique for the propagation of somatic wheat embryos. Biotechnology Techniques 11(9): 633 – 636.

Tweddell RJ, Jabaji-Hare SH and Charest PM. 1994. Production of chitinases and 1,3-ß-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. Applied and Environmental Microbiology 60 (2): 489-495.

Vain P, McMullen MD and Finer JJ. 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Reports 12: 84 – 88.

Van Loon LC and Antoniw JF. 1982. Comparison of the effect of salicylic acid and epethon with virus-induced hypersensitivity and acquired resistance in tobacco. Netherlands Journal of Plant Pathology 88: 237 – 256.

Vasil IK. 1990. Transgenic cereals becoming a reality. Bio/technology 8: 797.

Vasil V, Clancy M, Ferl RJ, Vasil IK and Hannah LC. 1989. Increased gene expression by the first intron of maize *Shrunken*-1 locus in grass species. Plant Physiology 91: 1575 – 1579.

Vasil V, Brown SM, Re D, Fromm ME and Vasil IK. 1991. Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. Bio/technology 9: 743 – 747.

Vasil V, Castillo AM, Fromm ME and Vasil IK. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/technology 10: 668 – 675.

Vasil V, Srivastava V, Castillo AM, Fromm EM and Vasil IK. 1993. Rapid production of transgenic wheat plants by direct particle bombardment of cultured immature embryos. Bio/technology 11: 1553 – 1559.

Vierheilig H, Alt M, Neuhaus JM, Boller T and Wiemken A. 1993. Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae.* Molecular Plant-Microbe Interactions 6 (2): 261-264.

Viertel K and Hess D. 1996. Shoot tips of wheat as an alternative for regenerable embryogenic callus cultures. Plant Cell, Organ and Tissue Culture 44:183 - 188.

Viertel K, Schmid A, Iser M and Hess D. 1997. Regeneration of German spring wheat varieties from embryogenic scutellar callus. Journal of Plant Phisiology 152: 167 – 172.

Vigers AJ, Roberts WK and Selitrennikoff CP. 1991. A new family of antifungal proteins. Molecular Plant Microbe Interactions 4: 315 – 323.

Weeks JT, Anderson OD and Blechl AE. 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L.). Plant Physiology 102: 1077 – 1084.

Winter P and Kahl G. 1995. Molecular marker technology for plant iimprovement. World Journal of Microbiology and Biotechnology 11: 438 - 448.

Witrzens B, Brettell RIS, Murray FR, McElroy D, Li Z and Dennis ES. 1998. Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. Australian Journal of Plant Physiology 25: 39 - 44.

Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, Van den Elzen PJM and Cornelissen BJC. 1991. Pathogen-induced proteins with inhibitory activity towards *Phytophthora infestans*. Plant Cell 3: 619 – 628.

Wyatt SE, Pan SQ and Kuc J. 1991. ß-1,3-glucanase, chitinase and peroxidase activities in tobacco tissue resistant and susceptible to blue mold as related to flowering, age and sucker development. Physiological and Molecular Plant Pathology 39: 433-440.

Yamashita T, Iida A and Morikawa H. 1991. Evidence that more than 90% of ß-glucuronidase expressing cells after particle bombardment directly receive the foreign gene in their nucleus. Plant Physiology 97: 829 – 831.

Zhang YY and Punja ZK. 1994. Induction and characterization of isoforms in cucumber (*Cucumis sativus* L.): effect of elicitors, wounding and pathogen inoculation. Plant Science 99: 141-150.