GENETIC TRANSFORMATION OF WHEAT

(*Triticum aestivum* L.)

ZAINUDDIN

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TABLE OF CONTENTS

Abstract

Wheat (*Triticum aestivum* L.) was one of the earliest crops to be domesticated and is now becoming the world's most important food crop. The demand for this commodity has increased in parallel with the growth of world population. Thus, it is becoming increasingly important to secure the supply of wheat, and it is therefore crucial to continuously enhance worldwide wheat production. So far, the main strategy for improving wheat production has been through conventional breeding methods. However, it is becoming apparent that to maintain production targets it will be necessary to complement conventional breeding methods with genetic engineering technology.

The successful application of genetic engineering in wheat is dependent on the availability of suitable tissue culture and transformation methods, and the development of these technologies using elite Australian wheat varieties was the primary objective of experiments described here. The specific goals of this project were:

- to screen Australian wheat genotypes for *in vitro* culture responsiveness
- to transform the responsive wheat genotypes with selectable marker and reporter genes, and
- to study the inheritance and expression of transgenes in successive wheat generations.

In testing the responsiveness of wheat genotypes in culture, four Australian wheat genotypes (*cvs.* Hartog, Frame, Krichauff and Janz) were used. Immature scutella were cultured onto Murashige and Skoog (MS) basal medium containing different combinations and concentrations of 2,4-dichlorophenoxyacetic acid and

benzylaminopurine. Three genotypes (*cvs.* Hartog, Frame and Krichauff) grew well in culture and one genotype (*cv.* Janz) did not. Two genotypes, namely *cvs.* Hartog and Krichauff, responded well in a medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid, while another genotype (*cv.* Frame) needed the same concentration of 2,4-dichlorophenoxyacetic acid with the addition of 0.1 mg/l benzylaminopurine. With these media, almost all immature scutella produced embryogenic callus, which was subsequently regenerated into mature, fertile plants.

Regeneration systems developed for the three responsive genotypes were coupled with a microprojectile bombardment-mediated transformation method. By bombarding immature scutella of *cvs.* Hartog, Frame and Krichauff, or freshly isolated immature embryos of *cv.* Frame with a construct carrying the *bar* gene, with or without another construct carrying the *GUS* gene, four transgenic plants (*cv.* Frame) were produced. Three of the four transgenic plants were shown to carry two or more copies of the *bar* gene, and another plant carried one copy of the *bar* gene and three or four copies of the *GUS* gene. The introduced transgenes were expressed in the transgenic plants; *bar* gene expression was indicated by the presence of PAT activity and herbicide tolerance, whilst the expression of the *GUS* gene was followed by the presence of GUS activity in histochemical assays that led to blue staining in both vegetative and reproductive organs.

The *bar* and *GUS* transgenes integrated into the genome of transgenic wheat were transmitted to successive generations. The transmission of the transgenes showed a Mendelian pattern of inheritance, and a homozygous genotype was achieved at T2 progeny. This suggests that the transgenic wheat

analysed here had a heterozygous genotype of integrated *bar* and *GUS* transgenes. It was observed that the expression of the *GUS* gene was stable over several generations, but that the expression of the *bar* gene was inactivated in some progeny, as indicated by the loss of PAT activity and herbicide tolerance.

The phenotypic characteristics of the primary transformants were, in most cases, slightly inferior to non-transformed, control plants, but showed some improvement in subsequent generations.

As a result of the work, transformation of elite Australian wheat varieties should be achievable on a routine basis, albeit at relatively low transformation frequency. This opens the way for the insertion of potentially useful genes into wheat, with the longer term aim of enhancing productivity and/or quality characteristics.

Statement of Authorship

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Date: 08/12/2000

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Abbreviations

ABA	Abscisic acid
BA	Benzylaminopurine
GUS	β-glucuronidase
ha	hectare
Kb	kilobase
mA	milliampere
PAT	phosphinothricin acetyl transferase
PDS	particle delivery system
PIG	particle inflow gun
psi	pounds per square inch
rpm	revolutions per minute
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
2,4-D	2,4-dichlorophenoxyacetic acid

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Wheat is one of the most important cereal crops and was amongst the first plants to be domesticated. It belongs to the genus *Triticum* of the family Poaceae (Cornell and Hoveling, 1998). Wheat is widely grown in subtropical countries and is the staple food of nearly 35% of the world population (Braun *et al.*, 1998).

The current production of wheat in the world stands at 560 million tons per annum and demand for wheat has increased simultaneously with the growth of world population. It is estimated that the demand of wheat in the world will be around 840 (Rosegrant *et al.*, 1995) to 1050 million tons (Kronstad, 1998) for the second decade of the next century. To reach this demand, current global wheat production will need to increase at least 1.6% annually (Braun *et al.*, 1998).

So far, the main strategies for improving wheat production have been by increasing yield through conventional breeding methods and through the expansion of cultivated area. However, the implementation of these strategies faces a number of limitations that are imposed by biological, environmental and practical problems (Fry *et al.*, 1998).

The recent innovation in agricultural biotechnology through genetic engineering has provided a new tool for making more rapid improvements in wheat productivity and security of supply. The successful application of genetic engineering in wheat is largely dependent on the availability of suitable tissue culture techniques and transformation methods, and the development of these technologies using elite Australian wheat varieties was the primary objectives of experiments described in this thesis.

The literature review that follows lists the major steps in genetic engineering and highlights prior work relating to tissue culture and transformation of cereals,

predominantly focused on wheat. Further sections review the behaviour of inherited transgenes and somaclonal variation in wheat. Finally, the detailed aims of the project conducted in this study are presented.

1.2 Steps in genetic engineering

Genetic engineering has been developed to enhance the phenotypic performance and agricultural value of plants, and to facilitate the investigation of basic biological processes in genetically modified plants, such as gene expression. Engineering plants for desirable characters involves several distinct steps. Wilke-Douglas *et al.* (1986) have presented seven important steps in plant genetic engineering, as follows.

1) Identification of a gene of interest. Genes that are attracting special attention for use in plant transformation include genes that are involved in resistance to herbicides, antibiotics, diseases and viruses, in stress tolerance and in quality improvement. These genes can originate from bacteria, viruses, fungi, plants or animals.

2) Isolation and characterization of the gene. The isolation of a gene of interest begins with the construction of a gene bank or library. From the library, a gene (or genes) of interest is isolated and identified by nucleotide sequencing. The gene may be further characterized to provide basic information on the products, which are usually proteins, specified by the gene.

3) Cloning a functional gene. The isolated gene of interest is subcloned into appropriate vectors. Once the gene has been isolated and cloned, it can be modified to enhance or alter its function. For example, its promoter may be

changed so that the gene can be expressed in a particular host plant, or its sequences are modified to alter the biological function of the gene product.

4) Construction of a vector for gene transfer. The ability to join pieces of DNA together *in vitro* before insertion into an organism enables the construction of a vector for gene transfer. This process includes the assembly of a regulatory region (promoter), transcriptional and translational start signals, the coding region and a stop signal. The promoter may either be native or foreign to the gene. Such hybrid genes can specify the production of new proteins or proteins that are expressed at different times and levels or in distinct tissues.

5) Introduction of the gene into plant cells. This is known as transformation. After the identification, isolation and manipulation of the gene of interest, the gene is introduced into plant cells. The ability to incorporate the introduced gene into the plant genome is a key step in genetic engineering. Once the gene is incorporated into the plant genome, the gene should be expressed and stably inherited.

6) Selection of transformed cells. The selection of transformed cells is generally achieved through the use of a selectable marker in combination with the corresponding selective agent. During the selection process, non-transformed cells surrounding transgenic cells will die, whilst transgenic cells can grow further.

7) Regeneration of the engineered plant. The last step in plant genetic engineering is to regenerate intact, fertile plants from transformed cells. This is performed by inducing the transformed plant cells, under *in vitro* culture conditions, to form somatic cells from which transgenic plants are produced.

Beside the seven steps stated above, Mendel and Teeri (1995) added a further step which is important in establishing new transgenic cultivars of crop plants, namely that the introduced gene should be consistently transmitted to

future generations, in a predictable fashion. This is necessary not only for the demonstration of true integration of the introduced gene into the plant genome, but also for the practical maintenance of the transgenic trait.

1.3 Tissue culture of wheat

Tissue culture is a term used to describe a method for the cultivation *in vitro* of plant parts at the cell, tissue or organ level. It is carried out under aseptic conditions, where nutrients and environmental conditions are controlled. The application of this technique has provided a system that allows the investigation of physiological, biochemical, genetic and structural problems related to plants and has paved the way for the development of plant transformation technologies (Lazzeri and Shewry, 1993).

The successful application of tissue culture or *in vitro* culture techniques is dependent upon the production of competent cultures which can regenerate into intact plants. Vasil (1987) has pointed out several factors that control regeneration in cultures. These include the source of explants, the requirement for growth regulators and the plant genotypes.

In vitro culture technology for wheat and other cereal plants has progressed rapidly through the identification of such factors. Much research has been focused on the responsiveness of explants, on favourable growth regulator requirements for cultures and on genotypic effects. These studies are reviewed briefly in the following subsections.

1.3.1 Explant sources

The source of explants is one of the most important factors which determines the production of callus and embryogenic callus. Terminologically, callus refers to the proliferation of de-differentiated cells arising from cultured explants, and embryogenic callus describes the ability of the callus to form an embryo which will subsequently regenerate into an intact, fertile plant under appropriate conditions (Dodds and Roberts, 1995).

In wheat, a number of explant sources have shown competence to produce embryogenic cultures and these include shoot tips (Dudits *et al.*, 1975; Viertel and Hess, 1996), anthers (Shimada and Makino, 1975; Lazar *et al.*, 1985, 1987; Armstrong *et al.*, 1987; Jones and Petolino, 1987;1988; Zhou and Konzak, 1989; Zhou *et al.*, 1991; Ekiz and Konzak, 1997; Zheng and Konzak, 1999) and leaf bases (Ahuja *et al.*, 1982; Zamora and Scott, 1983; Wernicke and Milkovits, 1984; Rajyalakshmi *et al.*, 1991). Some research groups have reported difficulty in regenerating intact plants from these explants and, in addition, many have observed the production of albino and infertile plants.

Other explants that have been used in tissue culture of wheat include mature embryos (Eapen and Rao, 1982; Mohmand and Nabors, 1990, 1991; Özgen *et al.*, 1998), immature inflorescences (Ozias-Akins and Vasil, 1982; Maddock *et al.*, 1983; Barcelo *et al.*, 1991; Sharma *et al.*, 1995; Marcinska *et al.*, 1995), zygotes and ovules (Kumlehn *et al.*, 1997[a];1997[b]). The use of such explants in wheat tissue culture, however, has limitations, mainly associated with difficulties in initiating suitable cultures. Furthermore, the latter methods are labor intensive during the maintenance and regeneration stages.

So far, immature embryos have been the most successful and most frequently-used explant in wheat tissue culture. This was demonstrated by early reports that highly regenerable callus could be easily produced from immature embryos (Chin and Scott, 1977; Gosch-Wackerle *et al.*, 1979; Sears and Deckard, 1982). In the following reports, the superiority of immature embryos over other explants was clearly demonstrated for the generation of embryogenic cultures and for the recovery of fertile plants (He *et al.*, 1986; Papenfuss and Carman, 1987; Qureshi *et al.*, 1989; Redway *et al.*, 1990; Ben Amer and Börner, 1997). Based on these reports, it is clear that immature embryos are currently the explant of choice for initiating regenerable cultures of wheat.

1.3.2 Plant growth regulators

Growth regulators play a crucial role in inducing explant growth in culture. The most common growth regulator used in tissue culture is synthetic auxin, especially 2,4-dichlorophenoxyacetic acid (2,4-D). The use of 2,4-D in tissue culture of wheat has been reported by many research groups (Ozias-Akins and Vasil, 1982;1983; Galiba and Erdei, 1986; Wernicke *et al.* 1986; Kaleikau *et al.* 1989; Mohmand and Nabors, 1991; Ben Amer and Börner, 1997). In other cases 2,4-D has been combined with a low level of abscisic acid (ABA) (Brown *et al.* 1989; Qureshi *et al.* 1989). A few researchers have reported the use of Dicamba (3,6-dichloro-o-anisic acid) (Dudits *et al.*,1975) or picloram (4-amino-3,5,6-trichloropicolinic acid) (Collins *et al.*, 1978) for inducing callus and regenerating wheat plants.

It has also been noted that some wheat cultivars required cytokinins (kinetin), in combination with synthetic auxins such as 2,4-D (Lazar *et al.*, 1988) or

dicamba (Papenfuss and Carman, 1987), to produce regenerable callus. In other cases the inclusion of cytokinins in the culture medium had a negative effect on callus growth (Dudits *et al.*, 1975). Based on these studies, it has become crucial to evaluate the requirements of growth regulators in culture, especially when testing new wheat cultivars for tissue culture responsiveness, that is the efficiency of callus induction, callus growth and regeneration frequency.

1.3.3 Plant genotypes

The effect of genotypes on tissue culture responsiveness has been demonstrated in several prior studies. The efficiency of callus induction, callus growth rate and regeneration of wheat are all highly genotype dependent (Sears and Deckard, 1982; Lazar *et al.*, 1983; Maddock *et al.*, 1983; Ou *et al.*, 1989; Bohorova *et al.*, 1995). Work by Mathias and Simpson (1986) suggested that genotype was the single most important factor affecting culture response of wheat explants.

Several groups have investigated the nature of tissue culture response differences arising from genotype. Higgins and Mathias (1987), Kaleikau *et al.* (1989) and De Buyser *et al.* (1992) concluded that nuclear genomes play a crucial role in controlling the response of explants in culture, and this factor they claimed to be the basis of genotypic differences in wheat tissue culture. In other studies, Mathias and Fukui (1986), Mathias *et al.* (1986) and Felsenburg *et al.* (1987) reported that tissue culture responsiveness of wheat was determined by cytoplasmic genes, or by interaction between cytoplasmic and nuclear genes. From all these studies, it is apparent that the response of wheat in tissue culture is probably influenced by both nuclear and cytoplasmic genes.

In contrast, it has been argued that different responses of cultured explants in *in vitro* culture largely result from physiological phenomena, and are independent of the genetic background of the cultured explants. For example, Close and Ludeman (1989) showed that the induction of somatic embryogenesis in maize could be achieved by simply manipulating the composition of the culture medium. Duncan *et al.* (1985) confirmed that cultured immature embryos of maize obtained from well-nourished donor plants show a better response in culture than those from nutrient-deficient plants. Similar reports on barley (Hanzel *et al.*, 1984) and sorghum (Ma *et al.*, 1987) indicated that explants excised from plants grown under different climatic conditions also responded differently in culture. It is concluded therefore that the response of explants in culture is determined by many genotypic and environmental factors, each of which plays an important role in explant response in *in vitro* culture. The understanding of such factors is important for establishing a regenerable culture, which is required for genetic transformation.

1.4 Genetic transformation of wheat

Genetic transformation is broadly defined as the transfer of foreign genes isolated from bacteria, viruses, plants or animals into a new genetic background (Webb and Morris, 1992). It will also include the transfer of a gene which might have been isolated from the host species, manipulated *in vitro*, and reincorporated into the same species. In plants, successful genetic transformation requires the production of fertile plants which express the inserted genes.

Focussing on the transformation of wheat, the production of transgenic wheat plants carrying foreign genes finally became a reality after reliable

transformation methods were developed and gene expression cassettes containing suitable promoters and marker genes were constructed.

In the subsections below, several methods are presented, together with a description of promoters and marker genes that have been successfully used in wheat transformation.

1.4.1 Methods of wheat transformation

Until recently, three transformation methods were found to be successful in the transformation of wheat, namely those effected by *Agrobacterium tumefaciens*, direct gene transfer into protoplasts or by microprojectile bombardment.

1.4.1.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a soil bacterium which can infect plant cells, especially dicotyledonous plant species, resulting in crown gall disease. The infection mechanism of *Agrobacterium tumefaciens* has been manipulated to facilitate the transfer of interest genes into plant cells.

In this transformation procedure, target tissue is coated with a suspension of bacterial cells, which is known as co-cultivation. During the transformation process, a specific segment of DNA in a tumor inducing (Ti) plasmid, called T-DNA, is transferred from *Agrobacterium* to the plant cells and subsequently inserted into the nuclear genome of the infected plant. These functions require a set of virulence genes, which also reside in the Ti plasmid (Chilton *et al.*, 1977; Sheng and Citovsky, 1996). The T-DNA can be engineered *in vitro* to contain a selectable marker and/or genes of interest. The *Agrobacterium*-based

transformation system has been facilitated by the use of a binary vector, where the T-DNA region and the virulence genes are placed in two different plasmids. In this system, the transfer of T-DNA or other inserted genes located in one plasmid is mediated by activity of the virulence region from another plasmid (Webb and Morris, 1992).

So far, *Agrobacterium tumefaciens*-mediated transformation has been the most common and widely-used method for transformation of dicotyledonous plants, mainly because the method is simple, inexpensive and efficient (De Block, 1993). Because of its simplicity, efforts have been made to extend the method for transforming cereal crops, including wheat. Initially, its application for cereal transformation was believed to be limited because monocotyledons were considered to be outside the host range of *Agrobacterium* (Potrykus, 1990).

The first successful attempt to transform wheat plants using *Agobacterium* was reported by Hess *et al.* (1990). In this work, a suspension of *Agrobacterium tumefaciens* was pipetted into spikelets of wheat and several kanamycin-resistant plants were obtained from the grains. In the following year, Mooney *et al.* (1991) infected immature embryos of wheat with *Agrobacterium tumefaciens* and a few kanamycin-resistant callus lines were produced.

Recently, Cheng *et al.* (1997) produced transgenic wheat plants showing herbicide resistance *via Agrobacterium tumefaciens*. In these experiments, immature embryos and embryogenic callus were co-cultivated with *Agrobacterium tumefaciens*. A surfactant (Silwet) was routinely added to the *Agrobacterium* suspension prior to inoculation of explants. The addition of the surfactant was shown to increase the transient expression of *GUS* gene. This probably enhanced *Agrobacterium* attachment to and infection of the co-cultivated explants.

The presence of transgenes in the transgenic wheat plants recovered in this work was confirmed by Southern hybridization analysis and by genetic analysis.

The use of *Agrobacterium*-mediated transformation has been reported in other cereals such as rice (Chan *et al.*, 1993; Hiei *et al.*, 1994), maize (Ishida *et al.*, 1996) and barley (Tingay *et al.*, 1997; Qureshi *et al.*, 1997). It is believed that further improvement of this system will eventually make it more efficient and routine for cereal transformation. Because the successful application of the *Agrobacterium*-mediated method for wheat transformation was initially limited, direct DNA transfer methods using microprojectile bombardment have been developed concurrently.

1.4.1.2 Direct gene transfer into protoplasts

Protoplasts are single plant cells from which the cell wall has been removed. They are prepared by incubating cells or tissues in a solution containing a mixture of cellulolytic and pectolytic enzymes (Brettell and Murray, 1995). The success in establishing suspension cultures from wheat protoplasts from which plants have later been regenerated has been reported by several research groups (Yang *et al.*, 1991; He *et al.*, 1992). This success has indicated that transforming wheat through protoplasts is a viable method.

Foreign genes are introduced into protoplasts following treatment either with polyethylene glycol or by electroporation (Potrykus, 1991). Several groups have reported success in delivering foreign genes into wheat protoplasts and in obtaining transient expression (Lörz *et al.*, 1985; Ou-Lee *et al.*, 1986; Hauptman *et al.*, 1987; Oard *et al.*, 1989). Success in the recovery of stably transformed wheat callus from protoplasts has also been achieved, but subsequent plant

regeneration was not obtained in early experiments (Marsan *et al.*, 1993; Zhou *et al.*, 1993). However, He *et al.* (1994) demonstrated the transformation of wheat protoplasts and the production of stably transformed embryogenic colonies from which flowering wheat plants were regenerated. Although this method has successfully been applied to wheat transformation, the method is rarely used because the technique is technically demanding and transformation cannot be achieved on a regular basis.

1.4.1.3 Microprojectile bombardment

Microprojectile bombardment, or biolistics, is a DNA delivery system in which the genetic material is coated onto microparticles of tungsten or gold and propelled into the target cells (Sanford, 1990). Indeed, the microprojectile bombardment method of transformation has opened the door for cereal genetic manipulation. The technology was initially pursued and developed because of the limited success of *Agrobacterium*-based methods with cereals. Now that this hurdle has been cleared and *Agrobacterium*-mediated transformation of wheat has been demonstrated (Cheng *et al.*, 1997; Fry *et al.*, 1998), biolistics-effected transformation is being re-examined because often produces plants containing multiple and unwanted insertions. The regulating bodies are becoming more cautious and sophisticated in demanding transgenic plants with simpler insertions, if the plants are to be accepted for commercial release. Once again attention is focussed on *Agrobacterium* for producing simpler and more acceptable transformation events.

Christou (1992) outlined several theoretical advantages with the use of microprojectile bombardment for plant genetic transformation. These include;

1. an ability to transform intact tissues;

   microprojectile bombardment can be used to target a wide range of tissues or organs and avoids the use of protoplasts or suspension culture.

2. the rapid recovery of transformants;

   by bombarding primary explants such as immature embryos, immature scutella, microspores and meristem tissues, it is possible to accelerate and shorten the time required for obtaining transgenic cell lines and transformed plants.

3. the ability to use microprojectile bombardment as a universal delivery system in all plant species;

   microprojectile bombardment results in the production of transgenic plants from many species, including monocotyledonous and dicotyledonous plants, and this method can be used to deliver foreign genes into cells of most genotypes. However, because transformation relies on the production of intact, transgenic plants, the method is more successful in genotypes which display a vigorous tissue culture response.

4. its application in studies of basic plant developmental processes;

   microprojectile bombardment makes the rapid evaluation of gene function and of gene regulation during plant growth and development.

Bombardment device

A number of microprojectile bombardment devices have been described in the literature. In the first successful transformation of plant cells, which were epidermal cells of onion, a Biolistics^{TM} PDS accelerator (DuPont, Wilmington, DE, USA) was used (Klein *et al.*, 1987). In this device an acceleration force is

generated by the explosion of gun powder. The generated force accelerates a cylindrical polypropylene macroprojectile, which is loaded with DNA-coated microparticles, down a 0.22 calibre barrel towards the target cells. The polypropylene macroprojectile is stopped by a polycarbonate disk which has a small hole through which the microparticles continue towards the target cells. Sufficient particle velocity is achieved when the acceleration chamber is kept under a partial vacuum. Other bombardment devices with a similar fundamental design to the Biolistics device have been also developed, but have used compressed air or gas (nitrogen) to generate the accelerating force (Iida *et al.*, 1990; Oard *et al.*, 1990).

The electric discharge particle acceleration device differs in design from the above devices. In this device, a shock wave is generated by the discharge of a capacitor through a small water drop inside a polyvinyl chloride expansion chamber. The shock wave causes the upward acceleration of a thin mylar carrier sheet carrying the DNA-coated particles towards a retaining screen. When the sheet hits the retaining screen, the particles continue onward and penetrate into target tissues. The use of this device in plant transformation has been reported by several research groups (McCabe *et al.*, 1988; Christou, 1990).

The most common bombardment device currently used in plant transformation is the biolistic PDS-1000/He gun, which is distributed by BioRad Laboratories (Figure 1.1). In operation, the PDS-1000/He device is powered by helium gas, which builds up pressure behind a rupture disk. When the disk ruptures, a burst of helium gas accelerates a macrocarrier, upon which DNA-coated microcarriers have been dried. The accelerated macrocarrier is retained by a stopping screen, but the microcarriers are allowed to continue downward until

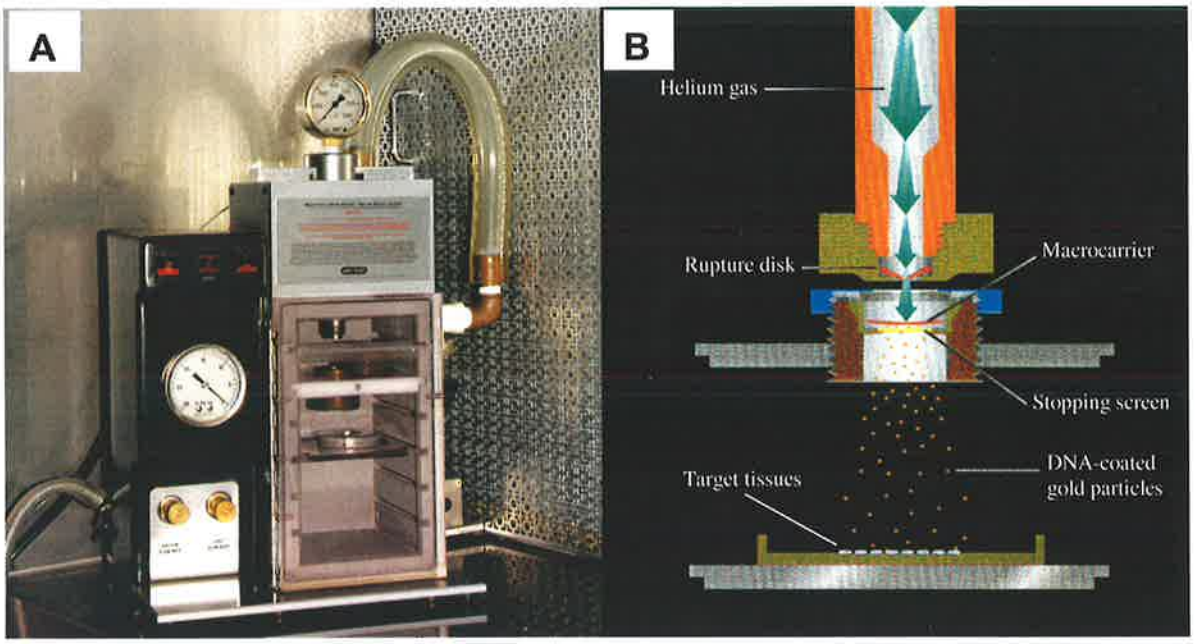
A
B
Helium gas
Rupture disk
Macrocarrier
Stopping screen
DNA-coated
gold particles
Target tissues

Figure 1.1 Microprojectile bombardment device. (A) the biolistic particle delivery system (PDS)-1000/He gun. (B) bombardment principles; rupture disk compressed by helium gas bursts at certain pressure and accelerates microparticle-coated DNA that is loaded onto a macrocarrier. The macrocarrier moves down and it is retained by a stopping screen, while microcarriers pass through and penetrate into target tissues (Source: modified from BioRad Products Catalog, 1998/99)

they impact and penetrate the target cells/tissues. The main chamber is maintained under reduced pressure to increase the velocity of the microcarriers (Kikkert, 1993). The PDS-1000/He device possesses several advantages over other models, such as a safer operation, more uniform distribution of microcarriers over the target cells/tissues, and better control of bombardment power which, in turn, enables the cells/tissues to be bombarded in a less harsh fashion (Kikkert, 1993). Until recently this bombardment device has proved to be the most versatile method for plant transformation and has been successfully used to transform all major cereal crops, including wheat (Vasil, *et al.*, 1992; Weeks, *et al.*, 1993; Becker, *et al.*, 1994; Karunaratne *et al.*, 1996; Takumi and Shimada, 1997).

Bombardment parameters

Since microprojectile bombardment-mediated transformation is a mechanical method for the introduction of DNA into the plant genome, several physical parameters should be considered in order to obtain a satisfactory result. Such parameters include helium pressure, the distance between the rupture disk and the macrocarrier and the flight distance of macrocarrier and microcarriers. With the PDS-1000/He gun, the gas pressure is generally about 1100 psi, the distance between the rupture disk and the macrocarrier is about 1 cm and the distances of macrocarrier travel and microcarrier flight are about 1.1 cm and 12 cm, respectively (Kikkert, 1993).

Although the physical parameters for bombardment have been defined, the method is still relatively inefficient, because only a few cells survive the physical disruption caused by bombardment (Hunold *et al.* 1994). The survival of cells after bombardment is probably affected by the number of particles used. Work by

Becker *et al.* (1994), Jahne *et al.*, (1995), Altpeter *et al.* (1996) and Rasco-Gaunt *et al.* (1999) demonstrated that small numbers of gold particles are more favourable for explant viability than large numbers. Higher particle densities (100-116 µg gold particles per bombardment) resulted in a significant reduction in embryogenic callus formation and plant regeneration.

Another factor which might affect the results is bombardment pressure. Koprek *et al.* (1996) demonstrated that transgenic plants were successfully recovered, even from genotypes showing low regeneration capability, by bombarding target tissues at low pressures (60-90 psi) using the particle inflow gun (PIG). A reduction in the production of transgenic plants was observed when target tissues were bombarded at high pressure (800-1200 psi using the PDS-1000/He device). Thus, physical factors can be critical for optimizing DNA delivery and for the recovery of transgenic plants.

DNA coating methods

Several groups have endeavoured to standardize DNA coating methods prior to microprojectile bombardment (Klein *et al.*, 1987, 1988; Russell, *et al.*, 1993). Usually, DNA is precipitated onto gold or tungsten particles in the presence of $CaCl_2$ and spermidine. Microparticles of gold are often preferred to tungsten, because gold particles are more spherical, more uniform in size and are biologically inert; they do not degrade DNA or harm the impacted cells (Kikkert, 1993; Sanford *et al.*, 1993).

Although the coating process has been standardized, different groups often employ slightly different procedures for DNA precipitation. For example, Perl *et al.* (1992) carried out DNA precipitation with silver thiosulfate instead of calcium

chloride, and eliminated spermidine from the DNA-microparticle mixture. They reported that their DNA precipitation procedure significantly increased transient GUS expression in bombarded tissues and surpassed the previous reports of Klein *et al.* (1988) and Vasil *et al.* (1991). In contrast, Vasil *et al.* (1993) argued that the exclusion of spermidine from the DNA precipitation procedure drastically reduces transgene expression.

1.4.2 Promoters and Markers used in wheat transformation

1.4.2.1 Promoters

One important determinant of transformation efficiency is the promoter used to direct expression of the transgene. Two fundamentally different kinds of promoter are known. One kind includes constitutive promoters, which induce the expression of transgenes in all or almost all tissues, regardless of developmental and environmental signals.

The most commonly used constitutive promoter in plant transformation studies to date has been the *CaMV* 35S promoter of the cauliflower mosaic virus 35S RNA gene (Bekkaoui *et al.*, 1990; Charest *et al.*, 1993). This promoter has been widely used in dicotyledons and, in some cases, in cereals such as maize (Rhodes *et al.*, 1988), rice (Shimamoto *et al.*, 1989), barley (Lazzeri *et al.*, 1991) and wheat (Vasil *et al.*, 1991; Ortiz *et al.*, 1997). However, levels of transgene expression driven by the *CaMV* 35S promoter are generally lower in monocotyledonous species than in dicotyledonous species (Fromm *et al.*, 1985; Hauptmann *et al.*, 1987). Consequently, efforts have been made to identify more efficient promoters for cereals.

Based on observations made by several research groups, it was noted that the alcohol dehydrogenase1 (*adh1*) promoter of maize could drive transgene expression at levels equivalent to or higher than those obtained with the *CaMV* 35S promoter (Zhang and Wu, 1988; Vasil *et al.*, 1992). It was also found that the addition of an *adh1* or a maize *shrunken1* intron between the promoter and the coding region greatly increased the level of transgene expression in cereal plant cells (Callis *et al.*, 1987; Vasil *et al.*, 1989).

So far, the promoters which are probably the most effective in expressing transgenes in cereals are the rice *Actin1* (*act1*) promoter, with its first intron (McElroy *et al.*, 1990; Zhang *et al.*, 1991; He and Lazzeri, 1998), and the maize ubiquitin1 *(ubi1*) promoter, along with its first intron (Christensen *et al.*, 1992; Ortiz *et al.*, 1997). The successful use of these promoters has been documented in wheat (Weeks *et al.*, 1993; Nehra *et al.*, 1994; Takumi *et al.*, 1994), barley (Wan and Lemaux, 1994) and rice (Toki *et al.*, 1992).

In other studies, it was found that cereal-derived promoters, such as the *adh1* promoter of maize (Ellis *et al.*, 1987), were not efficient in activating transgene expression in dicotyledonous plants. From these studies, it appears that dicotyledonous and monocotyledonous plants require specific promoters for high level transcription of transgenes in their genome.

Another group of promoters includes the non-constitutive promoters, which are activated in a tissue-specific and/or inducible fashion. These promoters specifically activate transgene expression in certain tissues, in response to physical stimuli, in response to biotic and abiotic stresses, or to physical damage. Examples of tissue-specific and inducible promoters are the tomato *rbc*S and rice *rbc*S promoters. These direct mesophyll-specific transcription and are induced by

light. Kyozuka *et al.* (1993) used these promoters to drive the expression of the *GUS* gene in transgenic rice and observed that the expression of the introduced gene was restricted to leaf blade and sheath mesophyll cells. Furthermore, expression was light dependent.

In wheat, Barro *et al.* (1997) reported success in using native promoters of 1Ax1 and 1Dx5 glutenin genes. These promoters, which are endosperm-specific, direct the expression of their own genes, namely 1Ax1 and 1Dx5 genes, and result in increased levels of glutenin in the grains of transgenic wheat plants and their progeny. Based on reports reviewed here, it becomes clear that the construction of an appropriate regulatory element or a promoter is crucial for driving transgene expression efficiently.

1.4.2.2 Markers

Marker genes, including selectable and scorable markers, are essential for the successful transformation of wheat. Such genes, especially the selectable markers, are important for the selection of transgenic cells from non-transgenic cells. In common transformation procedures, transgenic cells are recovered by culturing transformed cells on medium containing a selective agent, which corresponds to the selectable marker used. Transgenic cells expressing the acquired marker gene will survive on the medium, while non-transgenic cells will die or their growth will be severely retarded.

So far, the selectable markers extensively used in plant transformation have been genes that confer antibiotic or herbicide resistance on transgenic cells. For example, the *nptII* gene encodes neomycin phosphotransferase and confers resistance to aminoglycoside antibiotics such as kanamycin, geneticin and

paramomycin (Bevan *et al.*, 1983). This gene has been successfully used in transformation of wheat (Nehra *et al.*, 1994), rice (Uchimiya *et al.*, 1986; Raineri *et al.*, 1990), maize (D'Halluin *et al.*, 1992) and barley (Ritala *et al.*, 1994). Similarly, the *hpt* gene encodes the enzyme hygromycin phosphotransferase and confers resistance to hygromycin (Waldron *et al.*, 1985); it has been also used for wheat (Ortiz *et al.*, 1996), rice (Hayashimoto *et al.*, 1990; Hiei *et al.*, 1994) and maize transformation (Walters *et al.*, 1992).

The herbicide resistance genes *CP4*, encoding glyphosate tolerant enolpyruvyl shikimate phosphate synthase (EPSPS) and *GOX*, encoding glyphosate oxidoreductase, confer resistance to the herbicide glyphosate. The glyphosate normally inhibits EPSPS, which is a crucial enzyme for the biosynthesis of aromatic amino acids such as phenylalanine, tyrosine and tryptophan. The introduction of the EPSPS gene into the plant genome results in overproduction of EPSPS, so this could overcome the reduction in aromatic amino acid biosynthesis caused by the glyphosate (Shah *et al.*, 1986). The herbicide resistant gene *GOX* encodes glyphosate oxidoreductase, which degrades the herbicide glyphosate to form the non toxic compound, aminomethyl phosphonic acid. The presence of this enzyme in plant cells can therefore protect the plants from the toxicity of glyphosate.

Both the EPSPS and *GOX* genes have been used in the production of transgenic wheat plants (Zhou *et al.*, 1995; Qureshi *et al.*, 1995), but the most common selectable marker used for wheat transformation has been the *bar* gene (Vasil *et al.*, 1993; Becker *et al.*, 1994; Altpeter, 1996). The *bar* gene encodes phosphinothricin acetyl transferase (PAT) and confers resistance to the herbicides Basta® and Bialaphos. This is made possible by the ability of the gene product

(PAT) to acetylate and hence detoxify phosphinothricin, the active ingredient of the herbicide Basta® which inhibits plant growth by inactivating glutamine synthase. Phosphinothricin detoxified by PAT will therefore not interfere with the activity of glutamine synthase. Hence, assimilation of ammonia into L-glutamate by glutamine synthase can proceed and the death of plant cells by ammonia overload is prevented (De Block *et al.*, 1987).

Another selective marker used in wheat transformation is the *man*A gene. The *manA* gene encodes mannose-6-phosphate isomerase (MPI), which is an *E. coli* glycolytic pathway enzyme. This enzyme catalyses the interconversion of mannose-6-phosphate and fructose-6-phosphate (Miles and Guest, 1984). Normally, plants cannot use mannose-6-phosphate as a carbon source, but transgenic plants carrying the *manA* gene can grow on media containing mannose phosphate as the sole carbon source. This positive selection strategy has been shown to work for wheat (Reed *et al.*, 1999) and maize (Hansen and Wright, 1999), and has the potential to be a key component of future cereal transformation strategies.

Progress in plant transformation has also been achieved by improvement in the utilization of scorable markers or reporter genes. These genes are especially important for the early detection of transgene expression in transformed cells. Several reporter genes such as those linked to anthocyanin synthesis (Klein *et al.*, 1989), to chloramphenicol acetyltransferase (*cat*) (Kartha *et al.*, 1989), to firefly luciferase (Fromm *et al.*, 1990; Sadasivam and Gallie, 1994; Lonsdale *et al.*, 1998; Baruah-Wolff *et al.*, 1999; Harvey *et al.*, 1999) and to the green fluorescent protein (GFP) (Sheen *et al.*, 1995; Pang *et al.*, 1996; Fry *et al.*, 1998; Vain *et al.*, 1998; Elliott *et al.*, 1999), have been used in plant transformation.

Until very recently, the *GUS* (*uid*A) gene, encoding β-glucuronidase, has been the most common reporter gene used in wheat transformation (Nehra *et al.*, 1994; Becker *et al.*, 1994). This gene has been utilized both for the development of transformation systems and as a reporter of stable transformation. In plants, the expression of the this gene can be detected fluorometrically or histochemically. Of these two methods, the histochemical procedure is easier and more commonly employed.

The histochemical assay operates *via* the ability of the *GUS* gene product (β-glucuronidase) to catalyse the hydrolysis of the colourless compound 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid to release free indolyl derivatives which are blue in colour. The blue coloured products can be detected visually in plant tissues. The degree of *GUS* gene expression is determined by the metabolic status of the tissues (Jefferson, 1987; Jefferson *et al.*, 1987).

1.5 Transgene inheritance in transgenic wheat

Studies focused on transgene inheritance and stability in transgenic wheat plants are still few in number. The stable transmission and expression of transgenes in a predictable manner has been demonstrated by Vasil *et al.* (1993), where the inheritance of transgene expression showed Mendelian ratios of 3:1 in self-pollinated transgenic plants and 1:1 in cross-pollinated transgenic plants. In other studies, however, transgenes were unstable and were inherited in a non-Mendelian fashion (Srivastava *et al.*, 1996).

It has been suggested that instability of transgene expression in transgenic plants is partly attributable to transgene silencing. The silencing of transgenes could be affected by the surrounding chromosomal environment into which the

transgenes were integrated (Pröls and Meyer, 1992; Demeke *et al.*, 1998), as well as by the age and developmental stage of the cells (Myer *et al.*, 1992; Walter *et al.*, 1992).

Inactivation of transgenes in plants might also be influenced by the presence of multiple copies of transgenes in the plant genome (Finnegan and McElroy, 1994). However, transgene inactivation has been reported not only in transgenic plants carrying multiple copies of the transgene (Müller *et al.*, 1996; Alvarez *et al.*, 2000), but also in transgenic plants having a single copy of the transgene (Elmayan and Vaucheret, 1996).

Based on these reports, it is clear that more studies are needed for investigating the behaviour of transgene inheritance and expression in transgenic plants. Results from such studies are not only important for transgenic plant producers and plant breeders, but the results are also crucial for considering the reliable application of transformation technology in agricultural practice.

1.6 Somaclonal variation

Somaclonal variation is a term that refers to increased genetic variation in plants regenerated from tissue culture (Larkin and Scowcroft, 1981). In wheat, genetic variability resulting from tissue culture includes chromosomal aberrations, such as inversion and deletion of chromosomes (Ahloowalia, 1982; Karp and Maddock, 1984), and alteration in the expression of single genes (Larkin *et al.*, 1984). It was speculated that the occurrence of such variations arises from errors during *in vitro* cell division. The occurrence of somaclonal variation is generally higher in regenerants recovered from cells that have been in culture for a long period (Vasil, 1987).

Initially, much attention was devoted to the possible advantages of somaclonal variants for producing new cultivars. However, such expectations were not realised because most somaclonal variants showed poor agronomic performance and low fertility, as was reported for wheat by Ahloowalia (1982) and Ahloowalia and Sherington (1985). Qureshi *et al.* (1992) also provided evidence that superior characters of somaclonal variation-derived regenerants identified during *in vitro* culture were lost when the progeny of the regenerants were grown under field conditions. Similarly, Bozorgipour and Snape (1997) reported that somaclonal variants which showed herbicide tolerance in culture failed to transmit their characters to progeny.

In relation to transformation experiments, the effects of somaclonal variation have also been reported. Vasil *et al.* (1992) showed that many transgenic wheat plants exhibited poor growth and were mostly sterile. Furthermore, Bregitzer *et al.* (1998) reported that transgenic barley progeny exhibited retarded growth and low yields. All of these researchers believed that the presence of such aberrant agronomic characteristics was attributable to somaclonal variation.

To minimize the incidence of somaclonal variation during cereal transformation, it is therefore important to develop a simple regeneration system and to avoid prolonged culture of cells.

1.7 Aims of the project

The main aims of the current study were to screen four Australian wheat genotypes for *in vitro* culture responsiveness and to transform the responsive

genotypes using microprojectile bombardment. Within this overall aim, specific goals were:

- to assess the growth regulator requirements for each wheat genotype screened
- to develop regeneration systems for the responsive wheat genotypes. These experiments are described in Chapter 2
- to transform the responsive wheat genotypes with selectable marker and reporter genes. The results of this work are presented in Chapter 3
- to study the inheritance and expression of the transgenes in successive wheat generations. Results of these studies are described in Chapter 4

Finally, possible future experimental directions arising from work described here are outlined in Chapter 5.

CHAPTER TWO

TISSUE CULTURE OF WHEAT

2.1 Introduction

The availability of an efficient regeneration system is a prerequisite for the successful application of genetic transformation. In wheat, the efficiency of the regeneration system depends upon the capacity of cultured cells to produce embryogenic callus and the ability of this callus to regenerate into fertile plants.

One important factor influencing embryogenesis and regeneration in wheat is the source of explant. So far, the most common and reliable explant for inducing embryogenic callus and regenerating wheat plants has been the immature embryo (Fennell *et al.*, 1996). Besides the source of the explant, callus induction and regeneration of wheat are affected not only by the composition of the culture medium (Elena and Ginzo, 1988; He *et al.*, 1989), but also by the genotype (Sears and Deckard, 1982; Bohorova *et al.*, 1995). Mathias and Simpson (1986) and Maës *et al.* (1996) assessed the effects of culture medium and genotype on culture response for a range of wheat plants and suggested that genotype appeared to be more significant than the culture medium.

Similarly, genotype considerably influences the success of wheat transformation (Weeks *et al.*, 1993; Nehra *et al.*, 1994; Takumi and Shimada, 1997; Iser *et al.*, 1999). During the development of wheat transformation procedures, most research groups have chosen wheat genotypes which respond well in culture rather than genotypes of agronomic importance. Consequently, transformation procedures developed for wheat are still applicable to a limited number of genotypes, and many of these are of limited value in terms of agronomic performance and breeding potential.

In an attempt to broaden the range of wheat genotypes amenable to transformation and to demonstrate a wider application of microprojectile

bombardment-mediated transformation for wheat, it is crucial to find candidate genotypes that grow well in culture. While it has been argued that genotype is of secondary importance to culture "vigour" in cereal transformation, because transgenes in a stably transformed plant can subsequently be backcrossed into the desired genotype, cereal breeders generally support the direct incorporation of potentially useful transgenes into elite breeding varieties or lines (Professor A.R. Barr, Dr. A.J. Rathjen and Assoc. Prof. G.J. Hollamby, personal communication). This generally reduces the time required to produce the final variety for release, and allows the transgene to be evaluated in a more appropriate genetic background.

For these reasons, four Australian elite wheat varieties were screened for their tissue culture response. Three of the four screened varieties (*cvs.* Frame, Krichauff and Janz) are widely grown across Southern Australia and another genotype (*cv.* Hartog) is predomonantly planted in the north east of Australia. The latter cultivar has also been used by a number of laboratories across Australia for tissue culture and successful transformation of Hartog has been reported (He *et al.*, 1992; Witrzens *et al.*, 1998). All four Australian wheat genotypes used here are important hexaploid bread wheat cultivars. In the work described in this Chapter, experiments were undertaken to screen the four Australian wheat genotypes that were capable of producing regenerable callus in culture, and to determine the optimal requirements and concentrations of growth regulators for each responsive genotype.

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

Four elite Australian spring wheats (*cvs.* Hartog, Frame, Krichauff and Janz) were used in these experiments. Grains were kindly provided by Dr. A.J. Rathjen of the Department of Plant Science, University of Adelaide, Waite Campus.

Plants were grown in the glasshouse, in growth chambers or in the field. Temperatures in the glasshouse varied between 12°C and 30°C, with a 10-14 hour photoperiod, depending on the season. All grains were sown in eight-inch pots filled with Recycled Soil (RS) (see Appendix 1 for details). Seedlings of the four genotypes were grown in the glasshouse until ready for harvest, which was usually 80-95 days after sowing, depending on the genotype and environmental conditions.

Pots in the glasshouse were fertilized as follows: Osmocote® (15-4.8-10.8 for N-P-K, respectively) (Scotts Australia Pty. Ltd, Australia) and soluble John Innes (JI) feeder (36-11-11 for N-P-K, respectively) (formulated by South Australian Research and Development Institute; E. Nagy, personal communication) were applied simultaneously six weeks after the grains were sown, at a dosage of 2 and 0.045 kg/m^3 soil, respectively. Thereafter, plants were fertilized every ten days with soluble JI feeder, also at a dosage of 0.045 kg/m^3 soil. Any plants infected with fungi were sprayed at recommended dosages with fungicides such as Sulphine (3 g/l) or Bayfidan (0.4 ml/l). Mites were sprayed with miticides such as Omite (2 g/l) or Pyranica (1 g/l).

Some plants were grown in growth chambers, especially in the summer season when glasshouse temperatures regularly rose above 30°C. The

temperature in the chambers was maintained at 15°C during the day and 12°C during the night, with a 10/14 h day/night photoperiod. Plants were grown under these conditions for 50 days and transferred to another chamber where the temperature and photoperiod were increased to 19°C during the day and 16°C during the night, with a 16/8 h day/night photoperiod, respectively. The plants were grown in the second chamber until harvested. Plants were fertilized as described for plants grown in the glasshouse.

Plants of three wheat genotypes (*cvs.* Hartog, Frame and Krichauff) were also grown in the field at Charlick Experimental Station, Strathalbyn during the winter season. Fertilizer (Osmocote®) was applied at sowing at a dosage of 250 kg/ha. Plants grown in the field were watered as required. All plants grown in the glasshouse, growth chamber and field were tagged at the time of pollination.

2.2.2 Tissue culture media

The basic medium used during these experiments was Murashige and Skoog (MS) (1962) basal medium supplemented with 3% sucrose. The medium was solidified with 0.8% Bitek agar (DIFCO, Detroit, USA) and the pH was adjusted to 5.7 with sodium hydroxide prior to autoclaving (Appendix 2). The media were sterilised in the autoclave at 121°C for 20 min.

To test the *in vitro* culture response of the wheat genotypes, three varieties (*cvs.* Frame, Krichauff and Janz) were cultured on the MS basal medium supplemented with various combinations and concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BA) (see Table 2.1 for the combinations and concentrations of the two growth regulators tested in these experiments). Another genotype (*cv.* Hartog), which is reported to be responsive

Table 2.1 Combinations and concentrations of 2,4-D and BA tested on three Australian wheat genotypes (*cvs.* Frame, Krichauff and Janz)

		BA (mg/l)				
		0	1	2	3	5
2,4-D (mg/l)	0	MSD0BA0	MSD0BA1	MSD0BA2	MSD0BA3	MSD0BA5
	1	MSD1BA0	MSD1BA1	MSD1BA2	MSD1BA3	MSD1BA5
	2	MSD2BA0	MSD2BA1	MSD2BA2	MSD2BA3	MSD2BA5
	3	MSD3BA0	MSD3BA1	MSD3BA2	MSD3BA3	MSD3BA5
	5	MSD5BA0	MSD5BA1	MSD5BA2	MSD5BA3	MSD5BA5

Notes: MSD0BA0 is MS basal medium with no growth regulators.
MSD3BA2 is MS basal medium with the addition of 3 mg/l 2,4-D (D) and 2 mg/l BA.

in culture (Yang *et al.*, 1991; He *et al.*, 1992; A. Aryan, personal communication), was cultured on MS basal medium supplemented with 2,4-D at concentrations of 1, 2, 3 or 5 mg/l.

2.2.3 Isolation and culture of explant tissue

Developing grains from the four wheat genotypes were harvested ten to 14 days after pollination, when they were 1 to 2 mm in length (Figure. 2.1A) (Nehra *et al.*, 1994). The grains were stored at 4°C for a maximum of five days before use.

All grains were surface sterilised with 10% (v/v) Domestos® (ACI, Australia; with active ingredients 5.25% w/v sodium hypochlorite, 1.25% w/v sodium hydroxide and 0.5 g/l "alkaline salts") for 20 min and washed five times with sterile distilled water. Immature embryos were excised from the grains under a dissecting microscope (Nikon, Model C-PS) and embryonic axes were carefully removed from the immature embryos (Nehra *et al.*, 1994). Scutella isolated from the immature embryos were placed, scutellum face up, in 90 mm Petri dishes containing 30 ml solid agar MS medium supplemented with the various combinations and concentrations of growth regulators, as explained in Section 2.2.2 above. Petri dishes were sealed with Parafilm and wrapped in aluminium foil. Callus production was induced at 24-26°C for three to four weeks.

To assess the response of wheat genotypes to medium composition, ten scutella were cultured per Petri dish. Three Petri dishes were used for each treatment, bringing the total to 30 scutella per treatment. In the case of *cv.* Hartog, the total number of scutella used per treatment varied between 17 and 66 scutella (Table 2.2).

2.2.4 Induction of somatic embryos and plant regeneration

Callus formed during incubation in the dark was subsequently exposed to a 16 h day/8 h night photoperiod and maintained at 24-26°C for two to six weeks. After two to six weeks exposure to light, the response of explants in culture was assessed or scored for somatic embryo production. Scutella which failed to form callus, or formed callus which failed to produce somatic embryos, were scored as non-regenerable or non-embryogenic. Several parameters, such as the number and percentage of cultured scutella producing embryogenic callus and the number of somatic embryos or shoots formed per callus, were scored.

After recording relevant information regarding callus induction and somatic embryogenesis, somatic embryos or shoots from each embryogenic callus were picked individually and transferred to MS basal medium without 2,4-D or BA (MS hormone-free medium) for three to four weeks, to allow plantlet formation. Occasionally, some of the somatic embryos or the shoots were transferred to MS hormone-free medium for two weeks and subcultured onto fresh, identical medium for a further two weeks.

After three to four weeks on the growth regulator-free MS medium, the number of somatic embryos or embryos producing plantlets was recorded. Plantlets of around 3 cm in length and with a well-developed root system were removed from the Petri dishes, washed to remove traces of agar, and planted in three inch pots containing RS soil. The pots were placed in 37.5 x 25.0 x 25.0 cm plastic boxes and transferred to the glasshouse. The lid of the plastic box was closed for two weeks to avoid desiccation of plants. The pots were watered regularly to maintain a high humidity. After two weeks, the pots were taken out of the plastic boxes and placed on benches in the glasshouse. In the following four

weeks, the growing plants were transplanted into eight-inch pots and grown to maturity. Other control plants were grown in the same glasshouse. The phenotypic characteristics and fertility of the tissue culture-derived plants were recorded and compared with the control plants.

2.3 Results

2.3.1 Production of somatic embryos

Within four to five days of culture, scutella started to swell and cell proliferation commenced. Callus formed from cultured scutella was clearly identifiable within ten to 14 days after culture, but was allowed to grow for another two to three weeks in the dark (Figure. 2.1B). After this time, growing callus was examined for phenotypic characteristics. Variation was clearly evident in the texture of the callus. Callus produced from *cvs.* Hartog, Frame and Krichauff was hard and compact, whilst callus produced from *cv.* Janz was less compact and was sometimes friable.

The callus began to produce somatic embryos five to seven days after exposure to light. However, the response of different genotypes was variable. Three of the four genotypes tested (*cvs.* Hartog, Frame and Krichauff) had a high capacity to produce somatic embryos. One genotype, *cv.* Janz, showed a low capacity for the production of somatic embryos.

The experiments also revealed genotypic differences in response to growth regulators. Two genotypes, *cvs.* Hartog and Krichauff, produced somatic embryos after scutella were cultured in the presence of 2,4-D alone. Good results were obtained at low concentrations (1 and 2 mg/l) of 2,4-D, whilst concentrations of 3 and 5 mg/l 2,4-D led to a lower percentage of somatic embryo production. In these experiments, the best results were for *cvs.* Hartog and Krichauff, where scutella produced embryogenic callus at a frequency of 98 and 100%, respectively. No callus initiation or embryogenic callus production occurred when 2,4-D was excluded from the induction medium.

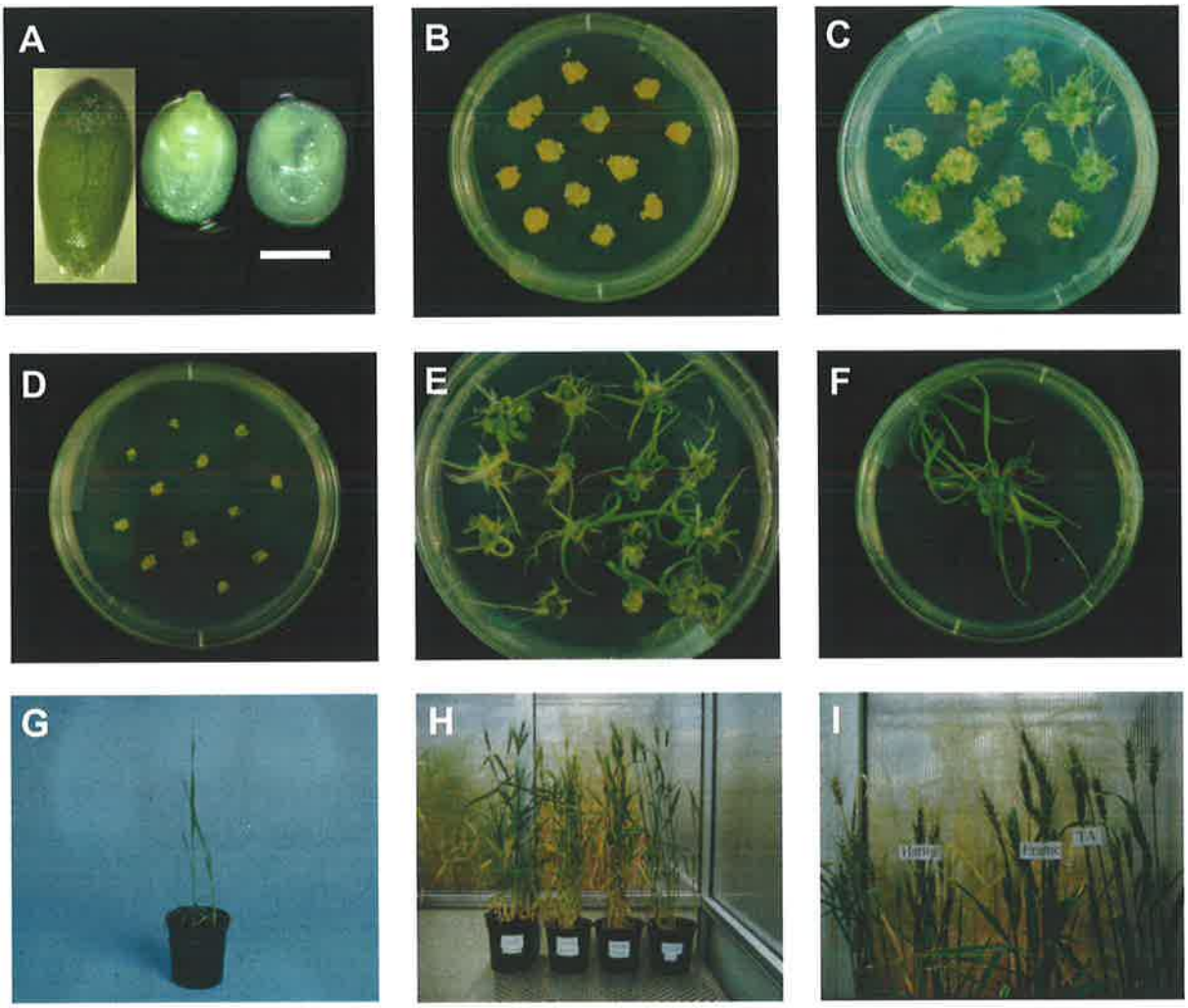
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Figure 2.1 Steps in tissue culture and regeneration of fertile wheat plants. (A) immature grain ten to 14 days after pollination (left), isolated embryo (centre) and isolated scutellum (right) (white bar represents approx. 1 mm); (B) callus production on induction medium containing 2 mg/l 2,4-D; (C) somatic embryo formation at seven to ten days after exposure to light; (D) separation of somatic embryos; (E) shoot and (F) plantlet formation in hormone-free media; (G) young, growing plants established in soil; (H) and (I) mature, fertile plants (*cvs.* Hartog, Frame and Krichauff [labelled as TA]) regenerated from tissue culture.

Addition of BA to the callus induction medium at all concentrations tested showed a negative effect on embryogenic callus production, for most genotypes. On BA-containing medium some scutella became necrotic and showed no further growth.

As shown in Table 2.4, the production of embryogenic callus for *cv.* Frame was less than 60% when the induction media was supplemented with 2,4-D only. Somatic embryo production increased when the induction media was supplemented with 1 or 2 mg/l 2,4-D combined with a low level of BA (1 mg/l). A more satisfactory result was obtained when scutella were induced on media containing 2 mg/l 2,4-D combined with a low concentration of BA (0.1 mg/l). It was observed that all cultured scutella produced regenerable callus on this medium. This contrasts with *cv.* Janz, which performed poorly under all conditions tested (Table 2.6). In Tables 2.2-2.6, the numbers and percentages of embryogenic callus production, and the number of somatic embryos and shoots formed per callus from the four genotypes tested are presented.

2.3.2 Factors affecting tissue culture response

In the initial experiments, which were conducted in 1995, it was demonstrated that the response of explants in culture was influenced by plant genotype, as well as by the type and concentration of growth regulators used. Given an appropriate medium, most genotypes showed a high *in vitro* culture response. However, in subsequent experiments in 1996 and 1997, the response of explants in culture from the three responsive genotypes was reduced dramatically. This was probably attributable to adverse environmental factors affecting the quality of donor plant material.

Table 2.2 The number and percentage of embryogenic callus produced by *cv.* Hartog in 1995

Treatment	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Number of callus producing somatic embryos and shoots			Total number of somatic embryos and shoots formed	Average no. of somatic embryos and shoots formed per callus
				1-4	5-7	8 or more		
MSD1	45	41	91	16	13	12	261	6.37
MSD2	66	65	98	34	26	5	315	4.85
MSD3	17	16	94	11	5	0	60	3.75
MSD5	21	14	67	11	3	0	49	3.50

Table 2.3 The number and percentage of embryogenic callus produced by *cv.* Hartog (callus broken into 2-3 pieces) in 1995

Treatment	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Number of callus broken into pieces	Total number of somatic embryos and shoots formed	Average no. of somatic embryos and shoots formed per callus
MSD2	46	44	96	35	318	9.09
MSD2Cas.50*	59	56	95	30	274	9.13
MSD2Cas.100**	65	62	95	45	427	9.49

Note:

*;** : MSD2 media with the addition of 50 and 100 mg/l Casein hydrolysate, respectively.

Table 2.4 The number and percentage of embryogenic callus produced by *cv.* Frame in 1995

Treatment	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Number of callus producing somatic embryos and shoots			Total no. of somatic embryos and shoots formed	Average no. of somatic embryos and shoots formed per callus
				1-4	5-7	8 or more		
MSD0BA0	30	0	0	0	0	0	0	0.00
MSD1BA0	30	16	53	10	4	2	63	3.94
MSD2BA0	30	17	57	10	3	4	79	4.65
MSD3BA0	30	15	50	9	6	0	63	4.20
MSD5BA0	30	14	47	11	3	0	45	3.21
MSD0BA1	30	0	0	0	0	0	0	0.00
MSD1BA1	30	21	70	11	9	1	87	4.14
MSD2BA1	30	21	70	16	5	0	79	3.76
MSD3BA1	30	20	67	12	6	2	86	4.30
MSD5BA1	30	6	20	4	2	0	17	2.83
MSD0BA2	30	0	0	0	0	0	0	0.00
MSD1BA2	30	5	17	4	1	0	12	2.40
MSD2BA2	30	10	33	9	0	1	25	2.50
MSD3BA2	30	14	47	13	1	0	32	2.29
MSD5BA2	30	6	20	6	0	0	15	2.50
MSD0BA3	30	0	0	0	0	0	0	0.00
MSD1BA3	30	5	17	5	0	0	12	2.40
MSD2BA3	30	10	33	10	0	0	30	3.00
MSD3BA3	30	6	20	6	0	0	11	1.83
MSD5BA3	30	8	27	7	1	0	21	2.63
MSD0BA5	30	0	0	0	0	0	0	0.00
MSD1BA5	30	6	20	6	0	0	17	2.83
MSD2BA5	30	6	20	6	0	0	13	2.17
MSD3BA5	30	11	37	10	1	0	31	2.82
MSD5BA5	30	11	37	8	3	0	34	3.09
Additional treatment								
MSD2BA0.10	20	20	100	3	6	11	162	8.10
MSD2BA0.25	30	21	70	9	8	4	109	5.19
MSD2Cas.100*	30	24	80	19	5	0	82	3.42

Note :

* : MSD2 media with the addition of 100 mg/l Casein hydrolysate.

Table 2.5 The number and percentage of embryogenic callus produced by *cv.* Krichauff in 1995

Treatment	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Number of callus producing somatic embryos and shoots			Total no. of somatic embryos and shoots formed	Average no. of somatic embryos and shoots formed per callus
				1-4	5-7	8 or more		
MSD0BA0	30	0	0	0	0	0	0	0.00
MSD1BA0	42	39	93	22	11	6	178	4.56
MSD2BA0	54	54	100	27	17	10	265	4.91
MSD3BA0	30	22	73	17	5	0	79	3.59
MSD5BA0	30	20	67	19	1	0	43	2.15
MSD0BA1	30	0	0	0	0	0	0	0.00
MSD1BA1	30	10	33	10	0	0	25	2.50
MSD2BA1	30	6	20	6	0	0	14	2.33
MSD3BA1	30	3	10	3	0	0	5	1.67
MSD5BA1	30	0	0	0	0	0	0	0.00
MSD0BA2	30	0	0	0	0	0	0	0.00
MSD1BA2	30	15	50	14	1	0	31	2.07
MSD2BA2	30	2	7	2	0	0	4	2.00
MSD3BA2	30	1	3	1	0	0	2	2.00
MSD5BA2	30	0	0	0	0	0	0	0.00
MSD0BA3	30	0	0	0	0	0	0	0.00
MSD1BA3	30	5	17	4	1	0	17	3.40
MSD2BA3	30	2	7	2	0	0	3	1.50
MSD3BA3	30	5	17	5	0	0	8	1.60
MSD5BA3	30	2	0	0	0	0	0	0.00
MSD0BA5	30	0	0	0	0	0	0	0.00
MSD1BA5	30	0	0	0	0	0	0	0.00
MSD2BA5	30	0	0	0	0	0	0	0.00
MSD3BA5	30	0	0	0	0	0	0	0.00
MSD5BA5	30	0	0	0	0	0	0	0.00
Additional treatment								
MSD2BA0.10	20	3	15	3	0	0	3	1.00
MSD2BA0.25	20	3	15	2	1	0	11	3.67
MSD2Cas.100*	30	6	20	5	1	0	11	1.83

Note :

* : MSD2 media with the addition of 100 mg/l Casein hydrolysate.

Table 2.6 The number and percentage of embryogenic callus produced by *cv.* Janz in 1995

Treatment	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Number of callus producing somatic embryos and shoots			Total no. of somatic embryos and shoots formed	Average no. of somatic embryos and shoots formed per callus
				1-4	5-7	8 or more		
MSD0BA0	30	0	0	0	0	0	0	0.00
MSD1BA0	30	5	17	2	3	0	24	4.80
MSD2BA0	30	0	0	0	0	0	0	0.00
MSD3BA0	30	0	0	0	0	0	0	0.00
MSD5BA0	30	0	0	0	0	0	0	0.00
MSD0BA1	30	0	0	0	0	0	0	0.00
MSD1BA1	30	2	7	1	1	0	7	3.50
MSD2BA1	30	5	17	4	1	0	19	3.80
MSD3BA1	30	3	10	3	0	0	3	1.00
MSD5BA1	30	3	10	3	0	0	6	2.00
MSD0BA2	30	0	0	0	0	0	0	0.00
MSD1BA2	30	4	13	3	1	0	17	4.25
MSD2BA2	30	2	7	2	0	0	3	1.50
MSD3BA2	30	0	0	0	0	0	0	0.00
MSD5BA2	30	0	0	0	0	0	0	0.00
MSD0BA3	30	0	0	0	0	0	0	0.00
MSD1BA3	30	2	7	2	0	0	2	1.00
MSD2BA3	30	2	7	2	0	0	4	2.00
MSD3BA3	30	0	0	0	0	0	0	0.00
MSD5BA3	30	0	0	0	0	0	0	0.00
MSD0BA5	30	0	0	0	0	0	0	0.00
MSD1BA5	30	0	0	0	0	0	0	0.00
MSD2BA5	30	1	3	1	0	0	2	2.00
MSD3BA5	30	0	0	0	0	0	0	0.00
MSD5BA5	30	0	0	0	0	0	0	0.00
Additional treatment								
MSD2BA0.10	30	0	0	0	0	0	0	0.00
MSD2BA0.25	20	0	0	0	0	0	0	0.00
MSD2Cas.100*	20	0	0	0	0	0	0	0.00

Note:

* : MSD2 media with the addition of 100 mg/l Casein hydrolysate.

Several additional experiments were therefore undertaken to improve the culture response, through manipulation of medium composition. The addition of ethylene inhibitors, examples of which are silver nitrate and copper sulphate (Table 2.7), and the use of different sources of starting material (glasshouse-, growth chamber- or field-grown) were attempted. It was found that despite all these treatments, including the use of growth chambers to grow plant material, a low percentage of embryogenic callus production was still observed (Tables 2.7 and 2.8) when compared with results achieved in 1995 (Tables 2.2-2.5). As a result, this problem brought about a significant negative impact on the transformation experiments conducted between 1996-1997. However, the problems were overcome in 1999 (see Chapter 3).

In late 1998, major modifications in conditions used to grow donor plants were made in collaboration with Mr. Keith T. Gatford (Department of Plant Science, University of Adelaide). Four different aspects of growing donor plants were changed, namely the soil used, the watering regime, growth conditions and fertilization protocols (Table 2.9). Recycled soil used in earlier experiments (1995-1997) was changed to Horsham soil mix in late 1998-1999, because the recycled soil showed some variation in texture between batches. The recycled soil also became hard and compacted after 2-2.5 months, and this could clearly interfere with root growth and plant development.

Furthermore, the tap water initially used was found to contain high levels of salt, especially in summer time, and was replaced with reverse osmotic water at this time. In the case of the modified growing conditions, the seed/seedlings were germinated and grown at low temperatures (10 ^{o}C) with a short photoperiod (8/16 h). The temperature and photoperiod were increased gradually, and plant growth

Table 2.7 The number and percentage of embryogenic callus produced from various additional treatments (*cv.* Hartog only) in 1996

Treatment	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Number of somatic embryos and shoots produced/callus
MSD2	100	23	23	1-3
MSD2Cas.100	60	14	23	1-3
MSD2Cas.1000	70	17	24	1-3
MSD2Cu1	70	18	26	1-4
MSD2Cu10	100	24	24	1-4
MSD2AgNO3	30	7	23	1-4
MSD2Cef	30	3	10	1-2
MSD2Cu10AgNO3	30	5	17	1-3
MSD2Cu10Cef	30	10	33	1-3
MSD2AgNO3Cef.	30	4	13	1-3
MSDic2.5	35	6	17	1-2
MSD2Malt.	30	5	17	1-3
LMD2	70	17	24	1-3

Notes:

Cu : CuSO4 (1 or 10µM/l) Cef : Cefotaxime (60µg/l) Dic : Dicamba (2.5mg/l)

Malt : Maltose (6%) Cas : Casein Hydrolysate (100 or 1000 mg/l) AgNO3 : 50mg/l

LMD2 : Lazzeri media with the addition of 2 mg/l 2.4-D (see appendix 2).

Table 2.8 The number and percentage of embryogenic callus produced by *cvs.* Hartog, Frame and Krichauff grown under different conditions in 1997

Cultivar	Source of explants	Number of scutella cultured	Number of scutella producing embryogenic callus	Percentage of embryogenic callus production	Total of somatic embryos and shoots/callus	No. of somatic embryos and shoots/callus
Hartog	Glasshouse	80	53	66	193	3.64
	Growth chamber	50	30	60	88	2.93
	Field	50	30	60	98	3.27
Frame	Glasshouse	80	50	63	188	3.76
	Growth chamber	50	30	60	92	3.06
	Field	50	23	46	66	2.86
Krichauff	Glasshouse	80	48	60	143	2.98
	Growth chamber	50	28	56	84	3.00
	Field	50	25	50	75	3.00

Note :
Immature scutella were cultured on standard media for each genotype (e.g. MSD2BA0 for *cvs.* Krichauff and Hartog, and MSD2BA0.1 for *cv.* Frame).

Table 2.9 Modified growth conditions of donor plants

Modifications	Initially	New conditions
Soil	Recycled soil	Horsham soil mix (appendix 3)
Watering	Tap water	Reverse Osmotic (RO) water
Germination and growing plants	Directly germinated in soil and grown in glasshouse, growth chamber or field until plants are ready to be harvested	Germinated in trays containing Horsham soil mix for four weeks and placed in a growth chamber with the temperature and photoperiod of 10°C and 8/16 h, respectively. The seedlings were repotted in 8" pots containing Horsham soil mix and transferred to growth rooms with temperature and photoperiod of 10°C and 8/16 h, respectively. The temperature and photoperiod were increased 1°C and 1 h per week, respectively. Thus, at the 8th week, the temperature and photoperiod reached 18°C and 16/8 h, respectively. In the 9th week, the plants were transferred to the glasshouse until ready to be harvested. The temperature and photoperiod in the glasshouse were maintained at 20°C and 16/8 h, respectively
Additional fertilizer	Using soluble John Innes fertilizer every 10 days during maintenance in the glasshouse	Using Aquasol™ fertilizer (23:4:18 for N-P-K, respectively) (Hortico Pty Ltd, Australia). The fertilizer was diluted in water (1.5 g/l) and sprayed onto the leaves of plants every 14 days during maintenance in the growth rooms and the glasshouse

and development improved significantly. Last, application of JI fertilizer to soil was superceded by spraying Aquasol onto the leaves of plants. The modified fertilizer regime was intended to prevent nutrient accumulation to toxic levels in soil, especially when the soil became hard and compacted. Foliar application of fertilizer was expected to increase the availability of nutrient to the plants.

In addition to the modifications listed in Table 2.9, immature embryos (ten to 12 DAA or 1 to 1.5 mm in length) (Pellegrineschi *et al.*, 1999) were used instead of isolated immature scutella (ten to 14 DAA or 1 to 2 mm in length) (Nehra *et al.*, 1994). The results of cultured immature embryos observed from non-bombarded (control) material in 11 bombardment experiments are presented in Table 2.10. Although the response of explants (*cv.* Frame) in culture improved up to 65%, these results were still low compared with the results obtained in early experiments (Table 2.4).

2.3.3 Organogenesis and plant regeneration

Somatic embryos produced from scutella-derived callus (Figure 2.1C) were allowed to germinate and to form shoots by transferring them individually to MSD0BA0 medium (Figures 2.1D-E). The shoots developed into plantlets within two to three weeks (Figure 2.1F), although not every somatic embryo could be induced to form a shoot or a plantlet. A variation in capacity to produce fully developed plantlets was noted among the genotypes tested. Wheat *cvs.* Hartog, Frame and Krichauff produced 4-17, 5-14 and 3-10 plantlets, respectively, from callus derived from a single cultured scutellum (data not shown). In these experiments, no albino shoots or albino plantlets were recovered from the genotypes tested. Once fully developed plantlets of around 3 cm in length were

Table 2.10 The number and percentage of embryogenic callus produced by *cv.* Frame grown under modified growth conditions (1999)

Number of immature embryos cultured	Number of cultured immature embryos producing embryogenic callus	Percentage of embryogenic callus production	Number of somatic embryos produced per callus
10	5	50	2-3
10	6	60	2-4
12	7	58	2-4
12	5	42	1-3
20	12	60	1-4
20	13	65	2-4
20	12	60	2-3
20	13	65	2-4
20	11	55	1-4
20	13	65	2-4
10	5	50	1-3
174	102	59	1-4

formed, these were transferred to soil and were grown under glasshouse conditions (Figure 2.1G).

2.3.4 Appearance of regenerated plants

In this study, 28 plantlets belonging to the three genotypes (*cvs.* Hartog, Frame and Krichauff) were transferred to soil. Eight of the 28 plants transferred to soil did not survive; the non-surviving plants were all of *cv.* Hartog.

Although the tissue culture-derived plants were healthy, morphologically normal and fertile (Figures 2.1H-I), they were relatively weak and showed low fertility compared with control plants. On average, every tissue culture-derived plant produced approx. 250 grains, whereas the control plants produced 300-375 grains. A few sterile heads were observed on the tissue culture-derived plants of all genotypes, but none were observed on the control plants. Table 2.11 shows results of the phenotypic assessment and fertility of the tissue culture-derived and the control plants.

Table 2.11 Phenotypic characteristics and fertility of tissue culture-derived and control plants

Cultivar	Source of explant	Plant grown/ regenerated	Plants survived to maturity	Average				
				Length of heads (cm)	No. of heads formed/ plant	No. of empty heads/ plant	No. of grains/ plant	Weight of grain (mg/grain)
Hartog	Grain (control)	2	2	7	10	0.00	352	37
	Tissue culture	22	14	5	13	0.21	252	30
Frame	Grain (control)	2	2	6	15	0.00	384	39
	Tissue culture	3	3	6	12	0.33	258	36
Krichauff	Grain (control)	2	2	5	15	0.00	294	40
	Tissue culture	3	3	5	10	1.33	250	38

2.4 Discussion

Four Australian wheat genotypes have been screened for their capacity to produce embryogenic callus from which viable plants could be recovered. The technique employed in these experiments involved culturing ten to 14 day old immature scutella from the four genotypes (*cvs.* Hartog, Frame, Krichauff and Janz) on a MS basal medium supplemented with two types of growth regulators, at a range of concentrations, both alone and in combination. The cultured scutella were subsequently induced by dark treatment to form callus. Callus formed during induction was later exposed to light for somatic embryo production.

The experiments demonstrated that there were differences among the wheat genotypes in the production of callus and in their ability to form somatic embryos (Tables 2.2-2.6). Cultured scutella of *cvs.* Hartog, Frame and Krichauff grew well in culture and produced embryogenic callus, while those from *cv.* Janz were poor in this regard. On average, each callus from *cvs.* Hartog, Frame and Krichauff produced 6, 8 and 5 somatic embryos, respectively. Additionally, using callus from *cv.* Hartog, the number of somatic embryos produced per explant could be increased if the original callus was broken into two to three pieces prior to exposure to light (Table 2.3). When an attempt was made to regenerate the embryogenic callus, it was noted that each callus from *cv.* Hartog, Frame and Krichauff could produce 4-17, 5-14 and 3-10 plantlets, respectively (data not shown).

The results also demonstrated that each wheat genotype had a specific growth regulator requirement (Tables 2.2-2.6). The optimum 2,4-D concentration for callus and embryogenic callus production was 2 mg/l for most genotypes examined. It was noted that the presence of 2,4-D at higher concentrations (3

and 5 mg/l) caused more vigorous growth of callus, but the production of embryogenic callus was reduced. Conversely, the presence of a lower level of 2,4-D (1 mg/l) frequently brought about direct shoot and/or root differentiation. The present study indicates that 2,4-D can stimulate callus formation and that the concentration of 2,4-D added to induction medium is critical (Tables 2.2-2.5), consistent with the widespread use of 2,4-D for the induction of embryogenic wheat callus (Ozias-Akins and Vasil, 1982; Sears and Deckard, 1982; Larkin *et al.*, 1984; Wernicke *et al.*, 1986 and Ben Amer and Börner, 1997).

Unlike 2,4-D, the presence of the cytokinin BA in the induction medium at any of the concentrations tested resulted in a reduction in callus formation and somatic embryo production for *cvs.* Krichauff and Janz (Tables 2.5 and 2.6). The reduction in callus or somatic embryo production appeared to be proportional to BA concentration.

In contrast, the presence of BA in the induction medium at a low concentration was beneficial for both callus and embryogenic callus production for *cv.* Frame. Induction medium containing low levels of BA combined with 1-2 mg/l 2,4-D resulted in higher embryogenic callus production than medium containing only 2,4-D. The best result was found with the medium consisting of 0.1 mg/l BA with the addition of 2 mg/l 2,4-D, where all cultured Frame scutella produced embryogenic callus (Table 2.4). Success in initiating embryogenic callus production has also been reported in wheat (Papenfus and Carman, 1987), barley (Rengel and Jelaska, 1986) and rice (Fatokun and Yamada, 1984) by using induction medium containing a combination of 2,4-D (2-2.5 mg/l) and low levels of cytokinins (kinetin or BA at 0.05-0.5 mg/l). The presence of cytokinins at high

levels (>2.5-10 mg/l BA) in induction medium tended to reduce the formation of regenerable callus (Bhaskaran and Smith, 1988; 1989).

These experiments clearly demonstrated a positive response of *cv.* Frame to BA (cytokinins) and a negative response for *cvs.* Hartog, Krichauff and Janz. Previous work has provided examples of positive (Bhojwani and Hayward, 1977; Papenfuss and Carman, 1987; Lazar *et al.*, 1983) and negative (Dudits *et al.*, 1975) responses of cultured explants to cytokinins. It is clear that differences in the responses of cultured explants result from genotypic effects, as reported by Ahloowalia (1982) and Carman *et al.* (1987). These authors speculated that tissue culture response was influenced by the status of endogenous phytohormones in the cultured explants. Ahloowalia (1982) cited several previous research groups that had reported differing levels of cytokinins and auxin in grain of different wheat cultivars (Gale, 1979; Mounla, 1979). Thus, differences in endogenous phytohormone content of the grain of wheat genotypes used in these experiments might have resulted in the observed differences in culture response.

An additional treatment was also included to test the effect of casein hydrolysate at concentrations of 50 mg/l or 100 mg/l in induction medium containing 2 mg/l 2,4-D (Altpeter *et al.*, 1996). The addition of casein hydrolysate in induction medium had no effect on embryogenic callus production when it was compared with induction medium containing only 2 mg/l 2,4-D (Tables 2.3-2.6).

During this study, 20 tissue culture-derived plants from three genotypes (*cvs.* Hartog, Frame and Krichauff) were successfully grown to maturity in the glasshouse. Under the regeneration system used, mature, fertile plants were obtained between 22 and 25 weeks after commencement of culture, depending on plant genotype and season. Mortality of regenerated plants on transfer to soil

was around 30%. Particular care was necessary during transplanting and maintenance of the young, growing plants, especially in the first two weeks after transplanting.

It was observed that tissue culture-derived plants generally showed retarded growth and low yield (Table 2.11). The latter was clearly affected by properties such as a short head, reduced numbers of grains, and shriveled grains, compared with control plants. Poor phenotypic characteristics and reduced fertility were reported previously for wheat plants recovered from tissue-cultured material by Ahloowalia (1982), Larkin *et al.* (1984), Ahloowalia and Sherington (1985) and Qureshi *et al.* (1992). It is believed that the reduction of vegetative and generative growth of regenerated plants results from *in vitro* culture effects (Vasil, 1988). To eliminate the negative effects of *in vitro* culture on tissue culture-derived plants, it is important to apply as short a tissue culture phase as possible and/or to undertake crossing between seed-grown plants and the tissue culture-derived plants to exclude any mutations or somaclonal variation.

During the current experiments, no albino shoots, plantlets or plants were regenerated from the genotypes tested. Previous work on wheat tissue culture has shown that the occurrence of albinos is influenced by genotype (Maddock *et al.*, 1983; Machii *et al.*, 1998), by the source of explants (Schaeffer *et al.*, 1979) and by tissue culture conditions (Ziegler *et al.*, 1990; Zhou *et al.*, 1991; Fellers *et al.*, 1995). It was noticed here that the response of wheat explants in culture varied considerably from one season to another in one particular year, or between the same season in different years. This was demonstrated by the high regenerability of explants in culture during the 1995 experiments (Tables 2.2-2.6), and the dramatic decrease in regenerability in later experiments (Tables 2.7 and

2.8). These results were similarly observed in barley by Lührs and Lörz (1987), where the response of explants in culture varied considerably between years. It can therefore be concluded that it is likely, in addition to genotype and growth regulator requirements, that aspects of plant growth conditions, in particular the physiological conditions of donor plants, is crucial factor in determining the behaviour of explants in tissue culture. Studies by Jones and Petolino (1987) and Hess and Carman (1998) have also highlighted the importance of optimizing donor plant conditions for maximizing *in vitro* culture response.

Having achieved high levels of embryogenesis in 1995, considerable efforts were made to reproduce this tissue culture response in later years. These efforts included the manipulation of media composition and the use of different sources of explants (Tables 2.7 and 2.8). However, these efforts had only a minor impact on tissue culture response. In further attempts, several additional modifications were therefore devised, and mainly related to the growth conditions of donor plants. These modifications did lead to a considerable improvement in culture response (Table 2.10), but culture response was still relatively lower than that achieved in early experiments (Table 2.4). Based on these observations, it appears that further investigation is needed to fully elucidate the effects of donor plant condition on tissue culture response.

The cumulative results of these experiments show conclusively that three of the four Australian wheat genotypes screened (*cvs.* Hartog, Frame and Krichauff) grew well in tissue culture, and that fertile plants could be recovered. Under appropriate tissue culture conditions, most cultured scutella from the three genotypes produced embryogenic callus, which could be induced to regenerate fertile plants. It is believed that the regeneration system developed in this study

could be adapted for other wheat genotypes and for wheat transformation. The application of the tissue culture and regeneration protocols in the transformation of wheat is described in the next Chapter.

CHAPTER THREE

TRANSFORMATION OF WHEAT

3.1 Introduction

The first successful effort to transform wheat plants was reported by Vasil *et al.* (1992). In this work, transgenic wheat plants carrying a herbicide resistance gene were produced *via* microprojectile bombardment-mediated transformation. This achievement has encouraged further attempts to generate transgenic wheat having other desired characters. Transgenic wheat plants carrying various agronomically-desirable genes, such as those for resistance to virus (Karunaratne *et al.*, 1996) and disease attack (Leckband and Lörz, 1998; Bliffeld *et al.*, 1999), for quality improvement (Shewry *et al.*, 1995; Blechl and Anderson, 1996; Barro *et al.*, 1997; Chibbar *et al.*, 1998), and those for male sterility (De Block *et al.*, 1997), have now been produced.

However, wheat transformation protocols have generally suffered from low efficiencies and a variety of DNA delivery systems have therefore been developed. So far, three transformation methods have been successfully used in wheat transformation. One is the use of direct gene transfer into protoplasts (He *et al.*, 1994). However, this technique is technically demanding and, owing to the long periods of tissue culture required, many of the resulting plants are found to lack vigour and fertility. Another method is the use of *Agrobacterium tumefaciens* (Cheng *et al.*, 1997). This method offers a number of advantages over other methods, but has not found wide use in wheat. Until recently, the most common transformation method used in wheat transformation has been microprojectile bombardment (Lörz *et al.*, 1998). The preference for microprojectile bombardment is presumably attributable to its relative ease of use, its high success rate, and because it can be used to deliver transgenes directly into a range of primary tissues. Therefore, in addressing the aim of transforming wheat

in the present project it was decided to use microprojectile bombardment as the method of choice.

Next, it was necessary to find suitable selectable marker and reporter genes for the transformation experiments. The most widely applied selectable marker gene in wheat transformation *via* microprojectile bombardment is the *bar* gene, which confers resistance to herbicides such as Bialaphos and Basta® (Witrzens *et al.*, 1998). In almost all transformation experiments, the *bar* gene has been co-introduced with reporter genes such as the *GUS* gene or other genes of interest (Becker *et al.*, 1994; Zhang *et al.*, 2000). In many cases, the introduction of two genes that reside either in the same or in different plasmids results in co-integration. Since the introduction of transgenes *via* microprojectile bombardment is an apparently random process (Sanford *et al.*, 1993; Pawlowski and Somers, 1996), the introduced transgenes are usually integrated as single or multiple copies in the plant genome (Blechl and Anderson, 1996; Kohli *et al.*, 1999).

In the current project, results presented in Chapter 2 demonstrated that three out of four elite Australian wheat genotypes screened grew well in culture and that fertile plants could be regenerated. Regeneration systems outlined in Chapter 2, in combination with the microprojectile bombardment method, were used for the successful transformation of an elite Australian wheat genotype (*cv.* Frame) with the *bar* and the *GUS* genes. The results of these experiments are presented in this Chapter.

3.2 Materials and Methods

3.2.1 Plant material

Three wheat cultivars (Frame, Krichauff and Hartog) were used in transformation experiments. These three wheat varieties for the most part, were grown in the glasshouse. Some material was also grown in growth chambers and some was grown in the field. The growth and maintenance conditions for the donor plants were essentially as described in Chapter 2 (Sections 2.2.1 and 2.3.2).

3.2.2 Isolation and culture of explant tissue

Grain sterilisation and scutellum culture conditions were as described in Section 2.2.3. Specifically, scutella isolated from immature embryos were cultured in 90 mm plastic Petri dishes containing the appropriate induction medium for each wheat genotype. The cultured scutella were subsequently incubated in the dark at 24-26°C for two to three days prior to bombardment (Nehra *et al.*, 1994).

Towards the end of this study, immature embryos instead of isolated scutella of *cv.* Frame were used for transformation experiments. These embryos were harvested from the plants grown under conditions as described in Chapter 2, Section 2.3.2. The immature embryos were isolated from surface sterilized grains and immediately cultured onto osmotic medium for bombardment (Pellegrineschi *et al.*, 1999).

3.2.3 Gene constructs

Plasmids pDM302 (Cao *et al.*, 1992), p*Act* 1-D *GUS* (McElroy *et al.*, 1990) and pAHC25 (Christensen and Quail, 1996) were used in these experiments

(Figure 3.1). Plasmids pDM302 and p*Act*1-D *GUS* were provided by Professor R. Wu (Cornell University, Ithaca, NY, USA) and pAHC25 was kindly donated by Dr. P.H. Quail (Plant Gene Expression Centre, Albany, CA, USA). Plasmids pDM302 and p*Act*1-D *GUS* consist of the *bar* and the *GUS* genes respectively, each under the control of the rice *Actin1* promoter and its first intron. Plasmid pAHC25 contains both the *bar* and the *GUS* genes, both under the control of the maize ubiquitin *ubi1* promoter and its first intron. The *bar* and the *GUS* genes encode phosphinothricin acetyl transferase (PAT) and β-glucuronidase (GUS), respectively.

Plasmid DNA of the three vectors was prepared using the following procedure. An *E. coli* stock maintained in glycerol at -80°C was transferred, using a sterile inoculating loop, into 5 ml LB medium (1 l of LB medium consists of 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 5 g NaCl, pH 7.5) containing 50 mg/l ampicillin. The culture was incubated overnight at 37°C with continuous shaking. Two 1.5 ml aliquots of the overnight culture were transferred to Eppendorf tubes and centrifuged at 13200 rpm for 30 sec at 4°C. The supernatant was removed and the tubes were placed upside down briefly to drain the pellets. The pellets were resuspended in 100 µl GTE solution (25 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM EDTA). After 10 min at 4°C, 200 µl denaturation solution {0.2 M NaOH, 1% (w/v) sodium dodecyl sulphate} was added and the solution was gently mixed. The tubes were incubated on ice for 5 min before 150 µl 3 M potassium acetate, pH 4.8 was added to precipitate chromosomal DNA. The tubes were held at -20°C for 15 min and centrifuged at 13200 rpm for 15 min. The supernatant was transferred to a fresh Eppendorf tube and 2 volumes absolute ethanol was added to precipitate plasmid DNA. The

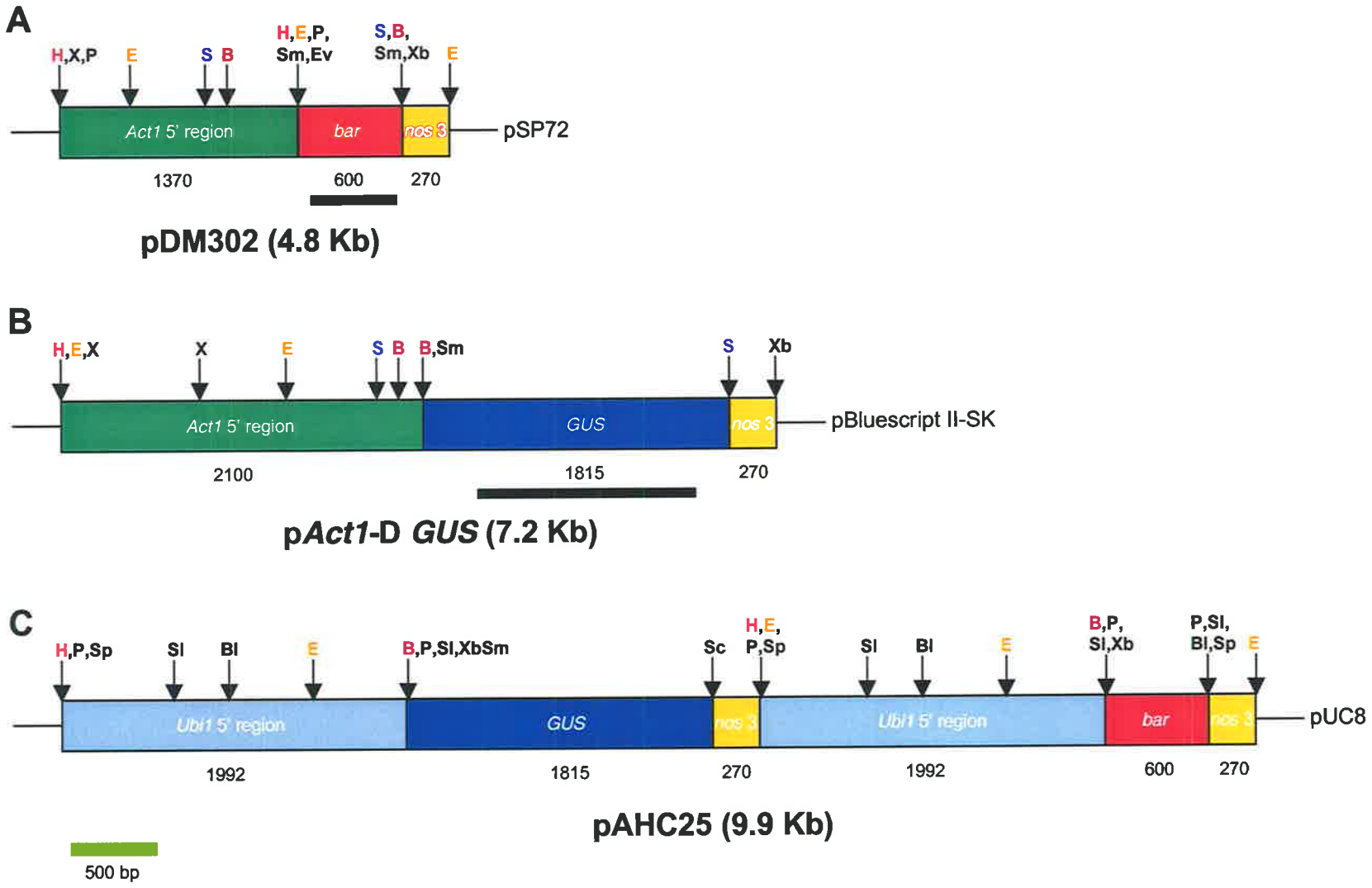

A
H,X,P
E
S B
H,E,P,
Sm,Ev
S,B,
Sm,Xb
E
Act1 5' region
bar
nos 3
pSP72
1370
600
270
pDM302 (4.8 Kb)
B
H,E,X
X
E
S B
B,Sm
S
Xb
Act1 5' region
GUS
nos 3
pBluescript II-SK
2100
1815
270
pAct1-D GUS (7.2 Kb)
C
H,P,Sp
SI
BI
E
B,P,SI,XbSm
Sc
H,E,
P,Sp
SI
BI
E
B,P,
SI,Xb
P,SI,
BI,Sp
E
Ubi1 5' region
GUS
nos 3
Ubi1 5' region
bar
nos 3
pUC8
1992
1815
270
1992
600
270
pAHC25 (9.9 Kb)
500 bp

Figure 3.1 Schematic representation of constructs used in transformation experiments. (A) pDM302; (B) p*Act*1-D *GUS*, and (C) pAHC25. Abbreviations: *Act*1: promoter, first exon and first intron of the rice *actin* gene; *bar* : coding region of *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene; *nos*: transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene; *Ubi*1: promoter, first exon and first intron of the maize *ubiquitin1* gene; and *GUS*; coding region of *E. coli* β-glucuronidase gene. Abbreviations of restriction sites: B: *Bam*HI; Bl; *Bgl*II; E: *Eco*RI; Ev: *Eco*RV; H: *Hin*dIII; P: *Pst*I; S: *Sst*I; Sc: *Sac*I; Sl; *Sal*I; Sm: *Sma*I; Sp; *Sph*I; X: *Xho*I and Xb: *Xba*I. The DNA fragments used as probes in DNA blot hybridization are indicated by black lines under the coding regions of the pDM302 and p*Act*1-D *GUS* constructs.

tubes were mixed vigorously by hand, stored at -20°C for 10 min and centrifuged at 4°C at 13200 rpm for 15 min. The supernatant was discarded and the plasmid DNA pellets were washed with 1 ml 70% (v/v) ethanol. The tubes were recentrifuged at 4°C at 13200 rpm for 5 min. The supernatant was removed and the pellets were dried under vacuum for 5 min. Plasmid DNA was dissolved in 50 µl R40 buffer (10 mM Tris-HCl buffer, pH 7.5, containing 10 mg/ml pancreatic RNaseA and 15 mM NaCl) and adjusted to a concentration of 1 mg/ml. The DNA was stored at -20°C until needed for bombardment.

3.2.4 Preparation of gold particles

The method used for preparation of gold particles followed the standard procedure outlined for the Biolistic® PDS-1000/He Particle Delivery System (BioRad Laboratories, Hercules, CA, USA). Gold particles (1 µm diameter, 25 mg) were weighed into a 1.5 ml Eppendorf tube and washed with 1 ml absolute ethanol. The tube was vortexed for 5 min, left for 10 min to settle, and centrifuged at 13200 rpm for 1 min. The ethanol was removed and the washing procedure was repeated twice. The gold particles were subsequently washed 3 times with 1 ml sterile water using the same procedure, and finally resuspended in 1 ml sterile water. Aliquots of 50 µl, containing approximately 1250 µg gold particles, were placed into clean 1.5 ml Eppendorf tubes.

3.2.5 DNA coating methods

DNA coating methods were as described in the Biolistic® PDS-1000/He Particle Delivery System User's Guide. To an Eppendorf tube containing 50 µl vortexed gold suspension, 3 µl each plasmid pDM302 and p*Act*1-D *GUS*, or 5 µl

plasmid pAHC25 were added. Gold particles were coated with the plasmid DNA by adding, in the following order, 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. The tubes were vortexed continuously for 3 min, left on ice for 10 min and centrifuged at 1000 rpm for 10 sec. The supernatant was removed and the DNA-coated gold pellet was washed with 250 µl absolute ethanol. The pellet was resuspended in 50 µl absolute ethanol. For each bombardment, 10 µl the DNA-gold suspension was placed in the centre of a macrocarrier, that is, approx. 250 µg gold particles per bombardment. In some bombardment experiments, the gold pellet was suspended in 250 µl absolute ethanol and 10 µl or 3.7 µl of the DNA-gold suspension was placed onto a macrocarrier, that is, approx. 50 µg or 18.5 µg gold particle per bombardment. For optimisation of bombardment conditions, 5 µl pAct 1-D *GUS* (1 µg/µl) was used and coated onto gold particles as described above.

3.2.6 Bombardment conditions

During these experiments, all materials were bombarded using the Biolistic® PDS-1000/He Particle Delivery System (Figure 1.1), as described in Section 3.2.2. Twenty immature scutella, when the immature scutella were shot using 250 and 50 µg gold particles per bombardment, or 10 immature scutella, when using 18.5 µg gold particles per bombardment, were placed in the centre of a 55x14 mm plastic Petri dish containing callus induction medium (MSD2 or MSD2BA0.1) supplemented with 0.4 M mannitol. Osmotic treatment was employed 4 h pre- and 16 h post-bombardment, as described by other groups (Zhou *et al.*, 1995; Altpeter *et al.*, 1996).

The immature scutella were bombarded under a vacuum of 28 inch Hg. The distance between the rupture disk and the macrocarrier was 2.5 cm and the macrocarrier flight distance was 1.1 cm. The distance between the stopping screen and the tissues was 6 cm or 9 cm, and the rupture disk pressure was 900-1350 psi. Immediately after osmotic treatment, the bombarded scutella were transferred to 90 mm plastic Petri dishes containing fresh induction medium and callus formation was induced for 20-25 days in the dark.

In all experiments conducted at the end of this study, freshly-isolated immature embryos were directly cultured onto osmotic medium and subsequently bombarded. Osmotic treatment and the handling of material after bombardment followed procedures described above.

For the optimisation of bombardment conditions, immature scutella pre-cultured for two days were used. All scutella were pre-treated with the mannitol osmotic media and bombarded at distances of 6, 9 and 12 cm, with pressures of 650, 900, 1100, 1350 or 1550 psi. Each bombardment was repeated twice. Thus, two replicates with a total of 40 scutella were bombarded using 250 µg and 50 µg gold particles per bombardment, and 20 scutella were bombarded using 18.5 µg gold particles per bombardment.

3.2.7 Somatic embryo induction and selection of transformants

Callus formed during induction was transferred to direct light for somatic embryo production, as described in Chapter 2, Section 2.2.4. Somatic embryos or shoot primordia produced during exposure to light were picked and transferred to the selection medium supplemented with 3 mg/l Bialaphos (Meiji Seika Kaisha, Japan). The cultures were kept under direct light for ten to 14 days, and shoots

growing on the selection medium were transferred to fresh selection medium for another ten to 14 days. Fast growing shoots were picked and transferred to the same medium without Bialaphos for further growth. Plantlets of around 5 cm in length were transferred to soil and grown to maturity in the glasshouse, as described in Section 2.2.4.

Towards the end of this study, all somatic embryos produced during exposure to light were subjected to a selection medium containing 5 mg/l PPT or 5 mg/l Bialaphos, instead of 3 mg/l Bialaphos.

3.2.8 Histochemical GUS staining

GUS activity was assayed histochemically using the following buffer: 0.1 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM ethylenediaminetetraacetic acid, disodium salt (EDTA) and 0.1% (v/v) Triton X-100. In this buffer, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc, Nalgene) was dissolved. The mixture was filter-sterilized and stored at –20°C.

For the transient expression studies, scutella induced for three days were bombarded with the p*Act*1-D *GUS* construct. Forty-eight hours after bombardment, the scutella were incubated in the X-gluc solution at 37°C for 24 h. GUS expression appeared as blue spots on the bombarded scutella. These blue spots were counted under a dissecting microscope. Each blue spot, including blue sectors that might have resulted from the fusion of several fine spots, was counted as one expression event. The results were expressed as the average number of blue spots observed on each scutellum.

GUS expression in other tissues such as roots, spikelets, anthers, ovaries and grains from putative transformants was studied in the same way as described for scutella. Leaf tissues, however, were incubated in the X-gluc solution for an extended period, which ranged from two to four days. In most cases it was essential to bleach the leaves before any blue colour could be observed. Bleaching was accomplished by soaking leaf pieces in 70% (v/v) ethanol for 48 h. The ethanol effectively extracted the chlorophyll in that period.

3.2.9 Phosphinothricin acetyl transferase (PAT) assay

The PAT assay follows procedures described by Spencer *et al.* (1990). One hundred to 150 mg fresh leaf sample from putative transformants and control plants were ground in 1.5 ml Eppendorf tubes with 100-150 µl extraction buffer (50 mM Tris-HCl buffer, pH 7.5, containing 2 mM Na_2-EDTA, 0.15 mg/ml leupeptin, 0.15 mg/ml phenylmethylsulphonyl fluoride, 0.3 mg/ml bovine serum albumin, and 0.3 mg/ml dithiothreitol). The tubes were centrifuged at 13200 rpm for 10 min at 4°C. The supernatants were transferred to clean 1.5 ml Eppendorf tubes and the tubes were recentrifuged under the same conditions. The leaf extract was transferred to fresh 1.5 ml tubes and the total protein content of the extract was measured in a spectrophotometer (Hitachi, Model U-1100) by the Coomassie Brilliant Blue reagent using bovine serum albumin as a standard (Bradford, 1976).

To each 1.5 ml eppendorf tube, 15 µl extraction buffer, 3 µl [^{14}C]acetyl-CoA (0.010 mCi/mmol, NEN^{TM}, Boston, MA, USA), 2 µl PPT (1 mM stock in 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM Na_2-EDTA) and an appropriate amount of the leaf extract (25 µg total protein in 5 µl extraction buffer) were added. The tubes containing the reaction mixture were incubated at 37 °C for 30 min and

dried in a vacuum drier for 20 min. The products of the reaction were resuspended in 3 µl extraction buffer and spotted onto a silica gel thin-layer chromatography plate (Silica gel 60, Merck, Germany). Ascending chromatography was carried out in a 1:1 mixture of n-propanol and NH_4OH. The chromatography plate was exposed to X-ray film (RX Fuji Medical X-Ray Film; RX-U, Japan) for three days and the film was developed in an X-ray developer (Curix 60 processor, type 9462/100/140) to reveal ^{14}C-acetylated PPT.

3.2.10 Herbicide leaf-dip test

Herbicide was applied by dipping leaf tips, around 5-7 cm long, of putative transformants and a control plant in Basta® (Hoechst Australia Limited) in which the active ingredient glufosinate ammonium was 50% (w/v). The concentrations of Basta® tested were 0.01% and 0.1% (or 0.005% and 0.05% glufosinate ammonium, respectively), and solutions were prepared in the presence of 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) to facilitate "wetting" of the leaves. A putative transformant recovered from the third successful experiment described in this thesis was subjected to herbicide application by spraying the whole plant with a 0.1% Basta® solution (or 0.05% glufosinate ammonium) containing 0.1% Tween 20.

3.2.11 Genomic DNA isolation

3.2.11.1 Small scale isolation of genomic DNA

The method of DNA extraction from wheat leaf tissues was derived from that described by Langridge *et al.* (1997). A 10 cm long piece of fresh leaf of approx. 100-150 mg was ground to a fine powder in a liquid nitrogen-chilled

mortar. The leaf powder was transferred to a 2 ml Eppendorf tube, and 600 µl DNA extraction buffer (100 mM Tris-HCl buffer, pH 8.5, containing 1% sarcosyl, 100 mM NaCl and 10 mM EDTA) was added and the sample homogenised using a stainless steel homogeniser. Phenol/chloroform (1:1, 600 µl) was added and the tube was vigorously shaken for 30 sec and placed in a rack for partial phase separation. The tube was centrifuged for 10 min at 13000 rpm to separate the phases. The upper aqueous phase was carefully removed with a pipette and transferred to a fresh tube. The phenol/chloroform extraction was repeated. The upper phase was mixed with 60 µl 3 M Na-acetate, pH 4.8, and 600 µl isopropanol. The tube was mixed gently by inversion and placed in a rack to precipitate the genomic DNA. The tube was centrifuged for 5 min at 13000 rpm, the supernatant was removed and the DNA pellet was washed with 1 ml 70% (v/v) ethanol. The supernatant was removed entirely and the DNA pellet recovered was allowed to air dry at 37°C for 30 min. The DNA pellet was resuspended overnight in 50 µl R40 buffer at 4°C.

3.2.11.2 Digestion of genomic DNA

Digestion of DNA was carried out by adding successively to a 1.5 ml Eppendorf tube: 1 µl the appropriate buffer (10x), 1 µl 1 mg/ml BSA (10x), 1 µl 40 mM spermidine, 1 µl the appropriate restriction enzyme (20,000 units/µl) and 6 µl DNA sample. All restriction enzymes and their buffers were supplied by New England Biolabs. The reaction mixtures were briefly vortexed and centrifuged for 5 sec at 13200 rpm, followed by incubation at 37°C for 5 hrs. After incubation, 2 µl 6X Ficoll dye (0.1 M Tris-HCl buffer, pH 8.0, containing 0.2 M EDTA, 30% Ficoll

type 4000, 0.25% bromophenol blue and 0.25% xylene cyanol FF) was added to the digested DNA samples.

3.2.11.3 Electrophoresis of DNA

Gel electrophoresis of digested DNA was performed in a 1% agarose gel using 1x TAE buffer (2 M Tris-acetate buffer, pH 8.0, containing 0.05 M EDTA). Digested DNA samples (12 µl) were loaded into each lane of the gel. A marker (λ *Hind*III; GIBCO-BRL®) was loaded in the end lane. Electrophoresis was performed at 35V and 31 mA for 16 h. After electrophoresis, the gel was stained with 1µg/ml ethidium bromide and DNA bands were examined on a UV transilluminator. Photographs were taken using a Watec (Hoya Skylight (1B), Japan) camera.

3.2.12 Southern blot hybridization

Southern blot hybridizations were performed as described by Southern (1975).

3.2.12.1 Membrane preparation

After staining with ethidium bromide as described in Section 3.2.11.3 above, the gel was soaked in 200 ml denaturing solution (1.5 M NaCl, 0.5 M NaOH) and agitated for 20 min. The gel was washed with 10x SSC solution for 2 min (1.5 M NaCl, 0.15 M trisodium citrate).

The Southern transfer apparatus was assembled as follows. A sponge, together with 2 sheets of Whatman 3MM filter paper, were soaked in 10x SSC solution. The sponge was placed on a plastic tray, followed by a sheet of

Whatman filter paper. A plastic screen covered the edges of the Whatman paper. The gel was placed upside down in the middle of the screen, and care was taken to avoid trapping air bubbles under the gel. The membrane (Hybond^{TM}-N^{+}, Amersham) was rinsed in 10x SSC solution, placed on top of the gel, and any air bubbles were removed. The membrane was covered with another sheet of Whatman paper, followed by a 5-6 cm thick stack of dry paper towels, a glass plate and a weight of approximately 0.25 kg on the very top. The tray below was filled with 10x SSC solution and transfer of DNA to the membrane was effected overnight.

After DNA transfer, the loading well positions and the side of the membrane closest to the DNA in the gel were carefully labelled. The membrane was rinsed in 5x SSC solution for 2 min and blotted dry on Whatman filter paper. The membrane, with the DNA side up, was placed on a stack of Whatman filter paper which had been soaked in 0.4 M NaOH for 20 min. The membrane was neutralised in 100 ml neutralising solution (0.5 M Tris-HCl buffer, pH 7.2, containing 1.5 M NaCl and 0.001 M EDTA) for 5 min, followed by washing in 2x SSC solution for 5 min. The membrane was blotted dry with a piece of Whatman filter paper, sealed in a plastic bag, wrapped in aluminium foil and stored at 4°C.

3.2.12.2 Preparation of radiolabelled DNA probes

Probe DNA fragment preparation

The primers were chosen to provide a probe of sufficient size for efficient labelling by random priming. Primers had a balanced G+C and A+T content with no obvious internal homologies. Probing sequence of DNA was prepared by the polymerase chain reaction (PCR), using approx. 100 ng DNA template, 5 µl 10x

PCR buffer (GIBCO-BRL®), 3 µl 25 mM $MgCl_2$, 2 µl 5 mM dNTPs mix, 5 µl dimethyl sulfoxide (for the *bar* gene only), 1 µl Taq polymerase (1.5 U/µl) (GIBCO-BRL®) and 1 µl of each primer, in a final volume of 50 µl. Primers used were:

a) *bar* gene:

Forward (5'-CTACCATGAGCCCAGAACGACG-3')

Reverse (5'-GAAGTCCAGCTGCCAGAAAC-3').

b) *GUS* gene:

Forward (5'-GGTGGGAAAGCGCGTTACAAG-3')

Reverse (5'-GTGATGATAATCGGCTGATGC-3').

The cycling parameters used were 94°C for 30 sec (denaturation), 52°C for 30 sec (annealing), 72°C for 2 min (extension). This was repeated for 35 cycles. A sample of the PCR reaction products was separated by electrophoresis on a 1% agarose gel. DNA bands were detected with ethidium bromide and the gel was photographed. The PCR product was cut out of the gel and purified using the BRESAclean™ procedure according to the manufacturer's instructions (Bresatec Ltd, PO Box 11, Adelaide, SA). This DNA was radiolabelled and used as a probe.

Radiolabelling of DNA probes

Radiolabelled probes were prepared using the Megaprime (Amersham, UK) Kit following the manufacturer's instructions. To an eppendorf tube, 3 µl probe DNA fragment (*bar* or *GUS*), 5 µl random hexamer primers, and 25 µl water was added. The tube containing this solution was heated to 100°C for 5 min and centrifuged for 5 sec. Ten µl labelling buffer, 2 µl Klenow DNA polymerase (1-2 units) and 5 µl α^{-32}P-dCTP (50 µCi) were added to the tube. The probe mixture was incubated at 37°C for 30 min. Subsequently, 50 µl fractionation dye {1X TE

buffer, pH 8.0, containing 1.5% (w/v) dextran blue and 0.5% (w/v) orange G} was added to the incubated probe and a Sephadex G-50 mini-column was used to separate the unincorporated radionucleotides from the labelled DNA fragments. The labelled DNA was heated to 100°C for 5 min and chilled on ice for 5 min before use.

3.2.12.3 Hybridization procedures

The membrane was prehybridized in a rotating, cylindrical glass bottle containing 10 ml hybridization solution consisting of 500 µl H_2O, 3 ml 5X HSB (0.1 M piperazine-N,N'-bis-2-ethanesulfonic acid [PIPES] buffer, pH 6.8, containing 3 M NaCl and 0.025 M Na_2EDTA), 3 ml Denhardt's III {2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinyl pyrollidone and 10% (w/v) sodium dodecyl sulfate}, 3 ml (25% w/v) dextran sulphate and 500 µl (5 mg/ml) carrier DNA in a hybridization oven (Ratek, Australia) at 65°C for 2 h.

The denatured DNA probe described in Section 3.2.12.2 was added to the bottle. Hybridization was performed overnight at 65°C in the same hybridization oven. After hybridization, the solution was removed from the glass bottle and the membrane was rinsed with 50 ml 2x SSC, 0.1% SDS at 65°C for 20 min. The membrane was transferred to a plastic tray and washed successively with 200 ml of the following solutions in a shaking waterbath at 65°C for 20 min each: 1) 1x SSC, 0.1% SDS; 2) 0.5x SSC, 0.1% SDS; and 3) 0.2x SSC, 0.1% SDS. The membrane was blotted dry on a clean Whatman paper sheet, wrapped in plastic film and exposed to a sheet of X-ray film (Fuji Medical X-Ray Film; RX-U, Japan) in an autoradiography cassette at -80°C for two to five days. The film was

developed in an automated X-ray developer (Curix 60 processor, type 9462/100/140).

3.2.12.4 Reprobing the membrane

It was possible to perform multiple probings of the membranes using both *bar* and *GUS* gene probes. Probes were stripped from membranes in 200 ml 10% SDS, 0.5 M EDTA at 100°C and membranes were agitated gently for at least 30 min at room temperature. After stripping, the membrane was blotted dry on Whatman 3MM filter paper and reprobed following the procedures described in subsections 3.2.12.2 and 3.2.12.3 above.

3.2.13 Statistical analysis

Two-way analysis of variance (ANOVA) was used to test for significant differences between rupture disk pressure (650 psi-1550 psi) and bombardment distance (6 cm-12 cm) for the means of blue spot counted on bombarded scutella. All analyses were performed in Genstat 5 release 4.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). To satisfy normality assumptions, the average number of blue spots was transformed on the log scale.

3.3 Results

3.3.1 Optimisation of bombardment conditions

Initial transformation experiments were undertaken to optimize bombardment conditions. Immature scutella were prepared and bombarded from various distances and at various rupture disk pressures. The results are summarised in Table 3.1. Bombardment conditions clearly affect transient *GUS* expression. Generally, an increase in bombardment pressure from 650 psi to 1550 psi resulted in an increased number of blue spots, and an increase in bombardment distance from 6 cm to 12 cm resulted in a reduction of *GUS* expression. Analysis of variance indicated that the interaction of rupture disk pressure and bombardment distance was significant to the 1% level.

In these experiments, the highest number of blue spots was observed using 1550 psi and 12 cm for bombardment pressure and distance, respectively. However, under these conditions, the bombarded scutella were occasionally knocked out of the Petri dish. It was also observed that bombardment at high pressure (1550 or 1350 psi), in combination with a short distance (6 cm), resulted in a significant reduction in the number of blue spots, probably because the spots were large and close together and multiple spots were counted as a single spot only. The large spots may also have resulted from damaged cells on the scutellum surface, allowing diffusion of GUS activity (blue colour) from damaged cells into the surrounding tissues.

These experiments also indicated that bombardment at 900-1100 psi and 6 cm, or 1350 psi and 9 cm, resulted in higher numbers of blue spots. Using these conditions, approx. 85-100 blue spots per scutellum were observed (Figure 3.2A).

Table 3.1 The average number of blue spots on log scale and original scale in bombarded scutella from various distances and rupture disk pressures

Rupture disk pressure (psi)	Distance (cm) 6	9	12	Average
650*	$_{a}$3.66 (46)^{a}	$_{b}$3.25 (27)^{a}	$_{c}$1.50 (6)^{a}	2.81 (26)
900*	$_{a}$4.42 (85)^{b}	$_{b}$3.59 (41)^{b}	$_{c}$2.65 (16)^{b}	3.55 (48)
1100*	$_{a}$4.41 (87)^{bc}	$_{b}$3.85 (51)^{b}	$_{c}$3.42 (36)^{c}	3.89 (58)
1350*	$_{a}$4.15 (71)^{cd}	$_{b}$4.50 (100)^{c}	$_{c}$3.60 (45)^{c}	4.08 (72)
1550*	$_{a}$4.12 (69)^{d}	$_{b}$4.46 (94)^{c}	$_{b}$4.52 (104)^{d}	4.37 (89)
Average	4.15 (72)	3.93 (63)	3.14 (41)	3.74 (58)
1100**	(103)	NT	NT	
1100***	(84)	NT	NT	

Notes :

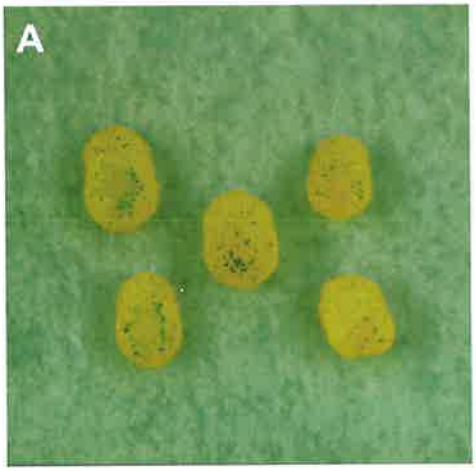
*, **, *** : bombarded with 250, 50 and 18.5 µg gold particles per bombardment, respectively.

( ) : The average number of blue spots on original scale.

Means with the same letters are not significantly different at the 5% level according to the Least Significant Difference test.

Superscript indicates differences at various bombardment pressures in the same distance, whilst subscript indicates differences at various bombardment distances in the same pressure.

NT : not tested.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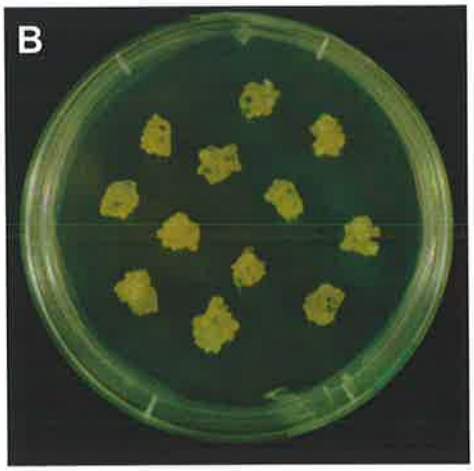
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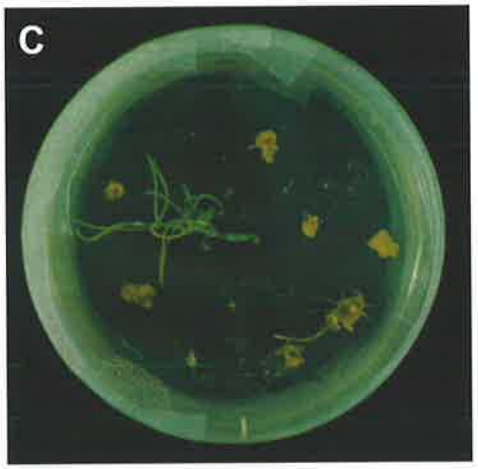
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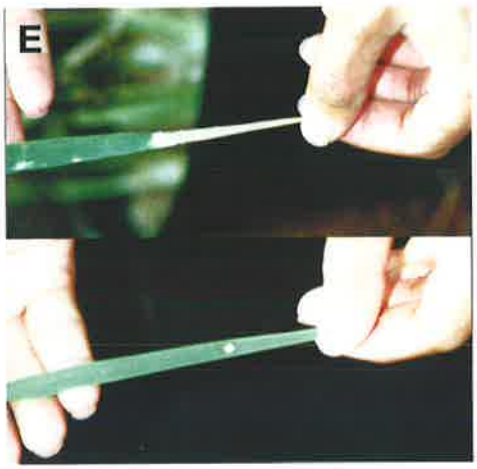
D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F

Figure 3.2 Transformation procedures. (A) transient GUS expression in bombarded scutella; (B) somatic embryo production from bombarded scutella; (C) shoot formation on hormone-free media containing 3 mg/l Bialaphos; (D) a fast growing plantlet recovered from selection media; (E) leaf dip test showing resistant leaf from W2 plant after 0.1% Basta® solution (or 0.05% glufosinate ammonium) application (bottom) and necrosis on control (top) plants; and (F) spraying test (0.1% Basta® solution) on transgenic (right, W9) and control (left) plants.

At 50 µg gold particles per bombardment, the number of blue spots was very high, approx. 103 blue spots/scutellum, with a pressure of 1100 psi and a distance of 6 cm. By reducing the amount of gold particles to 18.5 µg per bombardment while keeping all other conditions constant, the average number of blue spots observed was 84 per bombarded scutellum.

Following the optimisation of bombardment parameters, the following conditions were routinely used for bombardment transformation experiments (see Table 3.2 for details):

- 900 or 1100 psi, 6 cm (and occasionally 9 cm) distance with 250, 50 or 18.5 µg gold particles
- 1350 psi, 9 cm distance with 250 (or 50) µg gold

All other bombardment conditions were discarded due to either producing too few blue spots, or being too severe on the scutellar cells.

3.3.2 Production of somatic embryos

Immature scutella from three wheat genotypes or freshly isolated immature embryos of *cv.* Frame were bombarded as described in sections 3.2.5 and 3.2.6 (Table 3.2). After bombardment, the scutella or immature embryos were transferred to fresh induction medium and incubated in the dark for 20-25 days. Occasionally, some of the bombarded scutella were directly transferred to selection medium, which is induction medium containing 3 mg/l Bialaphos, prior to incubation. Following incubation in the dark, the plates were transferred to light for somatic embryo production (Figure 3.2B).

Table 3.3 shows the percentage of somatic embryo production from bombarded and non-bombarded (control) explants. It was generally observed that

Table 3.2 Summary of transformation experiments conducted during this study

Cultivar	Source of explant	Plasmid constructs	Bombardment conditions (pressure (psi)/amount of gold (µg/bombardment)/ distance from macrocarrier to target tissues (cm))	Number of scutella bombarded	Transformation events
Frame	Glasshouse	pAHC25	900/250/6	290	0
			900/50/6	100	0
			900/18.5/6	135	0
			1100/250/6	144	0
			1100/50/6	180	0
		pDM302 +	1100/250/6	1,142	1
		P*Act*1-D *GUS*	1350/250/9	120	0
	Growth chamber	pDM302 +	900/18.5/6	125	0
		p*Act*1-D *GUS*	1100/50/6	205	0
			900+1100/50/6	165	0
	Field	pDM302 +	900/50/6	75	0
		p*Act*1-D *GUS*	900+900/50/6	108	0
			1100/50/6	154	0
	New conditions*	pDM302	1100/250/6	1,695	3
Krichauff	Glasshouse	pAHC25	900/250/6	150	0
			1100/250/6	729	0
		pDM302 +	900/250/6	335	0
		p*Act*1-D *GUS*	900/18.5/6	100	0
			1100/250/6	1,358	0
	Growth chamber	pDM302 +	900/50/6	153	0
		p*Act*1-D *GUS*	1100/50/6	255	0
			900+1100/50/6	127	0
	Field	pDM302 +	900/50/6	76	0
		p*Act*1-D *GUS*	900+900/50/6	28	0
			1100/50/6	83	0
			900+1100/50/6	24	0
Hartog	Glasshouse	PAHC25	900/250/6	350	0
			900/250/9	72	0
			1100/250/6	637	0
			1100/50/6	200	0
			1100/50/9	180	0
		pDM302 +	900/250/6	400	0
		p*Act*1-D *GUS*	900/18.5/6	280	0
			1100/250/6	1,055	0
			1100+1100/250/6	100	0
			1100/50/9	420	0
			900/50/6	156	0
			1350/50/9	96	0
	Growth chamber	pDM302 +	900/50/6	58	0
		p*Act*1-D *GUS*	900+900/50/6	65	0
			1100/50/6	175	0
			900+1100/50/6	40	0
	Field	pDM302 +	900/50/6	36	0
		p*Act*1-D *GUS*	1100/18.5/6	50	0
			1100/50/6	152	0
Total scutella bombarded				12,578	

* : Bombardment experiments conducted in 1999 using donor plant material grown under growth conditions as explained in Section 2.3.2.

Table 3.3 Production of somatic embryos from non-bombarded and bombarded explants

Cultivar	Non-bombarded explants		Bombarded explants	
	Cultured	% EC	Cultured	% EC
Frame	13*	70	202[1st]	20
	44	30	639	11
	10*[(N)]	60	39[2nd]	20
	20*[(N)]	65	306[3rd]	22
	174[(N)]	59	1,695	18
Hartog	113	20	1,393	3
Krichauff	58	24	1,009	6

Notes:
% EC : Percentage of explants producing embryogenic callus.
* : Bombardment experiment leading to the production of transgenic plants.
(N) : Donor plants were grown in new conditions as explained in Section 2.3.2.
1st ; 2nd; 3rd: The first, second and third successful bombardment experiments.

somatic embryo production from bombarded explants was much lower than obtained from non-bombarded explants. From the results presented in Table 3.3, it is evident that there was a reduction of around 60-85% in embryogenic callus production from bombarded explants as compared with non-bombarded explants.

3.3.3 Plant regeneration and selection of transformants

Somatic embryos produced during exposure to light were picked from callus induction medium and transferred to selection medium, that is, growth regulator-free MS medium containing 3 mg/l Bialaphos (Weeks *et al.*, 1993) for experiments carried out in the early experiments, or containing 5 mg/l Bialaphos (Altpeter *et al.*, 1996) or 5 mg/l PPT (Becker *et al.*, 1994) for experiments conducted at the end of this study. The selection strategy employed in these experiments was to germinate the somatic embryos on selection medium (see Section 3.2.7 for details). It seems that this selection strategy could inhibit the growth of non bombardment-derived shoots, namely shoots grown from non-bombarded, control callus, as well as non-transformed shoots, namely shoots derived from bombarded, non-transgenic callus. However, in some cases "escape" plants, namely non-transformed shoots or plantlets regenerated on selection medium, were still recovered. Seven to ten days after the first selection cycle, the non-bombarded and non-transformed shoots were strongly inhibited by Bialaphos and became necrotic. The majority of shoots died during the second selection cycle and only a few fast-growing shoots with well-developed root systems survived after the second selection cycle (Figure 3.2C). These fast growing shoots, which were considered to be putative transformants, were transferred to hormone-free MS medium, without the presence of selective agent,

for plantlet formation (Figure 3.2D). Fourteen to 20 days after transferring to rooting medium, the putative transformants were transferred to soil. Embryogenic callus was not produced when bombarded scutella were immediately transferred to selection media; the scutella all died.

During this study, exciting results were obtained in three experiments, which are summarised in Table 3.4. In the early experiments, six putative transformants (*cv.* Frame) recovered from selection medium containing 3 mg/l Bialaphos were transferred to soil and grown to maturity for further analysis. These were labelled W1 to W6. In the later experiments, which were completed shortly before finishing this study, three putative transformants of *cv.* Frame were recovered from selection medium and transferred to soil. Two of the three plants, designated W7 and W8, were regenerated from the second successful experiment where selection medium contained 5 mg/l Bialaphos, and another plant, designated W9, was recovered from the third successful experiment, where selection was effected with 5 mg/l PPT (see Table 3.4). During this study, no transgenic plants were obtained with the other two genotypes (*cvs.* Krichauff and Hartog) tested.

3.3.4 PAT assay

The PAT assay was performed only on putative transformants recovered in early successful experiments. Leaf samples from six putative transformants, together with one control plant, were analysed for PAT activity. Four of the six putative transformants (W1, W2, W3 and W5) showed PAT activity, while no PAT activity was detected in the control plant and the other two plants (W4 and W6) (Figures 3.3A-B).

Table 3.4 Summary of transformation experiments leading to the production of transgenic plants (*cv.* Frame) with plasmids pDM302(*) and p*Act*1-D *GUS*(**)

No. of material bombarded	No. of plants recovered from selection medium	No. of PAT positive plants	No. of GUS positive plants	No. of plants carrying the *bar* and the *GUS* genes	Transformation frequency (%)
202**(1st)	6	4	1	1	0.5
39*(2nd)	2	NT	-	2***	5
306*(3rd)	1	NT	-	1***	0.3

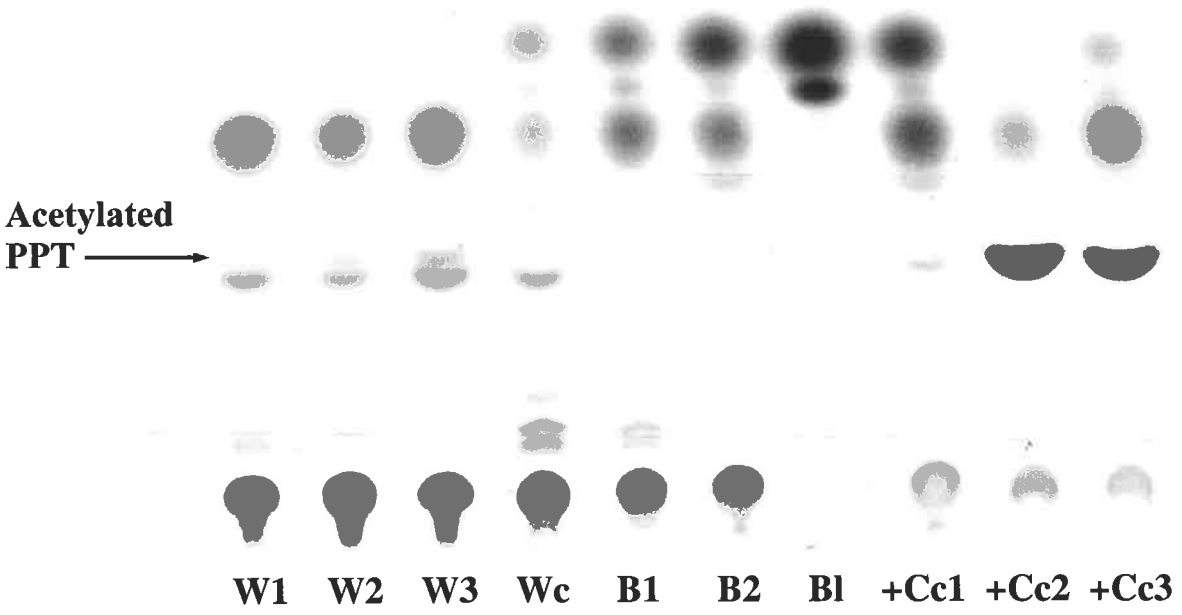
Notes:

NT : Not tested.

*** : Plants carried the *bar* gene only.

1st, 2nd,3rd: The first, second and third successful bombardment experiments.

A


Acetylated
PPT
W1
W2
W3
Wc
B1
B2
Bl
+Cc1
+Cc2
+Cc3

B

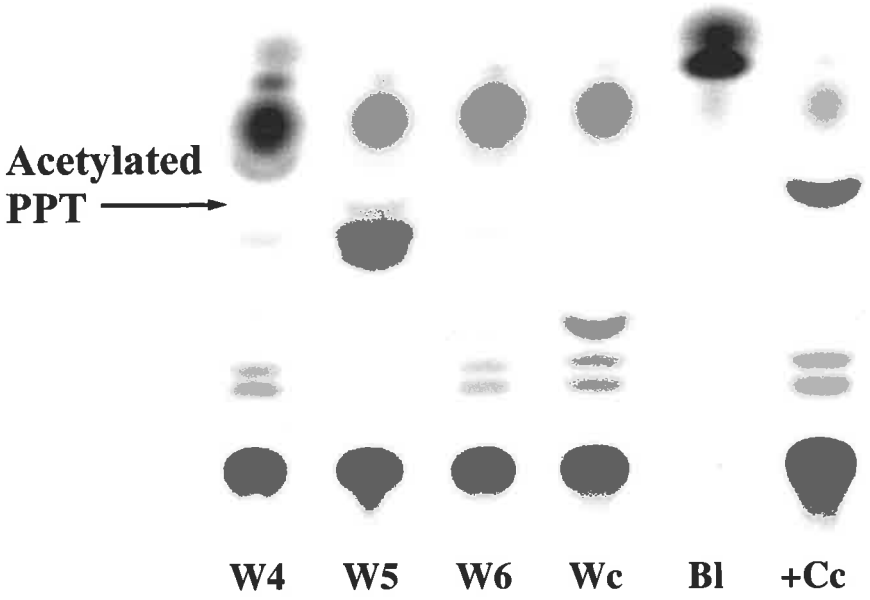

Acetylated
PPT
W4
W5
W6
Wc
Bl
+Cc

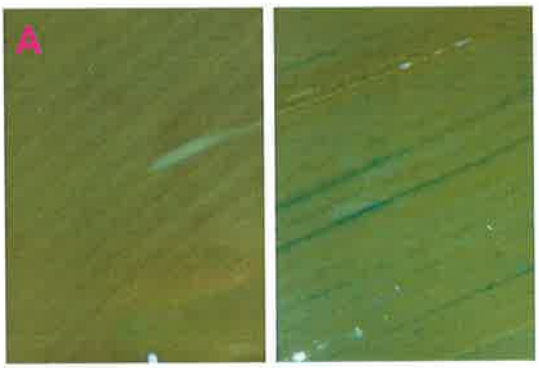
Figure 3.3 PAT assays of putative transformants. (A) and (B) PAT activity in leaf extracts of putative transformants (numbered W1-6) and a wheat control (WC). The position of acetylated phosphinothricin is indicated by an arrow. Abbreviations: W1-6: wheat plants with their code number recovered from selection media; WC: wheat control; B1 abd B2: samples of non-transformed barley callus; Bl: blank sample; +Cc, +Cc1, +Cc2: positive controls of a barley callus line transformed with pAHC25 (Christensen and Quail, 1996) (Callus provided by R. Singh).

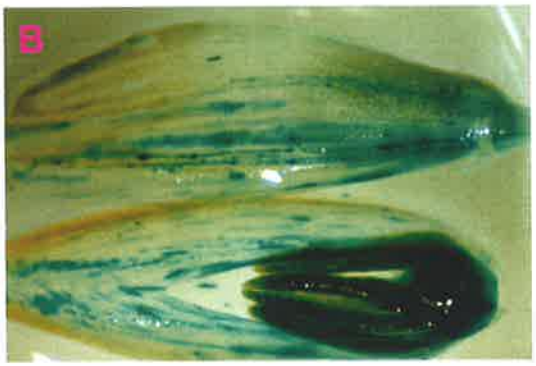
3.3.5 GUS staining

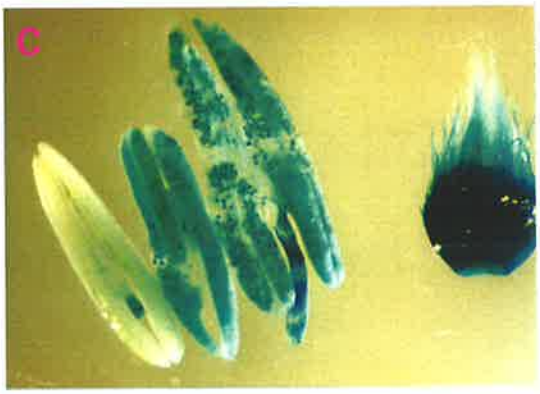
GUS was used as a reporter gene only in early transformation experiments. GUS activity was determined histochemically by staining roots, leaves, spikelets, ovaries, anthers, microspores and grains from six putative transformants and one control plant. Of the six putative transformants tested, only one plant (W2) showed GUS expression. High GUS activity was observed in spikelets, ovaries, anthers, microspores and grains (Figures 3.4B-E). Variability in the intensity of GUS expression was also identified in anthers and grains. Some of the anthers and the grains exhibited dark blue staining, some were light blue and some were only very faintly blue. Furthermore, GUS activity was generally very low in the leaves of this plant and was restricted to the veins (Figure 3.4A); no GUS expression could be detected in the roots. No GUS expression was ever observed in any tissues of the control plant.

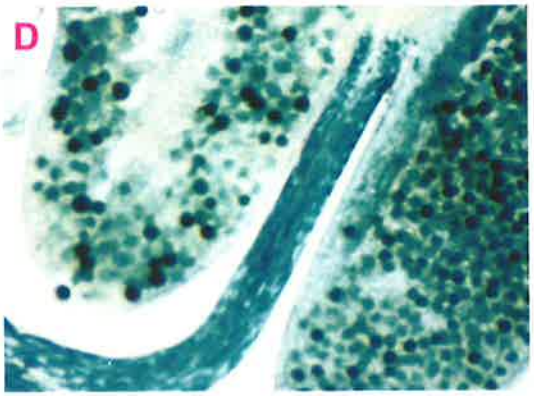
3.3.6 Response to the herbicide leaf-dip test

Two intact leaves from each of the six putative transformants from the first successful bombardment experiment were dipped into 0.01% and 0.1% Basta® solutions or 0.005% and 0.05% glufosinate ammonium, respectively. The effect of applying Basta® to the leaves of the six putative transformants was graded one week after herbicide application. The leaves showed a varying response to herbicide application. The leaves of one plant (W2) showed complete resistance to both concentrations of Basta® (Figure 3.2E). The leaves of plants W3 and W5 showed partial necrosis at a level of 0.01% Basta® (0.005% glufosinate ammonium) and leaves of the remaining three plants (W1, W4 and W6), together with a control plant, showed severe leaf necrosis at both concentrations.


A


B


C


D


E

Figure 3.4 Stable GUS expression in different tissues of a transgenic wheat plant. (A) GUS activity in leaves of transgenic (right) and control (left) plants; (B) in spikelets; (C) in anthers and ovary; (D) in microspores; and (E) in grains of transgenic (right) and control (far left) plants.

The entire plant recovered from the third successful experiment (W9) and a control plant were subsequently sprayed with Basta® at a concentration of 0.1% (or 0.05% glufosinate ammonium). A small amount of necrosis was detected on a few leaves of the putative transformant, whilst the control plant died one week after spraying (Figure 3.2F).

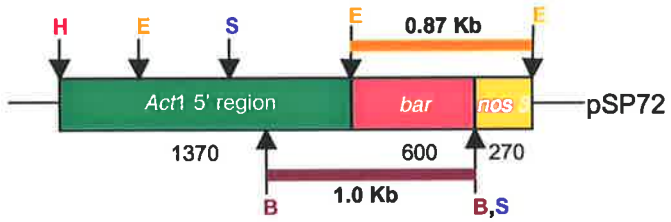
3.3.7 Southern blot analysis

Six putative transformants (W1-W6) recovered in the first successful experiment were analysed for the presence of the *bar* and *GUS* genes by Southern hybridization. The analysis indicated that only one of the six plants, designated W2, possessed the *bar* gene. There were two or more hybridizing bands observed in the *Eco*RI track, including the expected band of approx. 0.87 Kb (Figure 3.5). However, no discrete bands were identified in the *Xho*I track (Figure 3.6A).

To confirm this result, the Southern blot analysis was repeated using *Eco*RI and other two restriction enzymes (*Bam*HI and *Hin*dIII). In this case, only three primary transformants, namely W2, W3, W6 and control plants were analysed. Results from the second hybridization analysis again showed the presence of the *bar* gene in the W2 plant and not in the other two plants (W3 and W6). As predicted from the restriction map of plasmid pDM302 (Figure 3.1), the *bar* probe hybridized to fragments of the correct size in both *Eco*RI- (approx. 0.87 Kb) and *Bam*HI- (approx. 1 Kb) digested DNA samples (Figures 3.5 and 3.6B). Beside the expected bands, other bands were observed in both the *Bam*HI and *Eco*RI tracks.

Furthermore, hybridization of the *bar* probe to the uncut genomic DNA of the W2 plant confirmed the presence of the *bar* gene in the high molecular weight

A



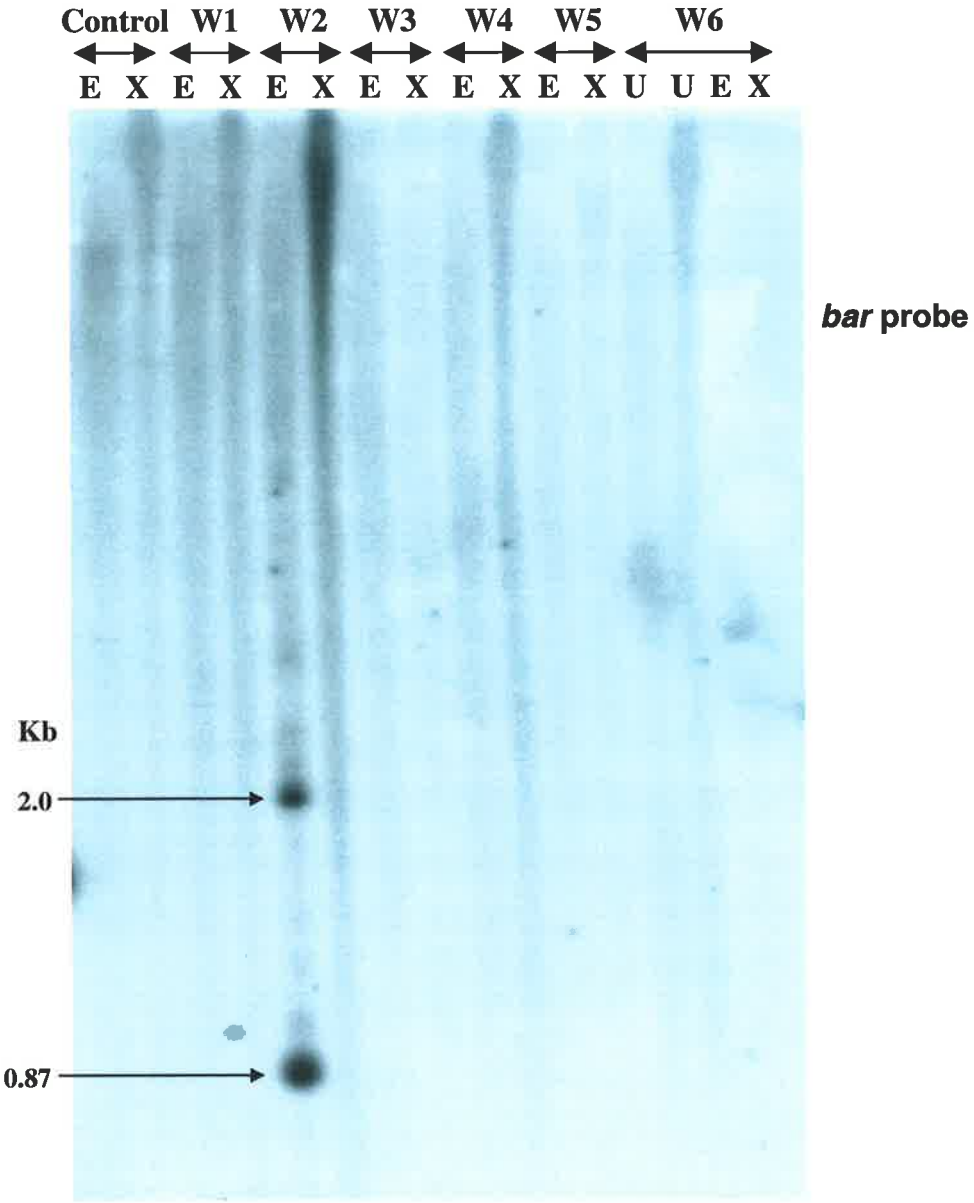
pDM302 (4.8 Kb)

B

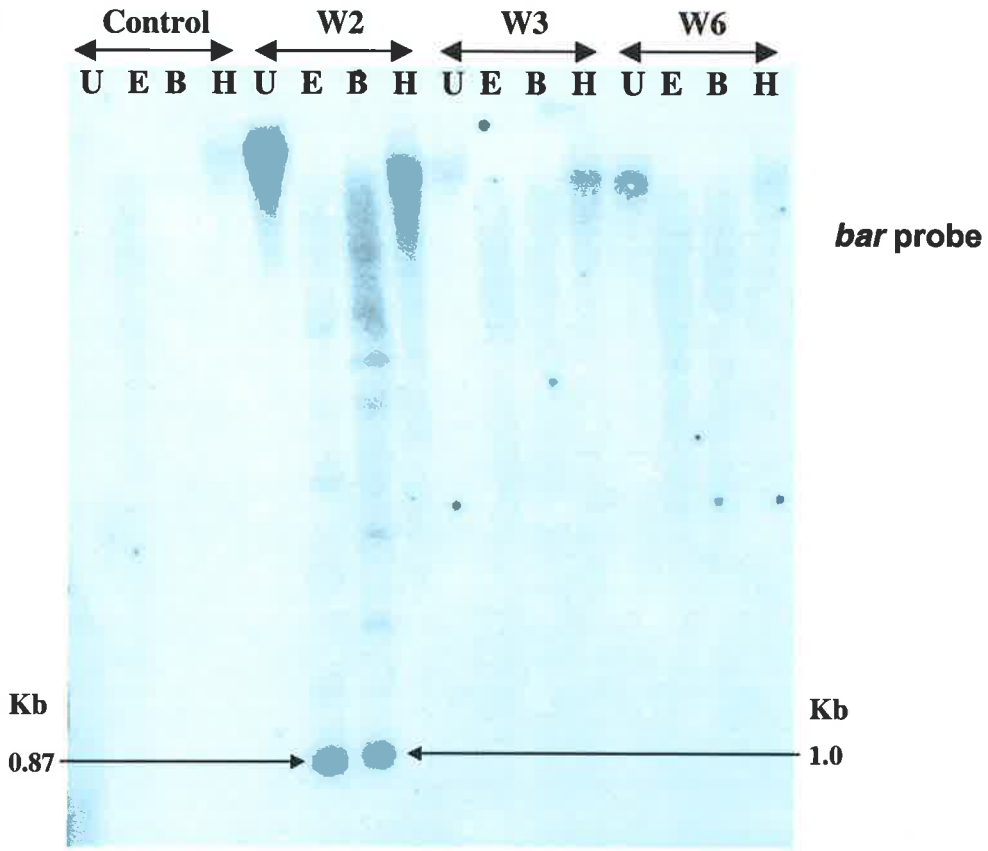
p*Act*1-D *GUS* (7.2 Kb)

Figure 3.5 Restriction map. Restriction maps of (A) pDM302 and (B) p*Act1*-D *GUS*. Enzymatic restriction sites used in these experiments are shown in coloured letters. Expected fragments from the enzymatic digestions used are indicated by coloured thick lines. Abbreviations of restriction sites: B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; S: *Sst*I; X: *Xho*I and Xb: *Xba*I.

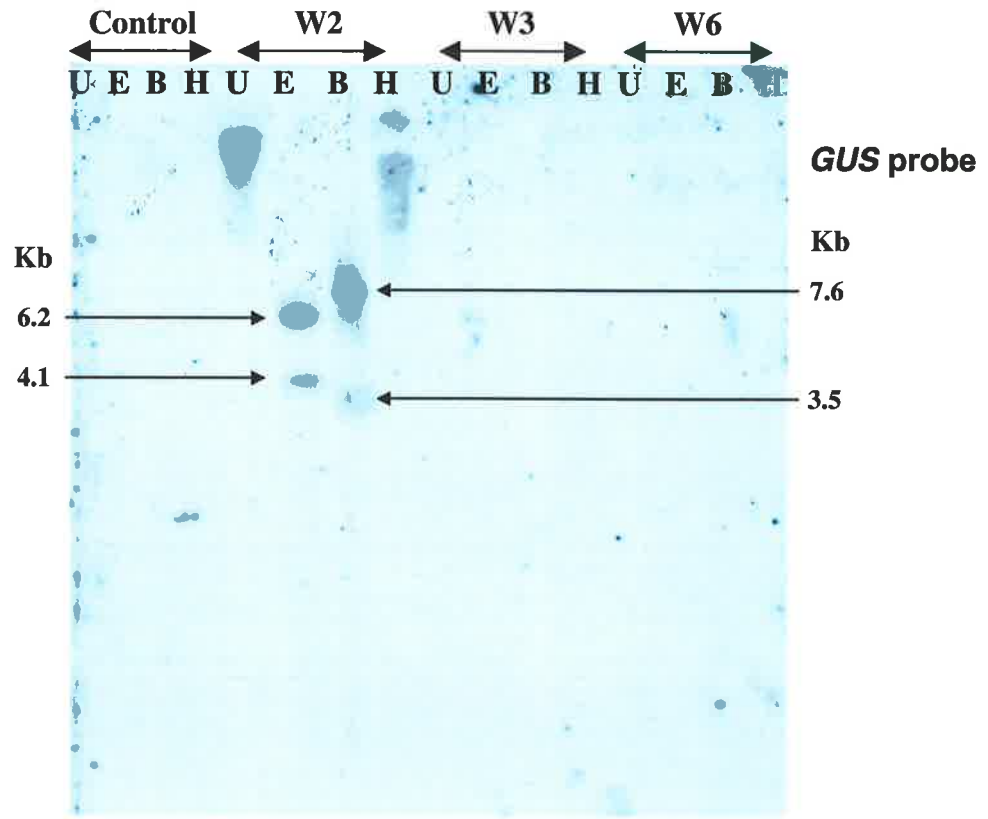
A


Control W1 W2 W3 W4 W5 W6
E X E X E X E X E X E X U U E X
bar probe
Kb
2.0
0.87

B


Control
W2
W3
W6
U E B H U E B H U E B H U E B H
bar probe
Kb
0.87
Kb
1.0

C


Control
W2
W3
W6
U E B H U E B H U E B H U E B H
GUS probe
Kb
6.2
4.1
Kb
7.6
3.5

D

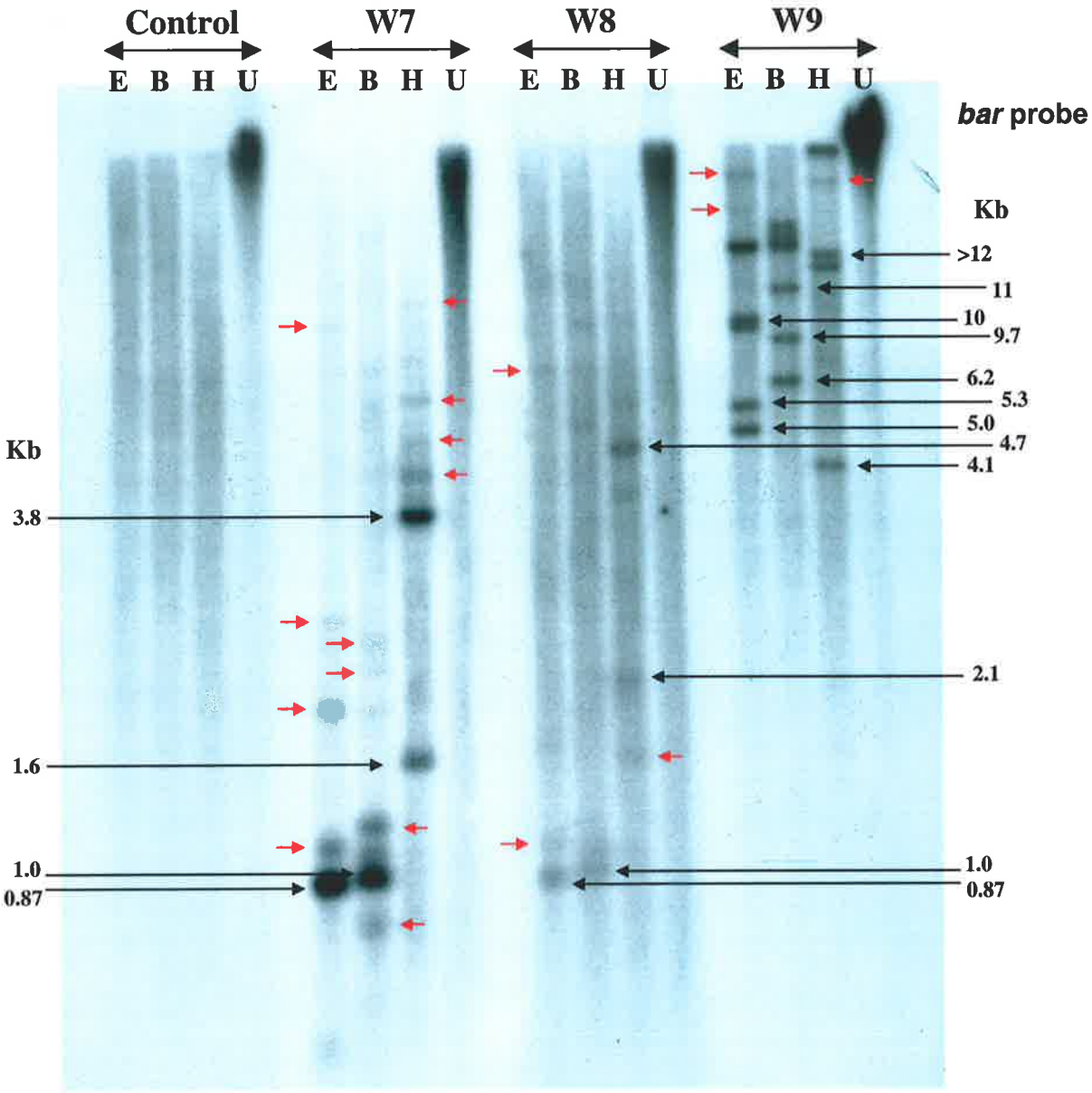

Control
W7
W8
W9
E B H U
E B H U
E B H U
E B H U
bar probe
Kb
>12
11
10
9.7
6.2
5.3
5.0
4.7
4.1
Kb
3.8
2.1
1.6
1.0
0.87
1.0
0.87

Figure 3.6 Southern blot analysis of transgenic wheat plants. (A) Genomic DNA isolated from primary wheat transformants (W1-6) and a control plant was digested with *Eco*RI and *Xho*I and probed with the *bar* probe, showing the expected fragment (0.87 Kb) and an additional intense band (approx. 2 Kb) in the *Eco*RI track. (B) Genomic DNA of W2, W3, W6 and a control plant was redigested with *Eco*RI, *Bam*HI and *Hin*dIII and probed with the *bar* probe, showing the predicted fragments in the *Eco*RI (0.87 Kb) and *Bam*HI (1.0 Kb) tracks (Figure 3.5), and (C) probed with the *GUS* probe, showing two hybridizing bands both in the *Eco*RI (approx. 4.1 and 6.2 Kb) and in the *Bam*HI (approx. 3.5 and 7.6 Kb) tracks. (D) Genomic DNA of W7, W8, W9 and a control plant was digested with *Eco*RI, *Bam*HI and *Hin*dIII and probed with the *bar* probe, showing the predicted fragments in the *Eco*RI (0.87 Kb) and *Bam*HI (1.0 Kb) tracks in W7 and W8, but the hybridizing bands were larger than expected in W9. Abbreviations : W1-9: primary (wheat) transformants; Control: wheat control; U: uncut genomic DNA; Kb: kilobase. Abbreviations of restriction enzymes used: B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; X: *Xho*I.

DNA of this plant (Figure 3.6B). However, no discrete bands were observed in the *Hin*dIII track, which may indicate failure of the enzyme to properly restrict the DNA.

After probing for the *bar* gene, the primary transformants were subsequently analysed for the presence of the *GUS* gene. Of the six putative transformants stained (W1-W6), only the W2 plant showed GUS activity. Hence, Southern blot analysis was performed using the membrane containing genomic DNA from this plant, together with DNA from W3 and W6. The membrane was therefore stripped after probing with *bar* and subsequently probed with the *GUS* fragment.

Based on Southern blot hybridization, it was found that only the W2 plant, which had also been shown to carry the *bar* gene, carried the *GUS* gene, whilst the other two plants (W3 and W6) did not have the *GUS* gene (Figure 3.6C). This was indicated by the hybridization of the *GUS* probe to uncut genomic DNA from the W2 plant. Furthermore, it was observed that two or more hybridizing bands could be identified in the *Eco*RI (approx. 4.1 and 6.2 Kb) and in the *Bam*HI (approx. 3.5 and 7.6 Kb) tracks. Since *Eco*RI and *Bam*HI cut outside the coding region of the *GUS* gene (Figure 3.5), this implies that more than one insertion of the *GUS* gene is present in the genome. Again no discrete bands were visible in the *Hin*dIII track (Figures 3.6B-C).

Based on this observation, it is clear that the GUS staining matched those from the molecular analysis, because the W2 plant showed GUS activity and also carried the *GUS* gene, whilst the other two plants, designated W3 and W6, did not have the *GUS* gene and had no GUS activity. No further effort was made to perform Southern blot analysis on the three remaining plants (W1, W4 and W5)

since none of these plants showed GUS activity on staining and were therefore considered to be GUS negative.

In these experiments, it was impossible to repeat the Southern blot analysis with other restriction enzymes which may have better indicated the number of insertions of the *bar* gene, since only a small number of leaves were collected for DNA extraction before the plant died.

The presence of the transgene (*bar*) in three putative transformants, namely two plants (W7 and W8) and one plant (W9) regenerated from the second and the third successful experiments, respectively, was also investigated by Southern blot analysis. Genomic DNA isolated from the three plants was again digested with *Eco*RI, *Bam*HI and *Hin*dIII. The *Hin*dIII enzyme was used, because *Hin*dIII generated good and consistent results when it was used in analysing the presence of transgenes in the progeny of the W2 plant (see Chapter 4). Newly purchased enzymes were used to avoid the possible risk of partially inactivated restriction enzymes, which were thought to cause the DNA smearing found in analysing the W2 plant with *Hin*dIII (Figures 3.6B-C).

Southern blot analysis showed that three plants (W7, W8 and W9) carried the *bar* gene (Figure 3.6D). The expected fragments of the *bar* gene could be easily observed in W7 and W8 plants, both in the *Eco*RI (0.87 Kb) and *Bam*HI (1.0 Kb) tracks. In W9, however, the fragments of the *bar* gene were larger than expected (>4 Kb in *Eco*RI and >6 Kb in *Bam*HI tracks).

In addition to the expected fragments of the *bar* gene in W7 and W8, extra bands were observed both in the *Eco*RI and *Bam*HI tracks. Multiple bands were also observed in both the *Eco*RI and *Bam*HI tracks of W9. Furthermore, the three plants showed multiple hybridization bands in *Hin*dIII tracks, suggesting multiple

integration sites of the gene. Plants designated W7 and W8 appeared to have two integration sites for the *bar* gene, based on the presence of two intensive bands (1.6 and 3.9 Kb in W7, and 2.1 and 4.7 Kb in W8) in the *Hin*dIII tracks. In W9, however, the *bar* gene appeared to have three or more integration sites (Figure 3.6D).

The integration of the *bar* gene into the high molecular weight DNA of the three plants was proven by the hybridization of the *bar* probe to uncut genomic DNA of these plants (Figure 3.6D). However, an intensive hybridizing band was also observed in uncut genomic DNA of a control plant, and this band is believed result from random hybridization of the *bar* probe with genomic DNA of the control plant (background), which accumulated in the top loading well.

3.3.8 Appearance of the primary transformants

The primary transformants (W2, W7, W8 and W9) regenerated in these experiments were normal in appearance and were fertile (Figure 3.7). Several notes relating to phenotypic characteristics and yields of the transgenic wheat plants, together with the other five regenerants recovered in the early experiment and control plants are presented in Figure 3.8 (Table 3.5). Transgenic wheat plant (W2) produced in the first successful experiment had only few tillers (Figure 3.7) and was shorter than the transgenic plants recovered from the second (W7 and W8) and the third (W9) successful experiments (Table 3.5). Furthermore, the total number of grains produced by the transgenic W2 plant was less than the other transgenic plants (W7, W8 and W9), and less than that seen in other five non-transgenic plants regenerated from the same experiment (W1, W3-6) and control

Figure 3.7 Mature transgenic wheat plants of *cv.* Frame. Primary transformants designated W2 (left), W7 (centre) and W8 (right) (see Figure 3.2F for W9).

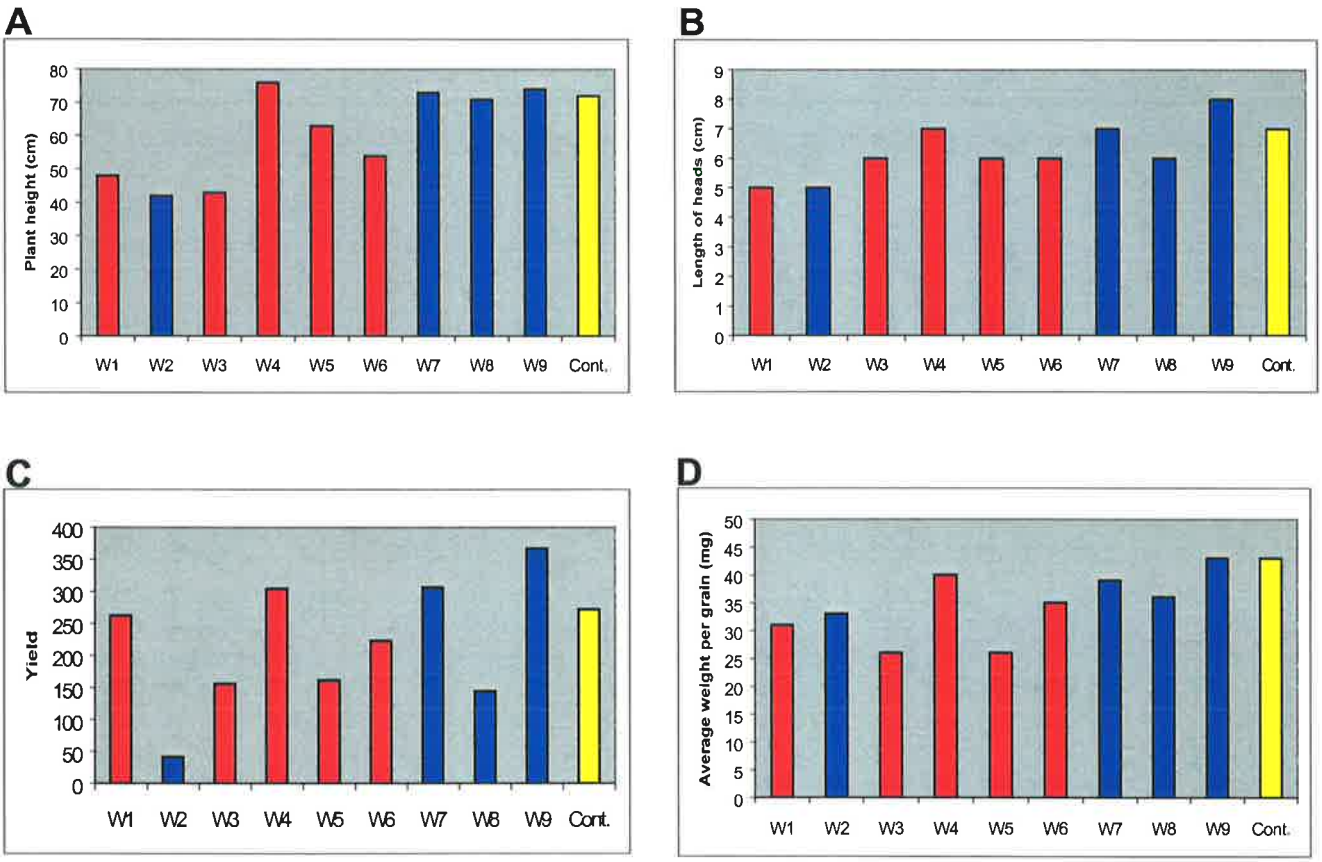


Figure 3.8 Phenotypic characteristics of transgenic (blue bars), non-transgenic (red bars) and control (yellow bars) plants. (A) height of plants; (B) length of heads; (C) yield (total number of grains formed/plant) and (D) average weight per grain.

Table 3.5 Comparisons of phenotypic characteristics and yield of the transgenic wheat plants (W2, W7, W8, and W9) with non-transgenic and control plants (*cv.* Frame)

Plant code	Height (cm) (average)	Length of heads (cm)	Yeld (No. of grains/plant)	Average weight per grain (mg)
W1*	48	5	262	31
W2	42	5	41	33
W3*	43	6	156	26
W4*	76	7	304	40
W5*	63	6	161	26
W6*	54	6	223	35
W7	73	7	306	39
W8	71	6	144	36
W9	74	8	367	43
Cont. 1	70	6	243	41
Cont. 2	74	8	300	44

Notes:
* : non-transgenic plants confirmed by Southern blot hybridization.
Cont : control plants.
Average yield of control plants : 272.
Average weight per grain of control plants (mg) : 43.

plants. Transgenic plants recovered in the second and the third successful experiments, however, usually showed similar characteristics to control plants.

3.4 Discussion

Early transformation experiments were devoted to finding the optimum conditions for bombardment by studying transient expression in bombarded scutella. Based on these experiments, it was observed that more severe bombardment conditions, such as high pressure and short bombardment distance, produced higher degrees of transient expression. However, such bombardment conditions resulted in more tissue damage and adversely affected subsequent embryogenic callus formation; this led to low transformation efficiencies. It is generally agreed that the degree of transient expression observed in a tissue is unrelated to the stable integration of the transferred gene (Becker *et al.*, 1994; Koprek *et al.*, 1996). During bombardment, a stable event is produced when the DNA coated particle penetrates the nucleus with minimal damage to the cellular machinery and the DNA is inserted into a transcriptionally active part of the genome. Stability is observed if the DNA is not methylated or inactivated in any other way. On the other hand, transient expression can be observed in most cells, even if they are damaged, because the introduced DNA can be transcribed for a short time.

During the present study, three sets of bombardment conditions were commonly used for transformation, namely 1) 1100 psi rupture disk pressure, 6 cm bombardment distance with 50 μg or 2) 250 μg gold particles/bombardment, and 3) 900 psi rupture disk pressure, 6 cm bombardment distance and 250 μg gold particles/bombardment. All results leading to the production of transgenic plants in this study were obtained from bombardment conditions using 1100 psi rupture disk pressure, 6 cm bombardment distance and 250 μg gold particles/bombardment, although this might be simply because these conditions

were more frequently used than other bombardment conditions during transformation experiments (Table 3.2). Because of the very low regeneration and transformation frequencies, it was not possible to make any correlations between transient expression and stable integration of transgenes.

The combination of a simple regeneration system developed for Australian wheat genotypes (see Chapter 2) with appropriate bombardment conditions, led to the production of fertile transgenic wheat plants, *cv.* Frame, which is an elite Australian genotype. During this study, three successful transformation events were achieved. In the early successful experiment, confirmation of the transgenic nature of the plants involved four steps. Firstly, fast-growing shoots were selected from the selection medium, which contained 3 mg/l Bialaphos. In this experiment, 202 immature scutella were bombarded with both the *bar* and the *GUS* genes, and six Bialaphos-resistant plantlets or putative transformants were recovered from the selection medium. Figure 3.2D shows an example of a putative transformant arising from the selection medium. The six putative transformants were subsequently transferred to soil for further analysis.

All six putative transformants were assayed for PAT activity as the second step in the confirmation of transformation status. This assay makes it possible to detect the PAT enzyme, which is the product of the *bar* gene, using a radiolabelled substrate followed by exposure to X-ray film. Of the six putative transformants analysed, four showed PAT activity. These results subsequently led to speculation that four of the six putative transformants may carry the *bar* gene in their genomes. Figures 3.3A and 3.3B show the results of the PAT assay from the six putative transformants.

The third test for confirmation of transformation status was the herbicide application test, which was performed by dipping leaf tips of the six putative transformants into various concentrations of Basta® solution (0.01% and 0.1%). The six treated plants showed variable tolerance towards Basta®. One of the putative transformants (W2) showed complete tolerance to both Basta® concentrations tested (Figure 3.2E). This suggested that the PAT enzyme activity protected the leaves of this plant from the toxicity of the Basta® herbicide. Two plants (W3 and W5) showed moderate tolerance at a concentration of 0.01% Basta® solution but they exhibited severe necrosis at a concentration of 0.1%. The remaining three plants behaved like non-transformed control plants and were sensitive to both concentrations of Basta® tested.

Southern blot analysis was performed as the fourth step to demonstrate the incorporation of the introduced genes into the plant genome. A Southern blot was carried out using genomic DNA isolated from all six plants (Figure 3.6A). As seen in Figure 3.6A, only one (designated W2) of the six plants showed a positive result. Undigested DNA from this plant hybridized with the *bar* gene probe (Figure 3.6B), suggesting that the *bar* gene was integrated into the wheat (W2) genome, while DNA from the other plants and a control plant did not.

DNA from the W2 plant was also digested with *Bam*HI and *Eco*RI, and gave the expected hybridizing band corresponding to that predicted from the restriction map (Figure 3.6B). In addition, the presence of extra bands in the *Eco*RI and *Bam*HI tracks at a much lower intensity than the expected band were also observed. These extra bands appear to represent incompletely digested DNA or additional insertions of *bar* gene fragments, and were also identified in the progeny of this plant (Chapter 4). The integration of gene fragments into the plant

genome is commonly observed in transgenic plants produced *via* microprojectile bombardment, because this method relies on physical force to deliver DNA into plant cells and the shearing of transgenes could occur before integration into the genome (Pawlowski and Somers, 1996).

In summary, the four criteria used for confirming the transformation status of the wheat plant designated W2 were met and it was concluded that W2 was truly transgenic for the presence of the *bar* gene. However, the observation that only one of the six putative transformants produced in this experiment was transgenic indicated that the selection strategy employed was still weak and failed to avoid the growth of "escape" plants. Moreover, the PAT assay was not always a reliable means to confirm transformation status and should be interpreted carefully, because several plants showed a positive result for enzyme activity but subsequent Southern analyses were negative for the presence of the *bar* gene. Srivastava *et al.* (1996) have also reported the presence of PAT activity in several putatively transformed wheat plants that were found to lack the *bar* gene when analysed by Southern hybridization.

In the experiments described here, all material bombarded with the *bar* gene was also co-transformed with a plasmid containing the *GUS* gene, and the six recovered plants were therefore examined for the presence of *GUS* gene expression. Histochemical analysis of GUS activity was carried out on various parts of the six putative transformants, but only the W2 plant showed GUS activity. GUS activity could be detected in both vegetative (leaves) and reproductive (anthers, ovaries, microspores and grains) tissues (Figures 3.4A-E), but activity in the reproductive tissues was much higher than that in vegetative tissues. Other

reports have also indicated a variability of GUS activity in different tissues of the same transgenic plant (Zhang *et al.*, 1991; Demeke *et al.*, 1998).

No GUS activity was found in the root system of W2. The absence of GUS activity in the roots of this plant raised the possibility that W2 might be chimeric with respect to incorporated *GUS* genes. Accordingly, additional tests were performed by staining anthers from every tiller of the transformed W2 plant. Results from these experiments showed that anthers from every tiller of the W2 plant exhibited GUS activity (data not shown). This suggested that the plants was not chimeric.

Although it was expected that the rice *Actin* promoter driving *GUS* gene in the p*Act*1-D *GUS* construct would be active in the roots (Zhang *et al.*, 1991), this plant showed no GUS activity in the roots, and the absence of GUS activity in the roots was also experienced in the transgenic progeny of this plant (Chapter 4). The reason for this is unclear, but may reflect the position in which this gene was integrated in the genome. Such a "position effect" could cause a tissue-specific block of GUS expression.

To confirm the presence of the *GUS* gene in putative transformants, it was decided to reprobe the membrane, which contained DNA samples of plants W2, W3 and W6, with the *GUS* probe. As predicted from the histochemical GUS staining results, only W2 was found to carry the *GUS* gene. These analyses also ruled out the possibility that the *GUS* gene had indeed been incorporated into the W3 and W6 plants, but was simply not being expressed (Figure 3.6C).

Two or more bands hybridizing to the *GUS* probe were detected in the *Eco*RI- and *Bam*HI-digested DNA from W2. This indicates that more than one integration site of the *GUS* gene is present. The presence of both *bar* and *GUS* in

the same plant (W2) indicates that co-integration of the introduced transgenes has obviously occurred. This result is in agreement with previous work using microprojectile bombardment, where two genes introduced *via* two independent plasmid vectors were co-integrated into the wheat plant genome (Becker *et al.*, 1994; Nehra *et al.*, 1994; Chibbar *et al.*, 1998).

As mentioned earlier, two successful bombardment experiments were achieved towards the end of this study (Table 3.3). In these experiments, 39 and 306 immature embryos were bombarded with the *bar* gene in the second and the third successful experiments, respectively. Because of the high incidence of 'escape' regenerants in the early experiments, efforts were made to refine the selection procedures. Selection pressure was increased to 5 mg/l Bialaphos for selection of W7 and W8, or to 5 mg/l of PPT for W9, compared with 3 mg/l Bialaphos used in earlier experiments. Putative transformants recovered from the selection medium were grown in soil and directly subjected to Southern blot analysis (W7 and W8), or subjected to the herbicide application test, followed by Southern blot analysis (W9).

To assess the effectiveness of the herbicide spraying test, it was decided to spray only a single putative transformant, together with a control plant. The single putative transformant (W9) from the third successful transformation experiment was used for this purpose. The W9 plant showed resistance to the applied herbicide, whilst the control plant died within one week (Figure 3.2F). This indicated that the *bar* gene was expressed and could protect the plant from herbicide toxicity.

Southern blot analysis showed that all three plants from the later experiments (W7, W8 and W9) carried the *bar* gene (Figure 3.6D). Thus, it was

demonstrated that by increasing the selection pressure and by performing a herbicide spraying test, the incidence of 'escape' regenerants could be reduced. The presence of 0.87 Kb and 1.0 Kb DNA fragment carrying the *bar* gene in *Eco*RI and *Bam*HI digests, respectively (Figures 3.6B and 3.6D), confirmed that intact copies of the *bar* gene had been integrated into the genomes of these plants. In the case of W9, the presence of hybridizing bands which were larger than the expected fragments (>4 Kb in both the *Eco*RI and *Bam*HI tracks) (Figure 3.6D) indicated that the *bar* gene in this plant might have undergone rearrangement when integrating into the plant genome. Since this plant showed resistance to herbicide application, the coding region of the *bar* gene must have been intact. The *Eco*RI and *Bam*HI restriction sites in the pDM302 (Figure 3.1A), might have been lost during integration.

Based on the Southern blot results, it was believed that the three plants had more than one integration site of the *bar* gene, which might also indicate the presence of multiple copies of transgenes, as indicated by the presence of multiple hybridization bands in all tracks of restriction enzymes used, particularly in *Hin*dIII tracks, since *Hin*dIII cuts outside the coding region of the *bar* gene. The results found here were similar to previous work reporting the integration of multiple copies of transgenes in transgenic wheat plants produced *via* microprojectile bombardment (Vasil *et al.*, 1992; Barro *et al.*, 1997; Lörz *et al.*, 1998).

Agronomic characteristics of all four transgenic plants produced from the three successful bombardment experiments were monitored. Generally, the transgenic wheat plants were healthy and fertile (Table 3.5). Maturity of the transgenic wheat plants was attained approx. 26-30 weeks after bombardment.

It was also noted that the transgenic wheat plant designated W2 showed a dwarf phenotype compared with the other three transgenic plants (W7, W8 and W9), which had physical characteristics similar to control plants (Figure 3.8). Differences were also noted in the number of grains produced from the four transgenic plants. The W2 plant produced only 41 grains as compared with over 100 grains produced from the other three transgenic plants.

During the course of this study, around 12,500 immature scutella and immature embryos were bombarded with various gene constructs in 77 experiments (Table 3.2). However, successful transformation was achieved in only three experiments. The transformation frequency was approx. 0.7%, based on the number of isolated scutella and immature embryos bombarded in the three successful experiments, or approx. 0.03% based on the total number of targets bombarded. The transformation frequency achieved in the successful experiments (0.7%) is still in the range of transformation frequency for wheat plants reported elsewhere (0.2-9.7%) (Weeks *et al.*, 1993; Vasil *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994; Ortiz *et al.*, 1996; Dobrzańska, *et al.*, 1997; Zhang *et al.*, 2000).

It is believed that by far the most limiting factor which hindered the production of transgenic plants on a regular basis was the condition of donor plants, as discussed in Chapter 2. In that Chapter, it was reported that cultured explants from the *cvs.* Frame, Krichauff and Hartog responded extremely well in initial experiments but that their growth rate in culture and regeneration capacity deteriorated dramatically in subsequent experiments. The first successful transformation outlined in this study was performed in 1995, when the response of explants in culture was still high. In subsequent experiments, the response of

bombarded explants in culture was very poor (see Chapter 2), so that although bombardment conditions had been manipulated (see Table 3.2), no satisfactory outcomes were achieved. Towards the end of this study in 1999, two further successful experiments were achieved. Their success was attributable to refinements in conditions for growing donor plants, which resulted in greatly improved culture response. Based on these observations, it must be strongly emphasized that a good explant response in culture is an important prerequisite for the successful transformation of wheat.

CHAPTER FOUR

TRANSGENE INHERITANCE AND EXPRESSION IN PROGENY OF TRANSFORMED WHEAT

4.1 Introduction

The production of fertile transgenic wheat plants carrying various genes of interest has already been reported in the literature (Chibbar *et al.*, 1998; Bliffeld *et al.*, 1999). All the indications are there to suggest that, in the near future, genetically engineered wheat will be available for breeding programs. However, before transgenic wheat plants can be confidently incorporated into wheat breeding programs, it is crucial to monitor the behavior of inserted transgenes, not just in primary transgenic wheat plants, but also in successive generations. The faithful inheritance and expression of transgenes are particularly important properties in the light of reports of gene silencing in transgenic plants, through mechanisms such as DNA methylation, co-suppression and genetic rearrangements (Meyer, 1995[a]; Vaucheret, *et al.*, 1998).

Until recently, few studies have been conducted to investigate the fate of inherited transgenes in the progeny of transformed wheat. This might be because inheritance studies are time-consuming and labour-intensive, or because insufficient plant material has been available for analysis. Indeed, the majority of work has been focussed on overcoming the technical barriers in producing transgenic wheat, and on improving transformation frequencies (Harvey *et al.*, 1999; Zhang *et al.*, 2000).

In the current project, the integration and expression of two transgenes (*bar* and *GUS*) have been studied in transgenic wheat plants, as presented in Chapter 3. In this Chapter, experiments are described to define how both introduced transgenes were transmitted to and expressed in the second and third generations after the initial transformation event.

4.2 Materials and Methods

4.2.1 Plant growth conditions and maintenance

To study transgene inheritance in successive generations (T1 to T3), grains produced from the self-pollinated transgenic W2 plant or its progeny plants were randomly picked and sown in soil. Plants arising from these grains were grown to maturity in the glasshouse as described in Chapter 2 (Section 2.2.1).

4.2.2 Histochemical GUS staining

GUS staining was performed as described in Section 3.2.8. In order to observe GUS expression in roots, grains from several T1, T2 and T3 plants were germinated in Petri dishes or in a vermiculite-filled tray. In the case of grains germinated in Petri dishes, the grains were initially sterilised in 15% (v/v) Domestos for 10 min and rinsed five times in sterile water. After surface sterilization, the grains were aseptically placed in Petri dishes on moist Whatman 3MM chromatography paper, and the seedlings were allowed to develop for two weeks.

Before the plants were transferred to soil, two root tips approx. 4 cm in length were cut from each seedling and stained in X-gluc solution (Section 3.2.8). The remaining seedlings were transferred to soil and grown in the glasshouse to maturity. Other plant parts such as leaves, ovaries, anthers and grains were stained as described in Section 3.2.8.

4.2.3 PAT assay and herbicide leaf-dip test

In analysing the expression of the *bar* gene in the progeny of the transgenic W2 plant, both PAT assays and herbicide leaf dip tests were performed, as

described in Sections 3.2.9 and 3.2.10, respectively. However, in leaf dip tests, two leaves from each plant were dipped into a 0.01% Basta® solution, and in one case, the leaves from a number of T1 plants (Section 4.3.1.2) were also dipped into 0.1% and 1% Basta®.

4.2.4 Southern blot hybridization

Southern blot analysis was carried out using procedures described in Section 3.2.12, with several modifications. Approximately 30 µg genomic DNA was used per restriction enzyme digest.

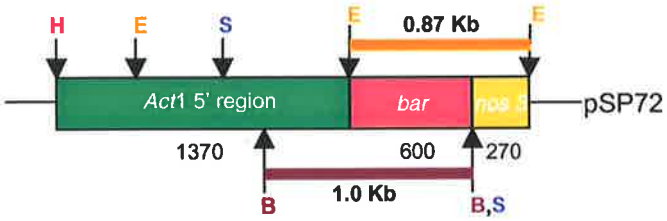
Based on the Southern blot analysis of T0 leaves of the primary transformant, it appeared that the W2 plant had multiple insertions of the *GUS* transgene, as indicated by the presence of two or more bands in the *Eco*RI and *Bam*HI tracks (Figure 3.6C). Thus, in analyzing the transmission of transgenes from the primary transformant to its progeny, efforts were made to provide an estimate of the copy number of the transgenes, especially the *GUS* gene, carried by each progeny. For this, both single and double restriction enzyme digests were used, as detailed in Table 4.1.

In these experiments, *Hin*dIII was used to provide the estimates of copy numbers of the *GUS* gene, because this restriction enzyme cuts at only one site in the p*Act*1-D *GUS* construct (Zhang *et al.*, 1991) (Figure 4.1). To overcome the problem of incomplete digestion of DNA, as encountered in analysing the primary transformant (W2) (Section 3.3.7), a newly purchased batch of each restriction enzyme was used, and some modifications were made to the DNA digestion procedure. In all DNA restriction mixtures, a 10x "Super-Duper" buffer (SDB) (Langridge *et al.*, 1999) (330 mM Tris-HCl buffer, pH 7.8, containing 650 mM

Table 4.1 The expected fragment sizes of the *bar* and *GUS* genes excised with various restriction enzymes

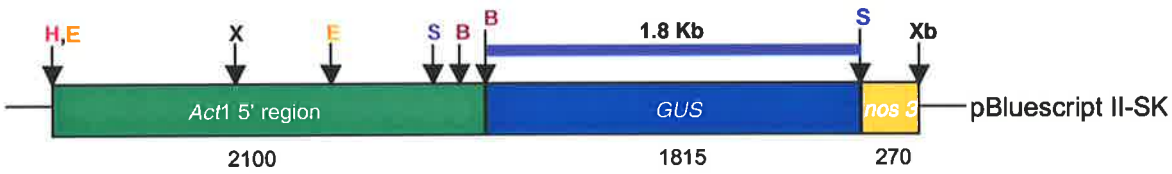
Constructs	Restriction enzymes	Expected fragment sizes (approx. Kb)
pDM302 (*bar* fragment)	*Hin*dIII	Unknown
	*Bam*HI + *Sst*I	1.0
	*Eco*RI	0.87
	*Nco*I	Unknown
p*Act*1-D *GUS* (*GUS* fragment)	*Hin*dIII	Unknown
	*Bam*HI + *Sst*I	1.8
	*Eco*RI	Unknown
	*Nco*I	Unknown

A



pDM302 (4.8 Kb)

B



p*Act*1-D *GUS* (7.2 Kb)

Figure 4.1 Restriction map. Restriction maps of (A) pDM302 and (B) p*Act1*-D *GUS*. Enzymatic restriction sites used in these experiments are shown in coloured letters. Expected fragments from enzymatic digestions used are indicated by coloured thick lines. Abbreviations of restriction sites: B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII; S: *Sst*I; X: *Xho*I and Xb: *Xba*I.

potassium acetate, 100 mM magnesium acetate, 40 mM spermidine and 50 mM dithiothreitol) was used. An appropriate amount of restriction enzyme (3 µl) was added and the mixture incubated at 37°C for 2 h. An additional 3 µl restriction enzyme was added and the reaction incubated for another 3 h.

A modification was also used during Southern transfer, where 0.4 M NaOH was used instead of 10x SSC solution.

4.2.5 Statistical analysis

The χ^2 (*Chi-square*) test (Russell, 1990) was conducted to aid the interpretation of transgene inheritance in transgenic progeny.

4.3 Results

4.3.1 Transgene inheritance in T1 plants

Seventeen of the 41 grains (T0 grains) produced from the self-pollinated primary transformant W2 were randomly chosen for analyzing transgene inheritance in T1 progeny. Four of the 17 grains were germinated in Petri dishes for analyzing GUS expression in the roots before transferring to soil, and the other 13 grains were directly sown in soil. All 17 grains germinated and subsequently grew and developed into mature plants.

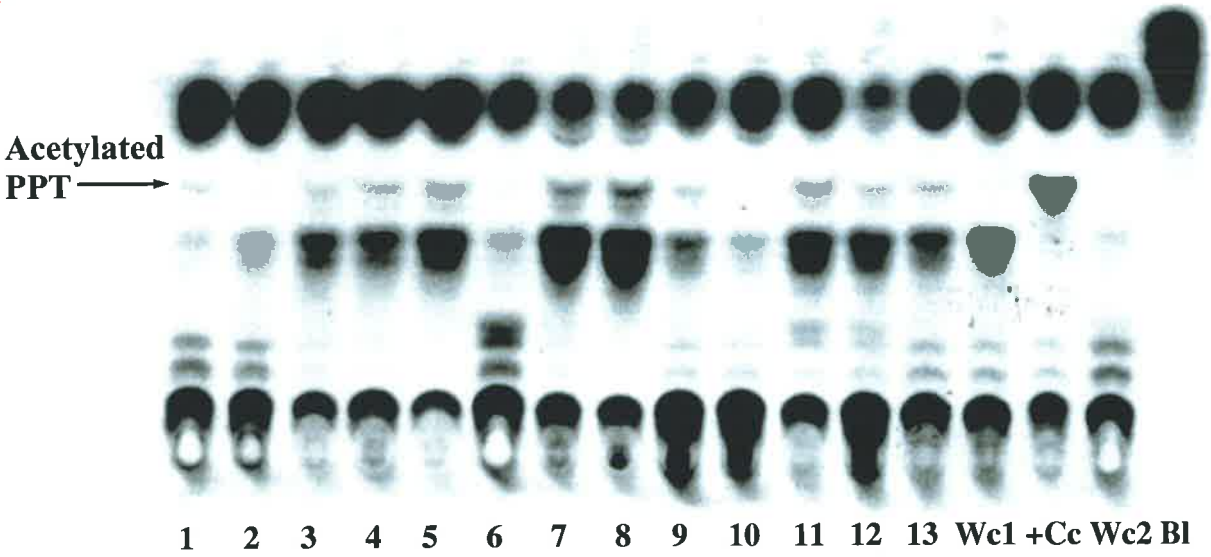
4.3.1.1 PAT assay

The expression of the *bar* gene in transgenic wheat progeny was analysed by performing PAT assays. Of the 17 plants tested, 14 plants showed PAT enzyme activity, but three plants (2, 6 and 10) did not show PAT activity (Figures 4.2A-B). Results from the PAT assays in leaves of T1 plants are summarized in Table 4.2.

4.3.1.2 Response to the herbicide leaf-dip test

Results from the leaf dip test indicated that 13 out of 17 T1 plants were tolerant to 0.01% Basta® solution (or 0.005% glufosinate ammonium), but the remaining four plants, no. 2, 6, 9 and 10, were as sensitive to the herbicide as control plants (Table 4.2). Furthermore, of the four T1 plants (no. 14-17) tested using high concentrations (0.1 and 1% Basta®), only one plant (no. 14) showed tolerance to 0.1% Basta®. Leaves from the other three plants (no. 15-17) became necrotic at this concentration. None of the four plants showed tolerance

A


Acetylated
PPT
1 2 3 4 5 6 7 8 9 10 11 12 13 Wc1 +Cc Wc2 Bl

B

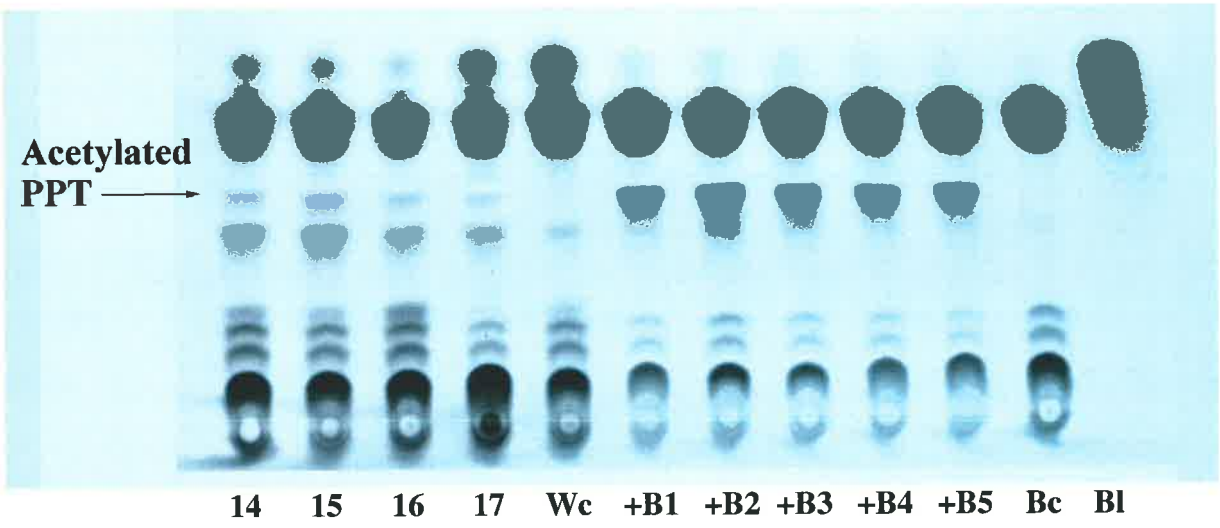

Acetylated
PPT
14 15 16 17 Wc +B1 +B2 +B3 +B4 +B5 Bc Bl

Figure 4.2 PAT assays of T1 progeny. (A) and (B) PAT activity in leaf extracts of T1 progeny (numbered 1-17) and in a control plant. The position of acetylated phosphinothricin is indicated by an arrow. Abbreviations: Bl: blank sample; Wc1-2; wheat negative control no. 1 and no. 2; +B1-5; positive control of transgenic barley plants no. 1-5 (provided by Dr. Javed Qureshi); Bc: barley negative control; +Cc: callus line positive control (provided by Mr. R. Singh).

Table 4.2 The presence and expression of the *bar* and *GUS* genes in T1 progeny of *cv.* Frame

Plant code	PAT assay	Leaf dip test for Basta® resistance	Southern blot		GUS staining	
			bar	*GUS*	Leaf	Anthers
1	+	+	+	+ (4*)	-	++
2	-	-	+	+ (3*)	+	+++
3	+	+	+	+ (3*)	-	++
4	+	+	+	+ (3*)	-	++
5	+	+	+	+ (2*)	-	++
6	-	-	+	+ (2*)	-	+
7	+	+	+	+ (2*)	+	++
8	+	+	+	+ (2*)	-	+
9	+	-	-	-	-	-
10	-	-	-	-	-	-
11	+	+	+	+ (4*)	+	++
12	+	+	+	+ (3*)	+	++
13	+	+	+	+ (2*)	-	++
14	+	+	+	+ (2*)	+	+++
15	+	+	+	+ (2*)	+	+
16	+	+	+	+ (2*)	-	+
17	+	+	+	+ (3*)	+	+++
χ^2 (for *bar* and *GUS*) = 1.59**			dF = 1		0.2 < P < 0.3	

Notes:
+ : shows positive result for the presence or expression of transgenes.
- : shows negative result for the presence or expression of transgenes.
* : numbers of hybridizing bands of the *GUS* gene observed.
** : Test of hypothesis for *bar* and *GUS* segregated in 3:1 ratio in T1 progeny (χ^2 = 1.59; 0.2<P<0.3), see Appendix 4.
χ^2 = *Chi-square*; dF = degree of freedom.

at 1% Basta® solution (Figure 4.3A). Based on results found here, it was decided to use a low concentration of Basta® solution (0.01%) when this herbicide was applied to T2 and T3 progeny.

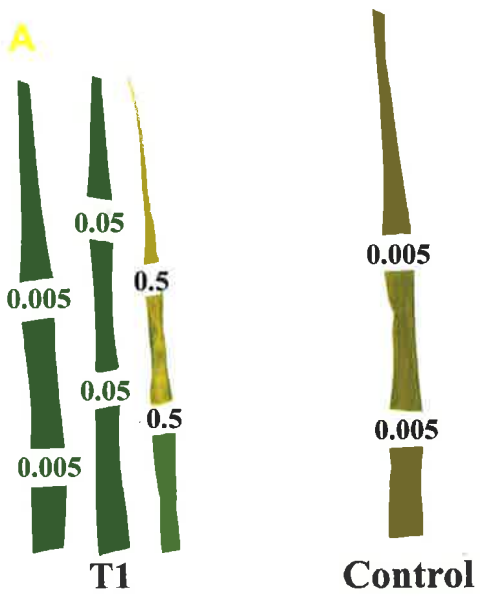
Overall, the results of leaf dip test did not exactly match those from the PAT assay, because one plant (no. 9) which showed PAT activity, proved to be sensitive to herbicide application (Table 4.2).

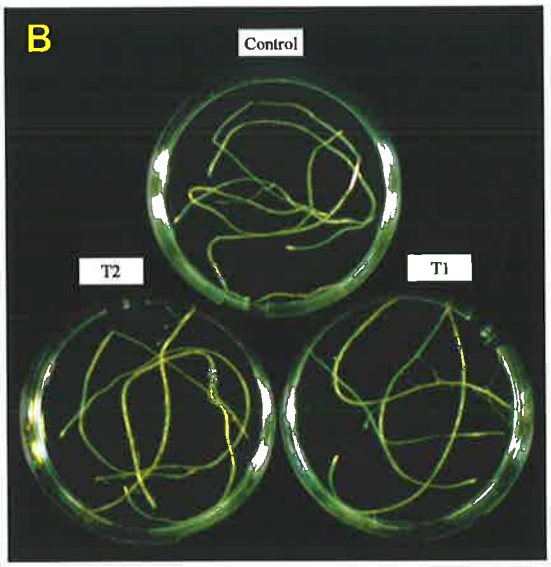
4.3.1.3 GUS staining

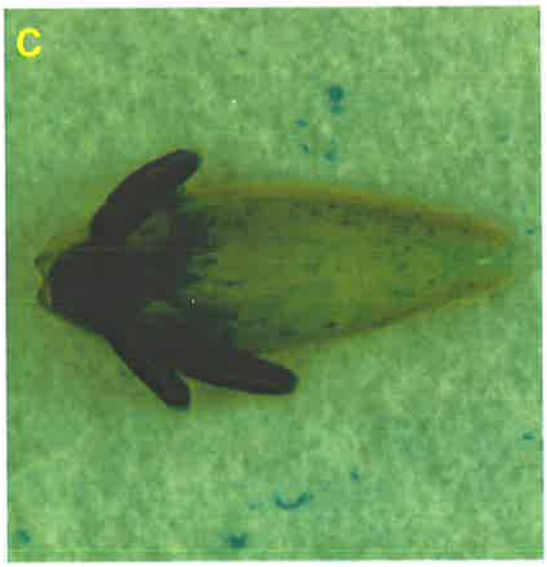
The expression of the *GUS* gene in T1 plants was analysed in roots, leaves, spikelets, anthers and grains. Variation in GUS activity could clearly be observed between tissues. Fifteen of 17 T1 plants showed high levels of GUS activity in anthers and spikelets. No GUS activity could be detected in the roots of the four plants tested (14-17) (Figure 4.3B). Furthermore, GUS expression in leaves was generally very low and could be detected in only seven of the 15 plants that showed activity in anthers. GUS activity could also be detected in several samples of grains (Figures 4.3C-E). It was noticed that the level of GUS expression in anthers varied from low (+) to high (+++) (Table 4.2; Figure 4.3D).

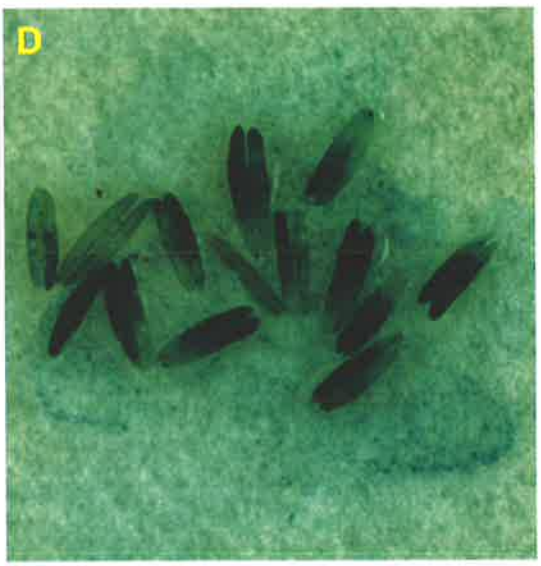
4.3.1.4 Southern blot analysis

Southern blot analyses showed that 15 of the 17 T1 plants analysed, namely the 15 plants showing GUS activity, carried the *GUS* gene (Figures 4.4B, 4.5B and 4.6B). It was noted that eight plants had two hybridizing bands (no. 5-8, 13-16), five plants with three hybridizing bands (no. 2-4, 12, and 17), two plants had four hybridizing bands (no. 1 and 11) and two plants (no. 9 and 10) had no detectable hybridizing bands (Table 4.2).


A
0.05
0.005
0.5
0.05
0.5
0.005
T1
0.005
0.005
Control


B
Control
T2
T1

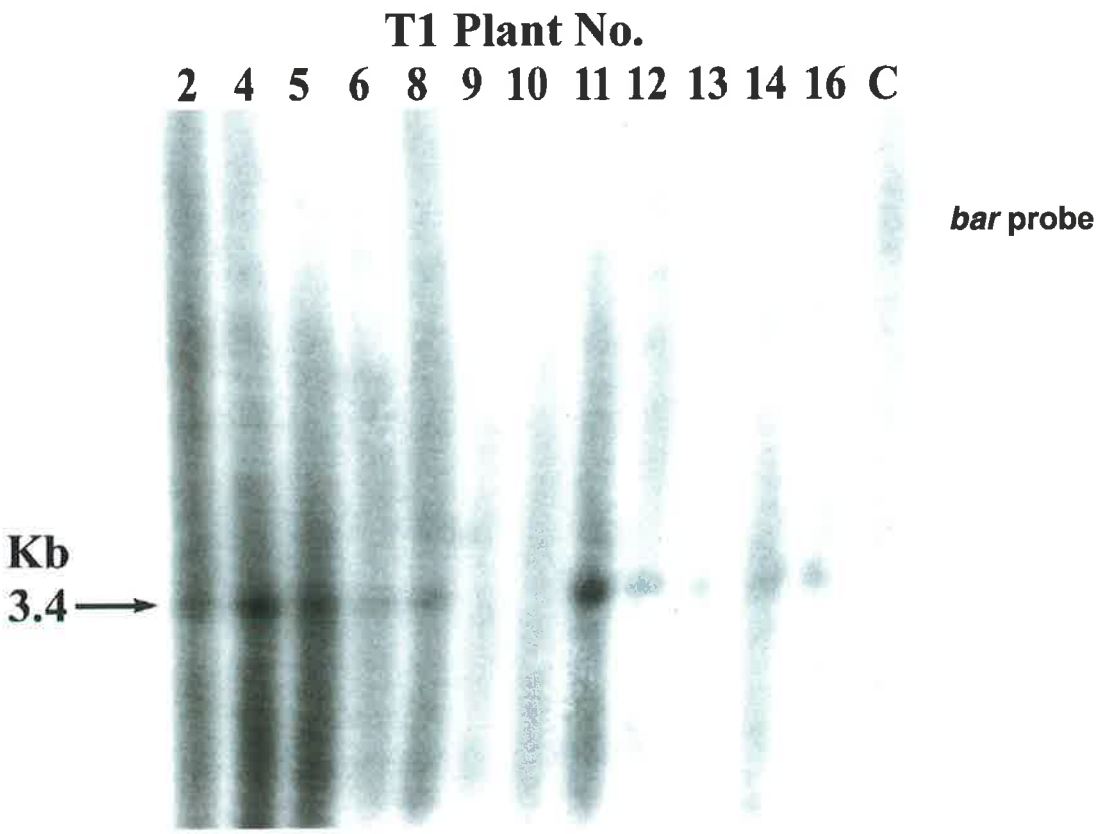

C


D


E
Control
T2 seeds

Figure 4.3 Analysis of transgene activity in T1 progeny. (A) The leaf dip test shows herbicide resistance at 0.1% and 0.01% Basta® solution (or 0.05% and 0.005% glufosinate ammonium, respectively) but necrosis at 1% Basta® solution or 0.5% glufosinate ammonium in leaves of transgenic plants (left). Leaves of control plants show necrosis at 0.01% Basta® solution (or 0.005% glufosinate ammonium) (right); (B) Absence of GUS activity in roots; (C) GUS activity in spikelet; (D) in anthers; and (E) in grains of transgenic (right) and control (left) plants.

A


T1 Plant No.
2 4 5 6 8 9 10 11 12 13 14 16 C
bar probe
Kb
3.4

B


T1 Plant No.
2 4 5 6 8 9 10 11 12 13 14 16 C
Kb
>12.0
9.0
7.9
6.0
GUS probe

Figure 4.4 Southern blot analysis of T1 progeny. Genomic DNA isolated from T1 plants was digested with *Hin*dIII. The digested DNA was hybridized with (A) *bar* probe, showing a single band (approx. 3.4 Kb); and (B) *GUS* probe, showing multiple bands (2-4 bands with sizes of approx. 6.0, 7.9, 9.0 and >12.0 Kb). Abbreviations : C: wheat control; T1: first transgenic wheat generation; Kb: kilobase.

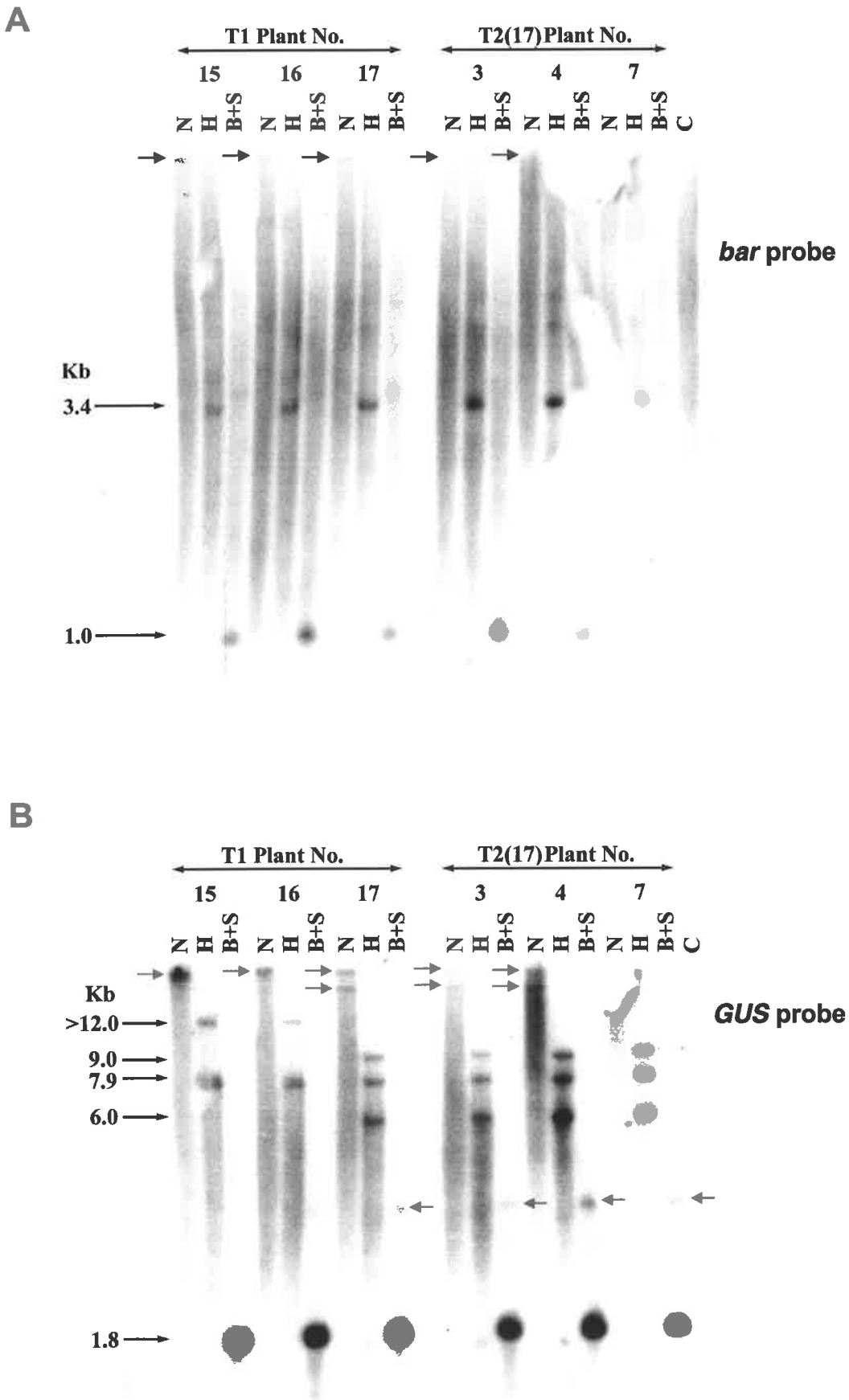

A
T1 Plant No.
T2(17) Plant No.
15
16
17
3
4
7
N H B+S N H B+S N H B+S N H B+S N H B+S C
bar probe
Kb
3.4
1.0
B
T1 Plant No.
T2(17) Plant No.
15
16
17
3
4
7
N H B+S N H B+S N H B+S N H B+S N H B+S C
GUS probe
Kb
>12.0
9.0
7.9
6.0
1.8

Figure 4.5 Southern blot analysis of T1 and T2 progeny. Genomic DNA isolated from T1 and T2 plants (derived from T1 plant no. 17) was digested with *Nco*I, *Hin*dIII and *Bam*HI + *Sst*I double digest. The digested DNA was hybridized with (A) *bar* probe and shows the predicted fragment of the pDM302 vector (approx.1.0 Kb) in the *Bam*HI+*SSt*I tracks, a single band in the *Hin*dIII (approx. 3.4 Kb) and in the *Nco*I (indicated by blue arrows, >12.0 Kb) tracks; and (B) *GUS* probe, showing the predicted fragment of the p*Act1*-D GUS vector (approx. 1.8 Kb) and an additional band (indicated by red arrows) in *Bam*HI+*Sst*I tracks, multiple bands (approx. 6.0, 7.9, 9.0 and >12.0 Kb) in *Hin*dIII tracks and one to two bands (indicated by pink arrows, >12.0 Kb) in *Nco*I tracks. Abbreviations: C: wheat control; T1 and T2: first and second transgenic wheat generations; Kb: kilobase. Abbreviations of restriction enzymes used: B+S: *Bam*HI and *Sst*I double digest; H: *Hin*dIII; N: *Nco*I.

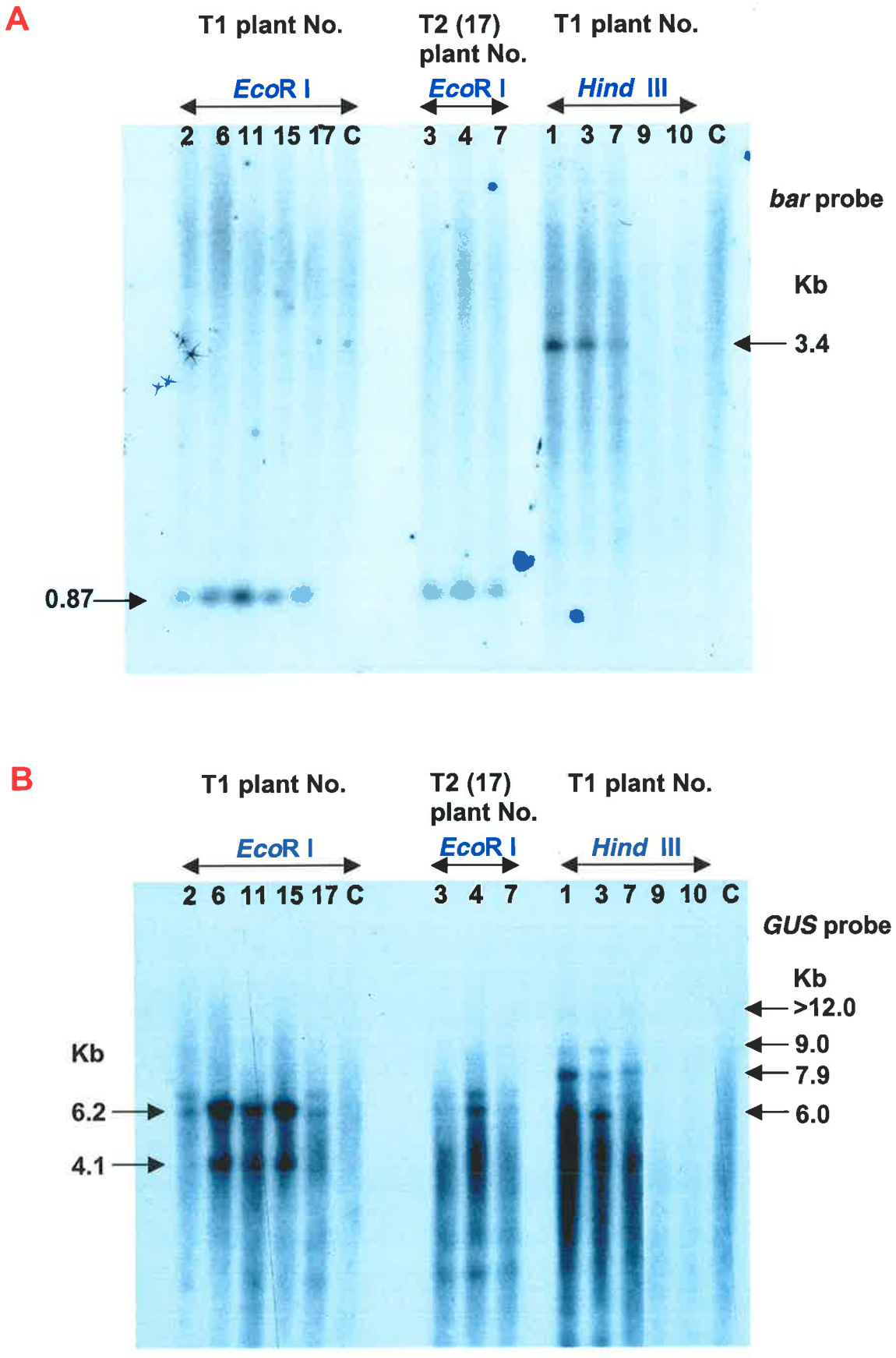

A
T1 plant No.
T2 (17) plant No.
T1 plant No.
EcoR I
EcoR I
Hind III
2 6 11 15 17 C
3 4 7
1 3 7 9 10 C
bar probe
Kb
3.4
0.87
B
T1 plant No.
T2 (17) plant No.
T1 plant No.
EcoR I
EcoR I
Hind III
2 6 11 15 17 C
3 4 7
1 3 7 9 10 C
GUS probe
Kb
>12.0
9.0
7.9
6.0
Kb
6.2
4.1

Figure 4.6 Southern blot analysis of T1 and T2 progeny. Genomic DNA isolated from T1 (2 sets of 5 plants) and T2 (3 plants derived from T1 plant no. 17) plants was digested with either *Eco*RI or *Hin*dIII. The digested DNA was hybridized with (A) the *bar* probe, showing the predicted fragment of the pDM302 vector (approx. 0.87 Kb) in *Eco*RI tracks; and (B) the *GUS* probe, showing two hybridizing bands in the *Eco*RI track. Genomic DNA from T1 plants no. 1, 3 and 7 digested with *Hin*dIII shows a single hybridizing band containing the *bar* gene (approx. 3.4 Kb) and multiple bands containing the *GUS* gene (2-4 bands with sizes of approx. 6.0, 7.9, 9.0 and >12.0 Kb). Plant no. 9 and 10 have not inherited the transgenes. Abbreviations: T1: first transgenic wheat generation; T2(17): second transgenic wheat generation derived from T1 plant no. 17; C: wheat control; Kb: kilobase. Abbreviations of restriction enzymes used: E: *Eco*RI; H: *Hin*dIII.

The presence of the inherited *GUS* gene in T1 plants was also confirmed by the digestion of genomic DNA derived from three T1 plants (no. 15, 16 and 17) with *Nco*I (Figure 4.5B). This enzyme cuts only in the flanking genomic DNA since no restriction sites are present in the p*Act1*-D *GUS* construct. It was noticed that T1 plant no. 17 had two distinguishable hybridizing bands (indicated by pink arrows) both of which were more than 12 Kb in size, suggesting that the copies of the *GUS* gene were integrated at different sites in the plant genome. In T1 plants no. 15 and 16, however, only one band was visible.

Further analysis using *Eco*RI on the DNA from five T1 plants (no. 2, 6, 11, 15 and 17), detected two hybridizing bands of the *GUS* gene in all plants (Figure 4.6B). One band of 6.2 Kb was common to all five plants, whilst plants no. 2 and 17 carried a fragment larger than this and plants no. 6, 11 and 15 shared a fragment of 4.1 Kb (Figure 4.6B).

Integrity of the *GUS* gene was checked by digesting genomic DNA from three T1 plants (no. 15, 16 and 17) with *Bam*HI and *Sst*I (Figure 4.5B). One intense band, which corresponds to the entire *GUS* gene with the size of approx. 1.8 Kb (Figure 4.1), could be easily detected in all tracks (Figure 4.5B). Moreover, it appeared that there was an additional fainter band (indicated by red arrows) in the digests of T1 (17) and T2 (17.3-4 and 17.7).

To analyse for the presence of the *bar* gene in the T1 progeny, the membrane was stripped and reprobed. All 15 plants carrying the *GUS* gene also had the *bar* gene (Figures 4.4A, 4.5A and 4.6A). Only one hybridizing band was detected in the *Hin*dIII (approx. 3.4 Kb), *Bam*HI+*Sst*I (approx. 1.0 Kb), *Nco*I (indicated by blue arrows, >12.0 Kb) and *Eco*RI (approx. 0.87 Kb) digests (Figure

4.1). From these results, it seems that only one copy of the *bar* gene is integrated in the genome of T1 plants.

The transmission of the *bar* and *GUS* genes from the W2 primary transformant to its T1 progeny resulted in 15 out of 17 T1 plants with both the transgenes. The segregation of the transgenes (*bar* and *GUS*) to T1 progeny suggested either a Mendelian pattern for two unlinked integration sites (15:1 ratio as compared to the 15:2 ratio observed), or a single integration site (3:1 ratio with χ^2= 1.59; 0.2<P<0.3; see Table 4.2 and Appendix 4).

Southern blot analyses were subsequently compared with histochemical GUS staining, the PAT assay and the herbicide leaf-dip test. It was found that all 15 plants carrying the transgenes showed GUS activity. However, only 13 out of these 15 plants were positive for PAT activity and the leaf-dip test. The remaining two plants (no. 2 and 6) lacked or had lost PAT activity, and showed sensitivity to herbicide application.

Of the two plants (no. 9 and 10) that did not show the presence of either transgene (Figures 4.4A-B and 4.6A-B), plant no. 9 showed PAT activity (Figure 4.2; Table 4.2). This result is similar to the analysis of putative primary transformants (Section 3.3.4) where some of the plants showed PAT activity without the *bar* gene being present.

4.3.1.5 Appearance of T1 plants

As stated earlier, 17 grains originating from the one T0 plant (W2) were successfully grown into mature plants. Visually, the majority of the T1 plants including both the transgenic progeny and non-transgenic segregants, showed poor phenotypic characteristics and low yield, which were relatively similar to the

primary transformant W2 (Figures 4.7 and 4.8). Fourteen of the 17 T1 plants showed a low yield of grain (no. of grains/plant), and one non-transgenic segregant, that is plant no. 9, was found to be completely sterile. Only two of the T1 plants (no. 3 and 15) showed a high yield (Table 4.3).

4.3.2 Transgene inheritance in T2 plants

In analysing transgene inheritance in T2 plants, three T1 plants showing different banding patterns of the *GUS* gene were chosen as parental plants, namely plants no. 11, 15 and 17. These plants showed 4, 2 and 3 hybridizing bands, respectively (Figures 4.4B and 4.5B), which suggests that these three plants had different numbers of insertions of the *GUS* gene. Thirty-three grains originating from these three plants (plants no. 11, 15 and 17 with 12, 6 and 15 grains, respectively) were randomly selected. All grains from T1 plants no. 11 and 15 were directly sown in the soil, while grains from T1 plant no. 17 were germinated in a vermiculite-filled tray for two weeks. Young seedlings from T1 plant no. 17 were subsequently transferred to soil and raised to maturity in the glasshouse. Prior to transferring to soil, two root tips from each plant were cut and stained for GUS expression.

4.3.2.1 PAT assay

PAT assays were performed on leaf extracts from all T2 plants (33 plants) (Figures 4.9A-C). PAT activity was observed in only seven of the 12 T2 (11.1-12), two out of six T2 (15.1-6) and six out of 15 T2 (17.1-15) plants (Table 4.4). These results clearly indicated that a considerable number of T2 plants did not show PAT activity. To confirm these results, one of the PAT negative plants, that is T2 plant

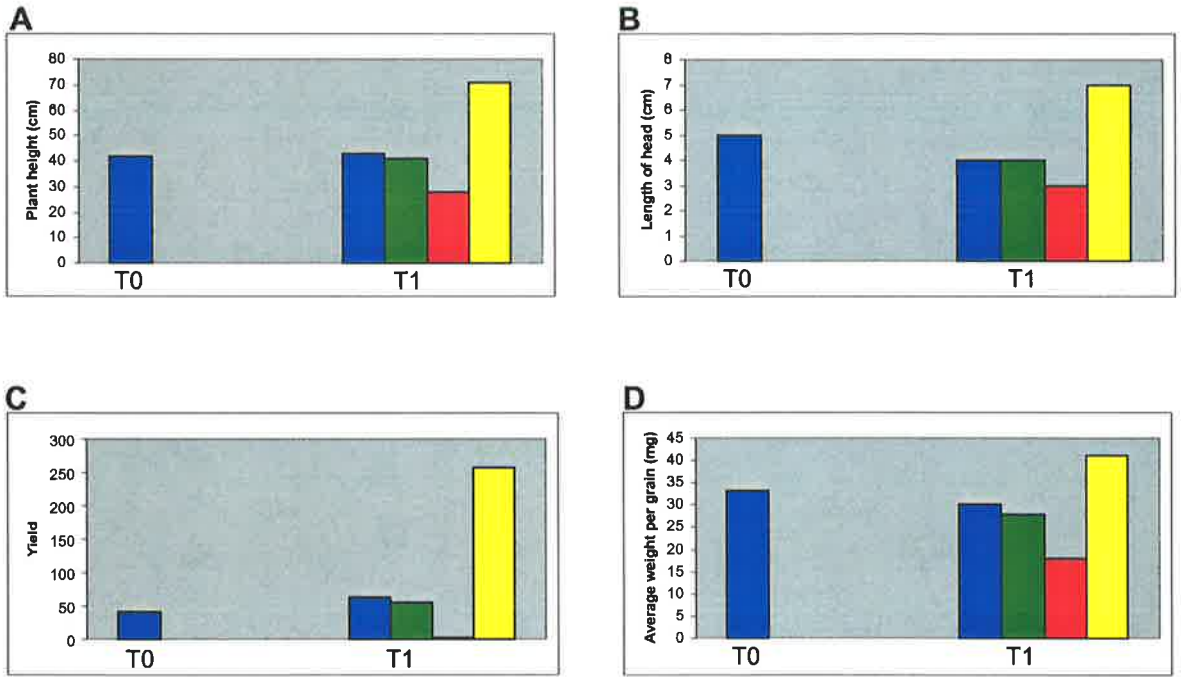


Figure 4.7 Phenotypic characteristics of transgenic plants (blue bars), non-transgenic segregants (red bars), T1 population (green bars) and control plants (yellow bars). (A) height of plant; (B) length of head; (C) yield (no. of grains/plant) and (D) average weight per grain.

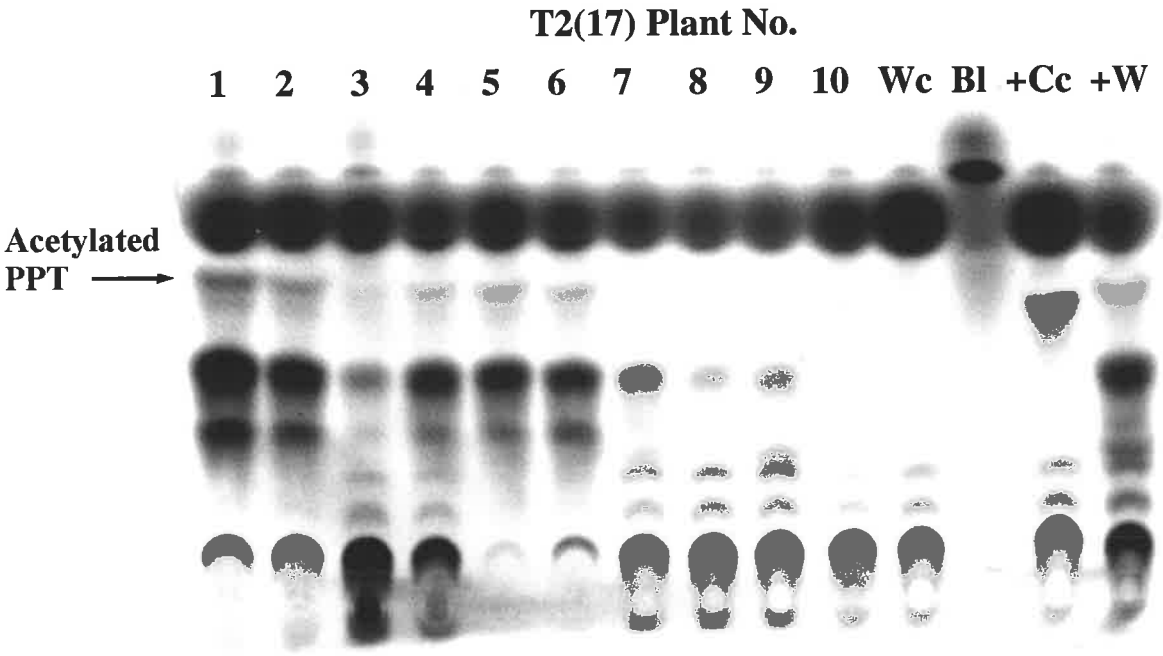
Figure 4.8 Mature, fertile transgenic wheat plants (*cv.* Frame) representative of T1 progeny.

Table 4.3 Phenotypic characteristics and yield of T1 progeny of *cv.* Frame

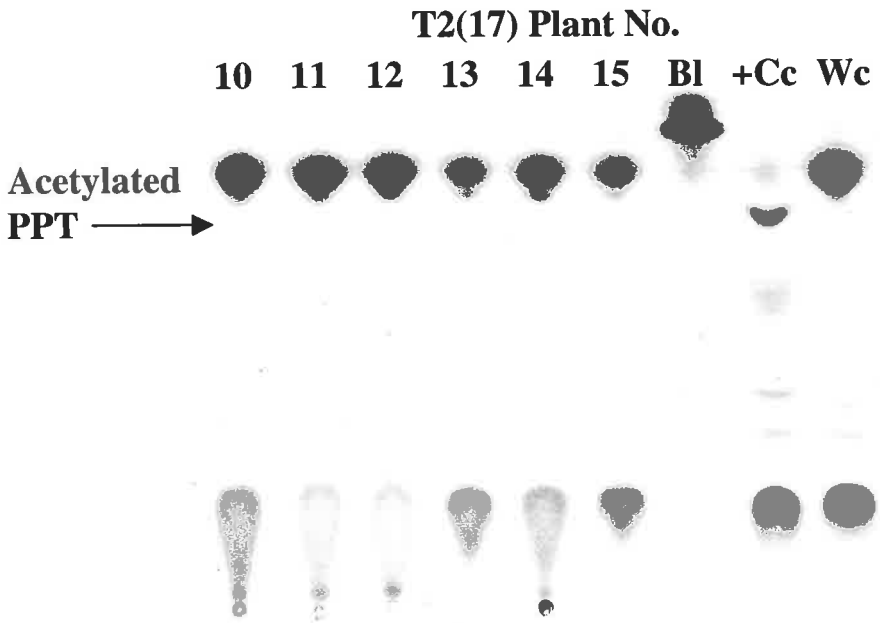
Plant code	Height (cm) (average)	Length of heads (cm)	Yield (No. of grains/plant)	Average weight per grain (mg)
1	46	4	43	21
2	41	3	49	22
3	48	4	100	25
4	46	4	53	34
5	43	3	24	36
6	41	3	49	37
7	48	4	64	35
8	44	3	34	33
9*	17	2	0	-
10*	39	3	4	36
11	44	4	29	34
12	32	3	38	36
13	47	4	55	28
14	33	4	26	32
15	47	4	224	21
16	48	5	64	29
17	39	4	86	22
Cont. 1	71	6	281	42
Cont. 2	68	6	199	40
Cont. 3	74	8	291	42

Notes:
* : non-transgenic segregants confirmed by Southern blot hybridization.
Cont : control plants.
Total grains produced by T1 progeny: 942.
Grains produced by transgenic progeny : 938.
Average weight per grain of transgenic progeny (mg) : 30.
Grains produced by non-transgenic segregants : 4.
Average weight per grain of non-transgenic segregants (mg) : 18.
Average yield of control plants : 257.
Average weight per grain of control plants (mg) : 41.

A


T2(17) Plant No.
1 2 3 4 5 6 7 8 9 10 Wc Bl +Cc +W
Acetylated
PPT

B


T2(17) Plant No.
10 11 12 13 14 15 Bl +Cc Wc
Acetylated
PPT

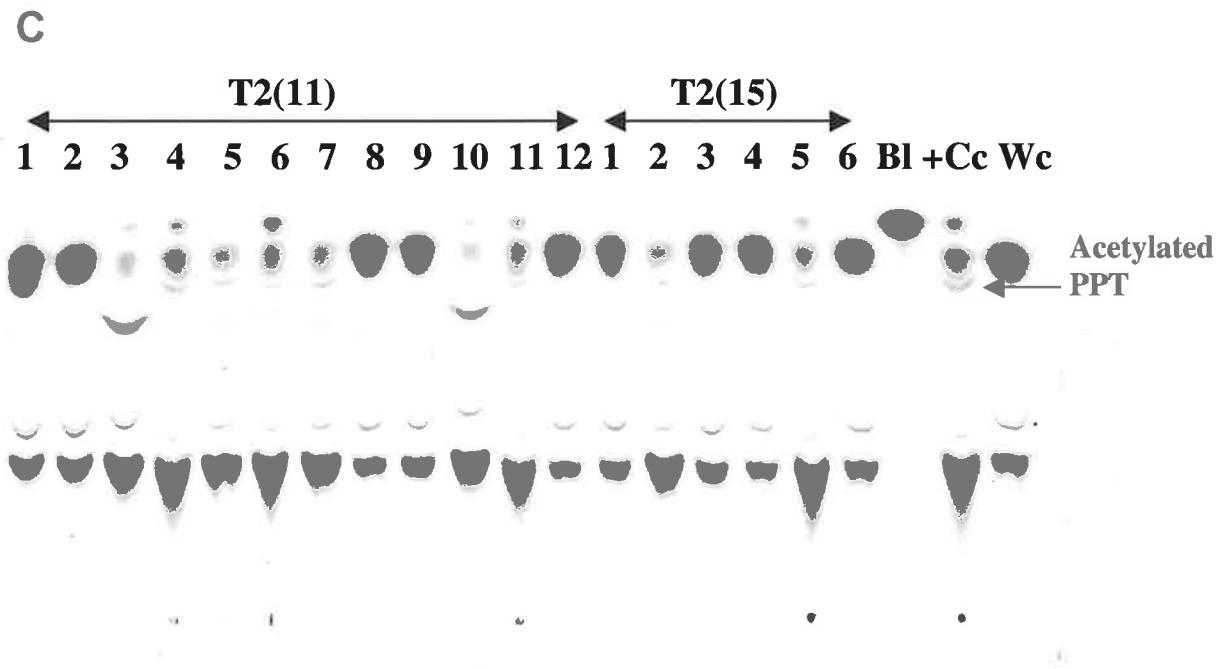


Figure 4.9 PAT assays of T2 progeny. (A) and (B) PAT activity in leaf extracts of T2 progeny (derived from T1 plant no. 17) and (C) T2 progeny (derived from T1 plants no. 11 and 15). The position of acetylated phosphinothricin is indicated by an arrow. Abbreviations: Bl: blank sample; Wc: wheat negative control; +Cc: callus line positive control (provided by Mr. R. Singh); +W: positive control of transgenic wheat progeny (T1).

no. 17.10, was analysed twice. Based on both PAT assays, it was concluded that this plant had lost PAT activity (Figures 4.9A-B).

4.3.2.2 Response to the herbicide leaf-dip test

As performed for T1 plants, two leaf tips from T2 plants were dipped into 0.01% Basta® solution or 0.005% glufosinate ammonium. Results from the leaf dip test showed that seven out of 12 T2(11.1-12) and two out of six T2(15.1-6) plants were tolerant to herbicide, while the other remaining plants were sensitive and responded similarly to the control plants (Figure 4.10A). In the case of T2 (17.1-15), six out of 15 plants showed tolerance and the other four plants showed very low tolerance to herbicide. In general, these results matched those of the PAT assay, where all the herbicide tolerant plants were PAT positive, whilst the herbicide sensitive plants were PAT negative. Results from the herbicide application test are summarized in Table 4.4.

4.3.2.3 GUS staining

Histochemical GUS staining in T2 plants was also carried out in roots, leaves, ovaries, anthers and grains. The results showed that nine out of 12 T2 (11), two out of six T2 (15) and all T2 (17) showed GUS activity. The expression of the *GUS* gene in these T2 plants was in most cases similar to the T1 plants in which no GUS activity could be detected in the roots (Figure 4.3B), low GUS activity was found in the leaves and high GUS activity was observed in ovaries, anthers and grains (Figures 4.10B-D). It was observed that the level of GUS activity in anthers of these T2 plants ranged from low (+) to high (+++) (see Table 4.4 for details).

Table 4.4 The presence and expression of the *bar* and *GUS* transgenes in T2 (11 and 15) progeny of *cv.* Frame

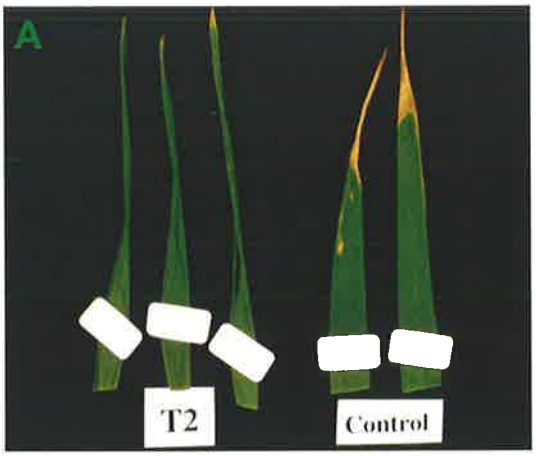
Plant code	PAT assay	Leaf dip test for Basta® resistance	Southern blot		GUS staining	
			Bar	*GUS*	Leaf	Anthers
T2(11)**						
11.1	-	-	-	-	-	-
11.2	-	-	+	+ (3*)	+	+
11.3	+	+	+	+ (4*)	+	++
11.4	+	+	+	+ (3*)	-	+++
11.5	+	+	+	+ (4*)	+	+++
11.6	+	+	+	+ (3*)	-	+++
11.7	+	+	+	+ (4*)	+	+++
11.8	-	-	+	+ (3*)	-	+++
11.9	-	-	-	-	-	-
11.10	+	+	+	+ (2*)	+	++
11.11	+	+	+	+ (2*)	-	++
11.12	-	-	-	-	-	-
T2(15)***						
15.1	-	-	-	-	-	-
15.2	+	+	+	+ (2*)	+	++
15.3	-	-	-	-	-	-
15.4	-	-	-	-	-	-
15.5	+	+	+	+ (2*)	+	++
15.6	-	-	-	-	-	-

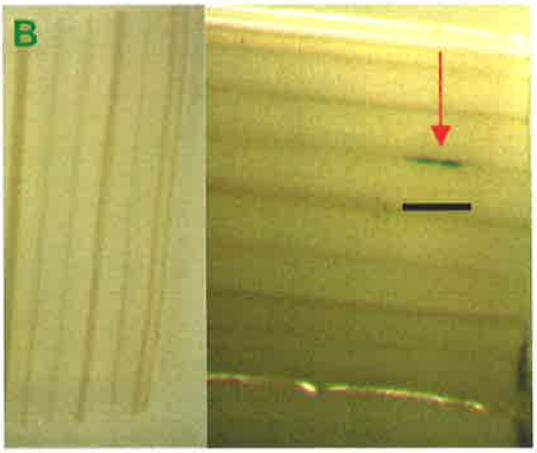
Notes:
+ : shows positive result for the presence or expression of transgenes.
- : shows negative result for the presence or expression of transgenes.
* : numbers of hybridizing bands of the *GUS* gene observed.
** : *bar* and *GUS* genes segregating 3:1 (or 9:3) ratio in T1(11)
*** : T2(15) was not analyzed using the χ^2 since the sample size was too small (6 plants).

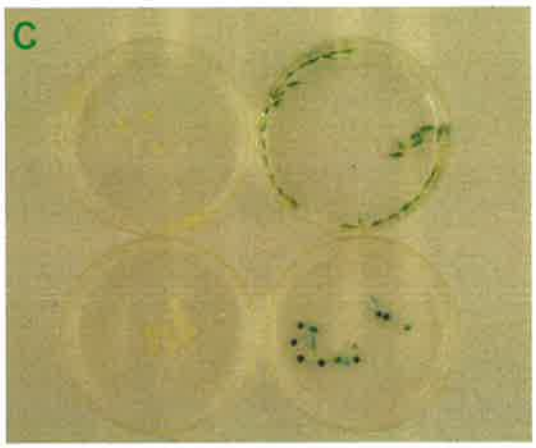
Table 4.4 (continued). The presence and expression of the *bar* and *GUS* transgenes in T2 (17) progeny of *cv.* Frame

Plant code	PAT assay	Leaf dip test for Basta® resistance	Southern blot		GUS staining	
			*Bar**	*GUS**	Leaf	Anthers
17.1	+	+	+	+	+	+++
17.2	+	+	+	+	+	+++
17.3	+	+	+	+	+	+++
17.4	+	+	+	+	-	+++
17.5	+	+	+	+	+	+++
17.6	+	+	+	+	-	+++
17.7	-	(+)	+	+	+	++++
17.8	-	(+)	+	+	-	+++
17.9	-	(+)	+	+	+	+++
17.10	-	-	+	+	+	++
17.11	-	-	+	+	-	++
17.12	-	-	+	+	+	+++
17.13	-	-	+	+	-	+++
17.14	-	-	+	+	+	+++
17.15	-	(+)	+	+	-	+++

Notes:
+ : shows positive result for the presence or expression of transgenes.
- : shows negative result for the presence or expression of transgenes.
* : hybridizing bands of the *bar* (1 band) and the *GUS* (3 bands) genes were similar in T1 plant no.17 and its progeny (T2 [17.1-15]).
( ) : plants showing low tolerance to herbicide application.


A
T2
Control


B


C

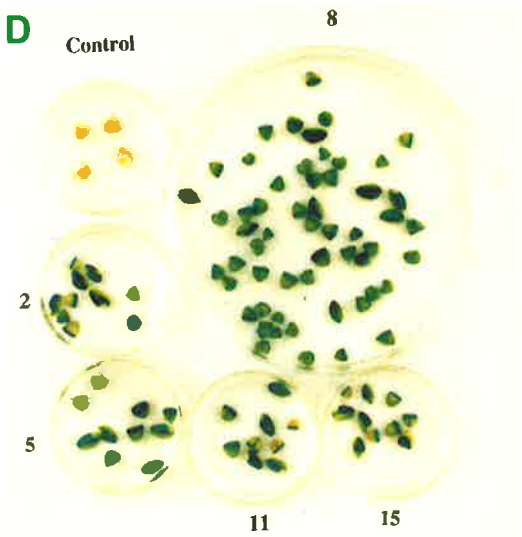

D
Control
8
2
5
11
15

Figure 4.10 Analysis of transgene activity in T2 progeny. (A) leaf dip test shows herbicide resistance at 0.01% Basta® solution in leaves of transgenic (left) and necrosis in leaves of control (right) plants; (B) GUS activity in leaf of transgenic (right, indicated by an arrow; a black bar represents approx. 1 mm) and control (left) plants; (C) in anthers and ovaries of transgenic (right) and control (left) plants; and (D) in grains of transgenic (indicated by plate number) and control (left, top) plants.

4.3.2.4 Southern blot analysis

Inheritance of the *GUS* gene in the T2 generation was confirmed by Southern blot analysis. Genomic DNA isolated from all of the T2 plants was digested with *Hin*dIII. Nine of 12 T2(11), two of six T2(15) and all T2(17) plants showed hybridization with the *GUS* probe, indicating the presence of the *GUS* gene in these plants (Figures 4.5B, 4.11B and 4.12B). These results matched those of GUS staining. The results also imply that the transmission of the *GUS* gene was still segregating in T2(11) and T2(15) progeny, but not in the T2(17) plants, suggesting that plants T1(11) and T1(15) were heterozygous and T1(17) was homozygous for the *GUS* transgene.

It was observed that the *GUS* genes in plant T1 no. 11 (this plant showed four hybridizing bands of the *GUS* gene) segregated in its progeny. Three T2(11) plants (no. 3, 5 and 7) had four hybridizing bands (approx. 6.0, 7.9, 9.0 and >12.0 Kb, as observed in the parental plant), and DNA from four plants (no. 2, 4, 6 and 8) produced three hybridizing bands (approx. 6.0, 7.9 and 9.0 Kb). Two plants (no. 10 and 11) possessed two hybridizing bands (approx. 7.9 and >12.0 Kb) and the remaining three plants (no. 1, 9 and 12) had no positive signal for the *GUS* gene (Figure 4.11B; Table 4.4).

In the case of progeny from plant T1 no. 15 (this plant showed two hybridizing bands of the *GUS* gene), Southern analyses of two T2 plants (no. 15.2 and 15.5) also showed two hybridizing bands of the *GUS* gene (approx. 7.9 and >12.0 Kb) (Figure 4.11B), whilst the remaining four plants have no bands. However, in plant T2 no. 17 (this plant showed three hybridizing bands of the *GUS* gene), all its progeny showed three hybridizing bands of the *GUS* gene (approx. 6.0, 7.9 and 9.0 Kb) in a pattern identical to the parental plant (Figure 4.12B).

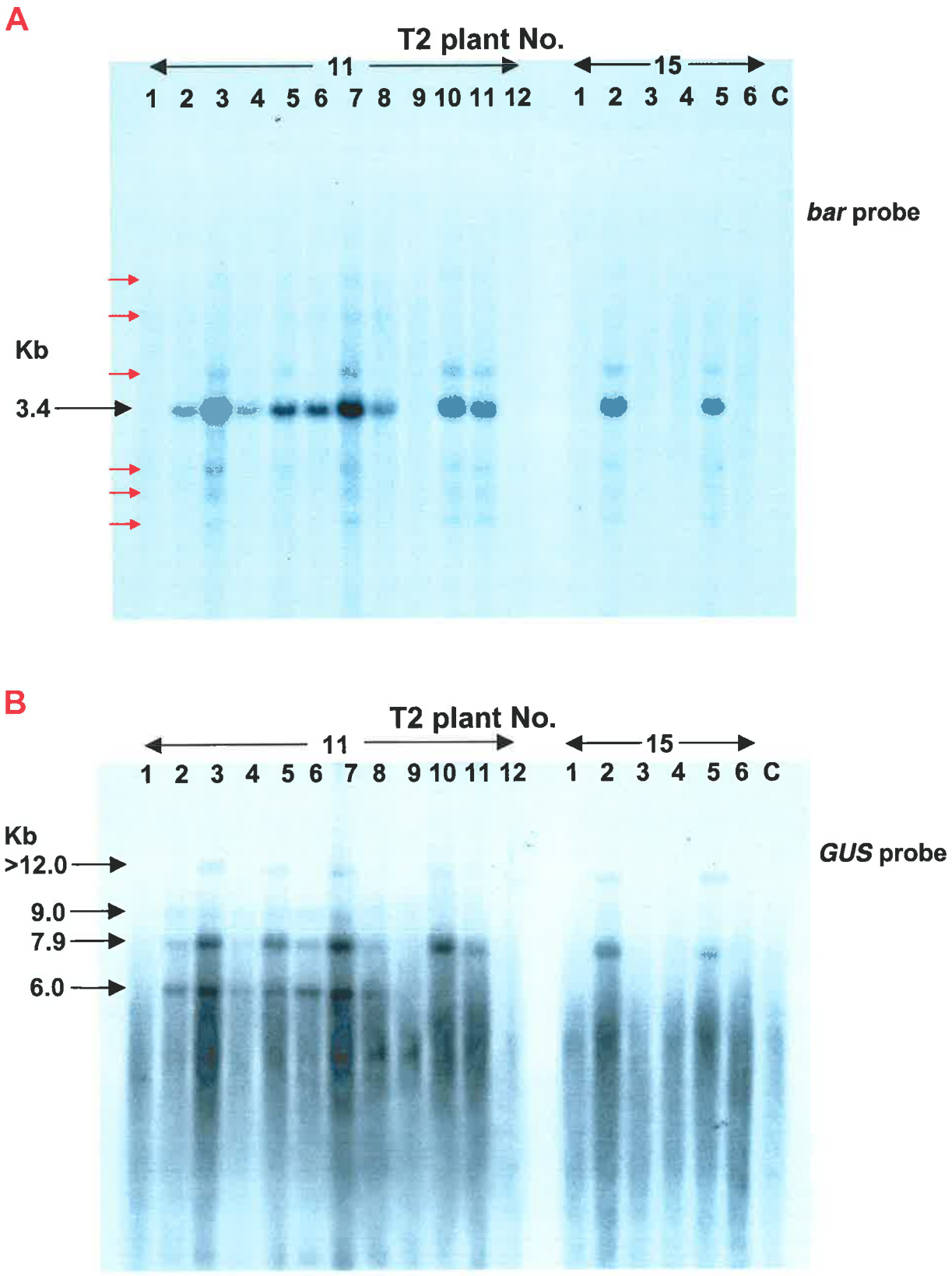

A
T2 plant No.
11
15
1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 C
bar probe
Kb
3.4
B
T2 plant No.
11
15
1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 C
Kb
>12.0
9.0
7.9
6.0
GUS probe

Figure 4.11 Southern blot analysis of T2(11 and 15) progeny. Genomic DNA isolated from T2 plants (derived from T1 plants no. 11 and 15) was digested with *Hin*dIII. The digested DNA was hybridized with (A) *bar* probe, showing an intense hybridizing band (approx. 3.4 Kb) with several additional faint bands (indicated by red arrows); and (B) *GUS* probe, showing variation in the number of hybridizing bands (2-4 hybridizing bands) in T2 plants derived from T1 plant no. 11 (T1 plant no. 11 had 4 hybridizing bands) and 2 bands in T2 plants derived from T1 plant no. 15 (T1 plant no. 15 also had 2 hybridizing bands). Abbreviations: C: wheat control; T2: second transgenic wheat generation; Kb: kilobase.

A


T2(17)Plant No.
1 2 5 6 8 9 10 11 12 13 14 15
bar probe
Kb
3.4

B

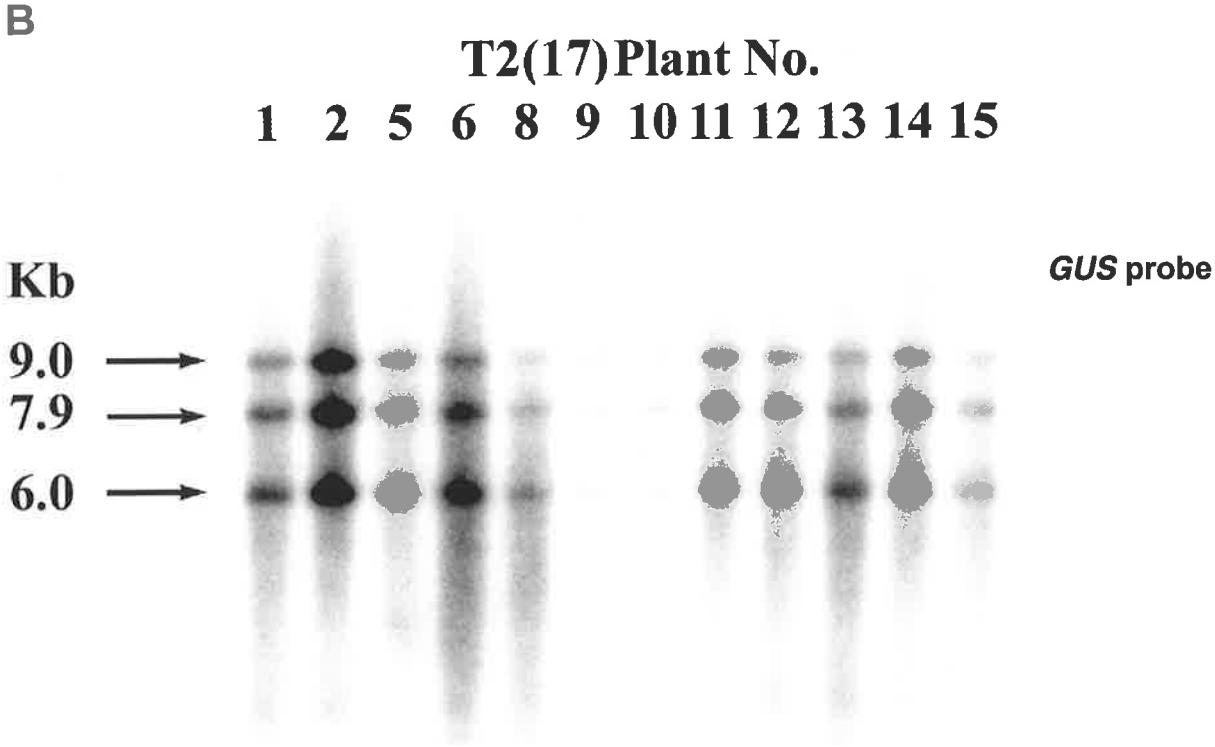

T2(17)Plant No.
1 2 5 6 8 9 10 11 12 13 14 15
GUS probe
Kb
9.0
7.9
6.0

Figure 4.12 Southern blot analysis of T2(17) progeny. Genomic DNA isolated from T2 plants (derived from T1 plant no. 17) was digested with *Hin*dIII. The digested DNA was hybridized with (A) *bar* probe, showing a single band (approx. 3.4 Kb); and (B) *GUS* probe, showing multiple bands (3 bands as exactly observed for the parental plant no. 17). Abbreviation : T2: second transgenic wheat generation; Kb: kilobase.

Genomic DNA from three T2(17) plants (no. 3, 4 and 7) was also digested with *Nco*I and *Bam*HI+*Sst*I double digest. Results from the Southern blot hybridization showed that two hybridizing bands (indicated by pink arrows, >12.0 Kb) in the *Nco*I tracks (Figure 4.5B). It should be noted here that these two bands could not be observed in T2 (17.7) because the gel was damaged near the *Nco*I track. It was also observed that a fragment of the *GUS* gene of approx. 1.8 Kb, with an additional band (indicated by red arrows) in the *Bam*HI+*Sst*I tracks, was present, as earlier seen in the parental plant (T1 no. 17) (Figure 4.5B). These results implied that the *GUS* genes integrated into the T1 (17) plant genome were transmitted to T2 (17.1-15) plants.

To confirm the presence of the *bar* gene in the T2 plants, the membrane used for analyzing the *GUS* gene was stripped and reprobed with the *bar* probe. Results showed that T2 plants carrying the *GUS* gene also had the *bar* gene. One intense band, approx. 0.87, 1.0 and 3.4 Kb in the *Eco*RI; *Bam*HI+*Sst*I, and *Hin*dIII digests, respectively, was identified in the T2 plants (Figures 4.5A, 4.6A and 4.12A). A single band was identified in *Nco*I tracks (Figure 4.5A, indicated by blue arrows). In Figure 4.11A, however, several fainter additional bands, which were not detected in the T1 plants, were identified in the *Hin*dIII tracks.

Results from the Southern analyses were subsequently compared with the results from the PAT assays and leaf dip tests. It was found that the *bar* gene inherited by T2 15.2 and T2 15.5 was functional, because the two plants showed herbicide tolerance and PAT activity (Table 4.4). In the case of progeny from T2(11) and T2(17), however, two out of nine T2 (11) and five to nine out of 15 T2 (17) plants having the *bar* gene showed no herbicide tolerance and/or PAT activity (Table 4.4).

Unlike the results observed for the expression of the *bar* gene, all plants carrying the *GUS* gene showed GUS activity (Table 4.4). The presence of GUS activity in T2(11.1-12) fitted a Mendelian inheritance pattern, where nine plants carried and expressed the *GUS* gene, while the other three plants did not (9:3 or 3:1 ratio). The T2(15.1-6) plants showed a 2:4 or 1:2 ratio, but in the T2(17.1-15) plants a homozygous phenotype apparently had been established, since all these progeny carried the *GUS* gene and showed GUS activity in a pattern identical to the parental plant.

4.3.2.5 Appearance of T2 plants

Thirty-three grains originating from three self-pollinated T1 plants (no. 11, 15 and 17) were successfully germinated and grown into mature plants. Visually, most of the T2 plants appeared normal. Compared with the T1 plants, the T2 plants showed improved vegetative growth (Figures 4.13 and 4.14). It was noted that the height of several T2 plants reached 70 cm and the length of head reached 6 cm. These characteristics were better than those for T1 plants where the tallest plant was 48 cm and the size of heads was around 3-4 cm. As observed for the T1 plants, several T2 plants produced low yields of grain, both in transgenic progeny and non-transgenic segregants. It was also noticed that grains produced by transgenic progeny and non-transgenic segregants were lighter than control plants (Table 4.5).

4.3.3 Transgene inheritance in T3 plants

For the analysis of transgene inheritance in T3 plants, T3 grains derived from T2 (17) plants were studied. This line appeared to be homozygous at the T2

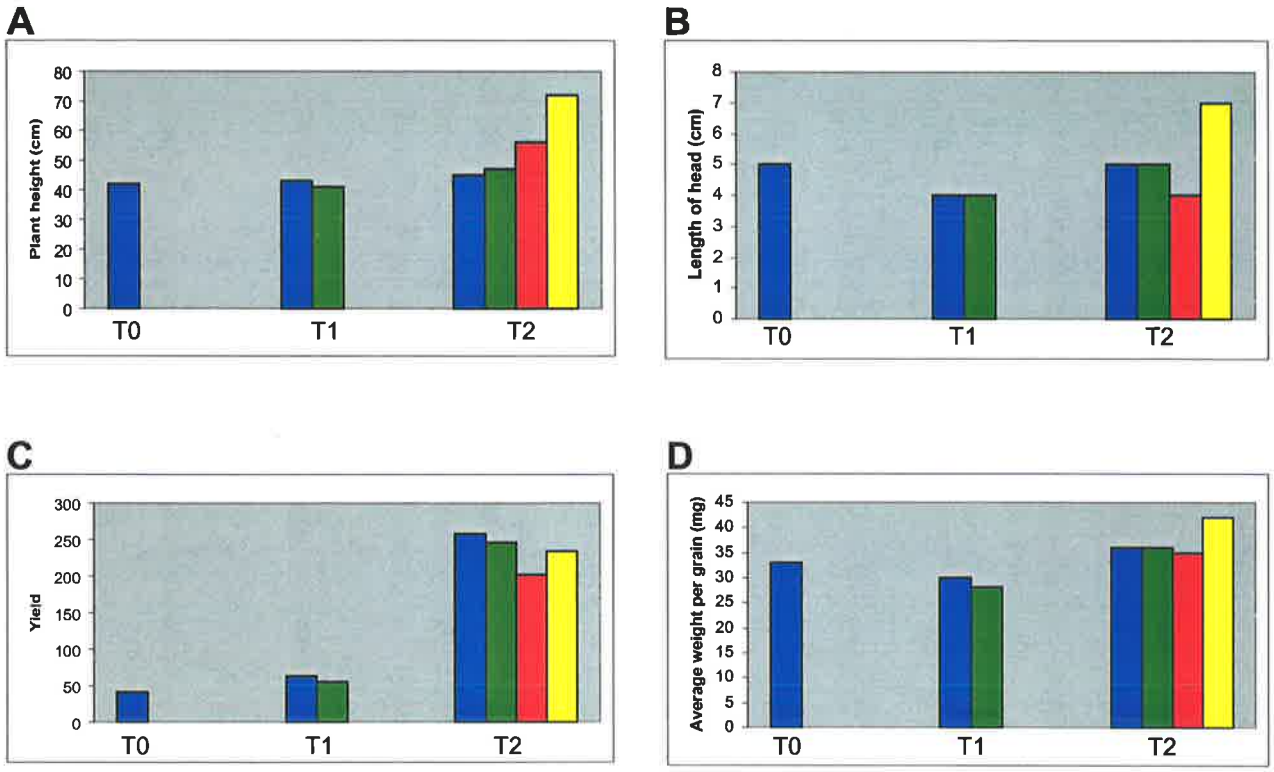


Figure 4.13 Phenotypic characteristics of transgenic plants (blue bars), non-transgenic segregants (red bars), T1 and T2 population (green bars) and control plants (yellow bars). (A) height of plant; (B) length of head; (C) yield (no. of grains/plant) and (D) average weight per grain.


T2
T2

Figure 4.14 Mature, fertile transgenic wheat plants (*cv.* Frame) representative of T2 progeny.

Table 4.5 Phenotypic characteristics and yield of T2 progeny of *cv.* Frame

Plant code	Height (cm) (average)	Length of heads (cm)	Yield (No. of grains/plant)	Average weight per grain (mg)
T2.11				
11.1*	53	4	203	33
11.2	27	3	13	33
11.3	55	5	79	41
11.4	44	4	186	41
11.5	42	4	282	33
11.6	44	4	130	40
11.7	44	4	166	29
11.8	53	5	183	35
11.9*	50	4	253	34
11.10	29	3	14	44
11.11	59	4	195	36
11.12*	70	6	389	40
T2.15				
15.1*	49	4	109	30
15.2	77	6	415	36
15.3*	46	4	53	36
15.4*	61	4	214	35
15.5	60	4	210	34
15.6*	65	5	194	35
T2.17				
17.1	45	6	681	33
17.2	51	6	332	29
17.3	43	5	205	34
17.4	34	5	341	41
17.5	38	6	316	42
17.6	41	6	498	43
17.7	40	5	263	39
17.8	41	6	601	39
17.9	36	5	226	38
17.10	32	4	172	26
17.11	48	5	265	33
17.12	41	5	351	30
17.13	45	5	109	32
17.14	47	5	215	39
17.15	48	5	268	35
Cont.1	65	5	154	38
Cont.2	75	7	247	44
Cont.3	77	8	301	44

Notes:
Cont: Control plants
* : Non-transgenic segregants confirmed by Southern blot hybridization.
Grains produced by transgenic T1 progeny : 6,716. Average weight per grain of transgenic T1 progeny (mg) : 36.
Grains produced by non-transgenic segregants : 1,415. Average weight per grain of non-transgenic segregants (mg) : 35.
Average yield of control plants : 234. Average weight per grain of control plants (mg) : 42.

stage. Six grains, each originating from self-pollinated T2 plants numbers 17.1 and 17.5, were selected and germinated in vermiculite for two weeks. Young seedlings were subsequently transferred to soil and grown to maturity in the glasshouse. Prior to transferring to soil, two root tips from each plant were cut and stained in X-gluc solution in order to test for GUS expression in the roots.

4.3.3.1 PAT assay

A PAT assay was performed on the 12 T3 plants. PAT activity was detectable in all plants (Figure 4.15 and summarized in Table 4.6), but for at least two of the 12 plants, activity was low.

4.3.3.2 Response to the herbicide leaf-dip test

Two leaf tips from the T3 plants were dipped into 0.01% Basta®. Ten of the 12 T3 plants were tolerant to herbicide, while the other two plants were sensitive. The herbicide sensitive plants and control plants showed necrosis within seven days after herbicide application (Figure 4.16A). The results from the herbicide leaf dip test on T3 plants are summarized in Table 4.6.

4.3.3.3 GUS staining

Histochemical GUS staining in T3 plants was also carried out for roots, leaves, ovaries, anthers and grains. The patterns of GUS activity in T3 plants were in most cases similar to the T1 and T2 plants in which no GUS activity could be detected in the roots, low GUS activity was found in the leaves and high GUS activity was observed in anthers, ovaries and grains (Figures 4.16B-D). It was

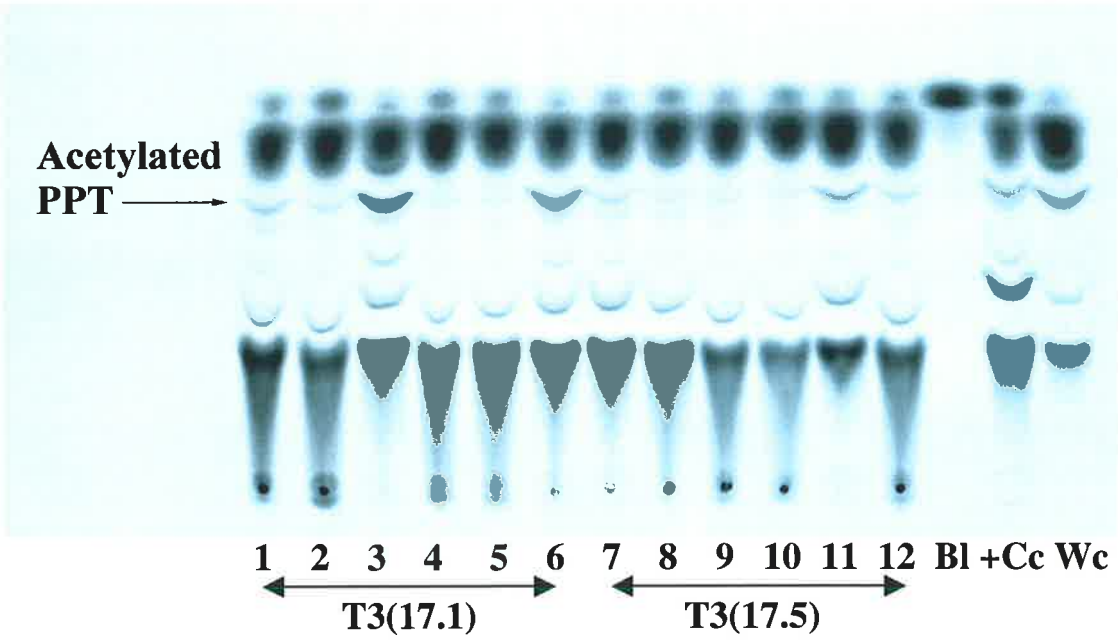


Figure 4.15 PAT assay of T3 progeny. PAT activity in leaf extracts of T3 progeny (derived from T2 plants no. 17.1 and 17.5) and a control plant. The position of acetylated phosphinothricin is indicated by an arrow. Abbreviations: Bl: blank sample; Wc: wheat negative control; +Cc: callus line positive control (provided by Mr. R. Singh).

Table 4.6 The presence and expression of the *bar* and *GUS* transgenes in T3 (17.1 and 17.5) progeny of *cv.* Frame

Plant code	PAT assay	Leaf dip test for Basta® resistance	Southern blot		GUS staining	
			*Bar**	*GUS**	Leaf	Anthers
T3(17.1)						
17.1.1	+	+	+	+	+	+++
17.1.2	+	+	+	+	+	+++
17.1.3	+	+	+	+	+	+++
17.1.4	(+)	-	+	+	+	++
17.1.5	(+)	-	+	+	+	+++
17.1.6	+	+	+	+	+	++
T3(17.5)						
17.5.7	+	+	+	+	+	+++
17.5.8	+	+	+	+	-	+++
17.5.9	+	+	+	+	+	+++
17.5.10	+	+	+	+	+	+++
17.5.11	+	+	+	+	+	++
17.5.12	+	+	+	+	-	+++

Note:
+ : shows positive result for the presence or expression of transgenes.
- : shows negative result for the presence or expression of transgenes.
* : hybridizing bands of the *bar* (1 band) and the *GUS* (3 bands) genes were similar in T2 (17.1 and 17.5) plants and its progeny (T3 [17.1.1-6 and 17.5.7-12]).
( ) : plants showing low PAT activity.

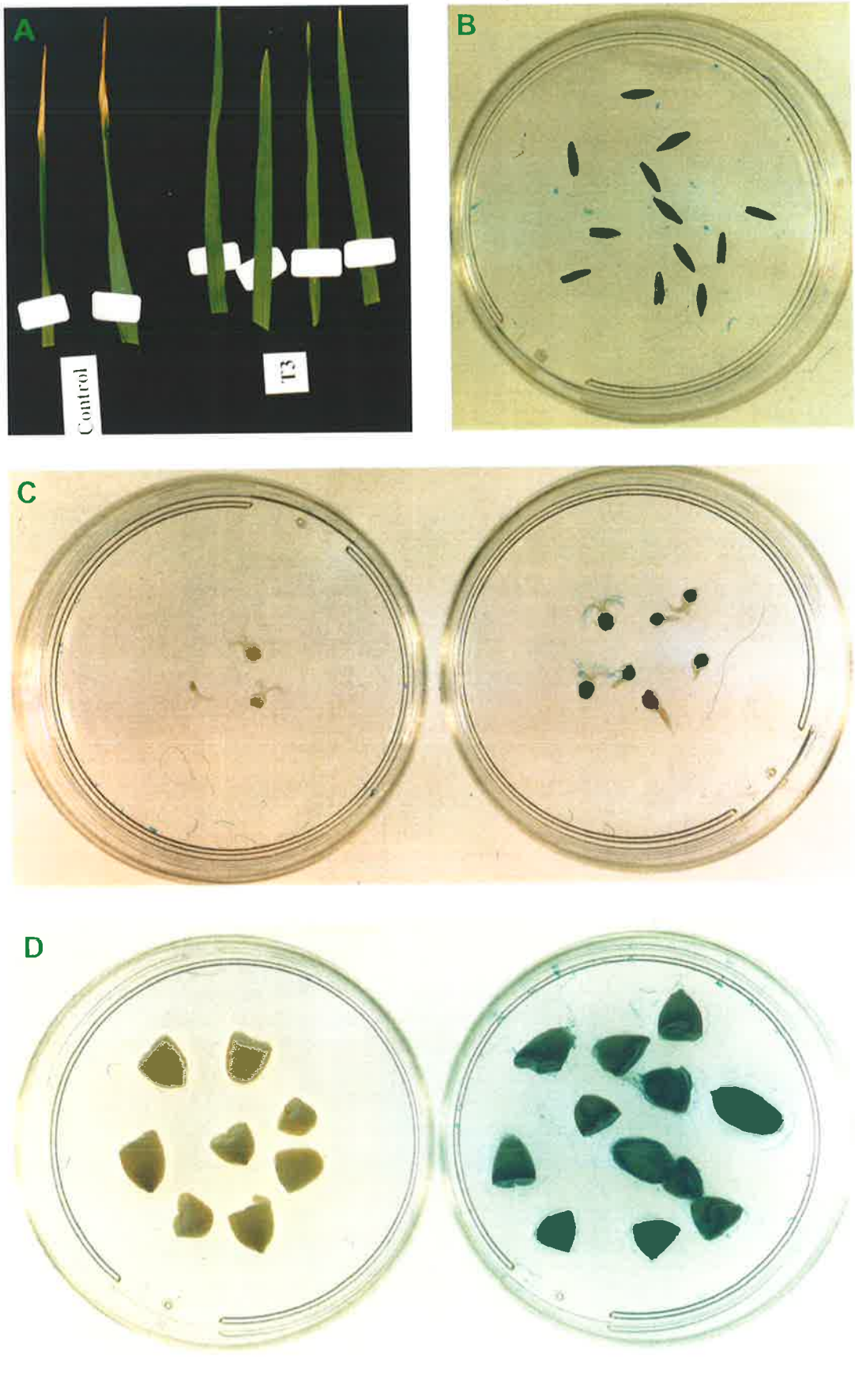

A
Control
T3
B
C
D

Figure 4.16 Analysis of transgene activity in T3 progeny. (A) leaf dip test shows herbicide resistance at 0.01% Basta® solution in leaves of transgenic (right) and necrosis in leaves of control (left) plants; (B) GUS activity in anthers; (C) in ovaries of transgenic (right) and control (left) plants; and (D) in grains of transgenic (right) and control (left) plants.

found that all the T3 plants showed GUS activity in their anthers and the levels ranged from medium (++) to high (+++) (Table 4.6).

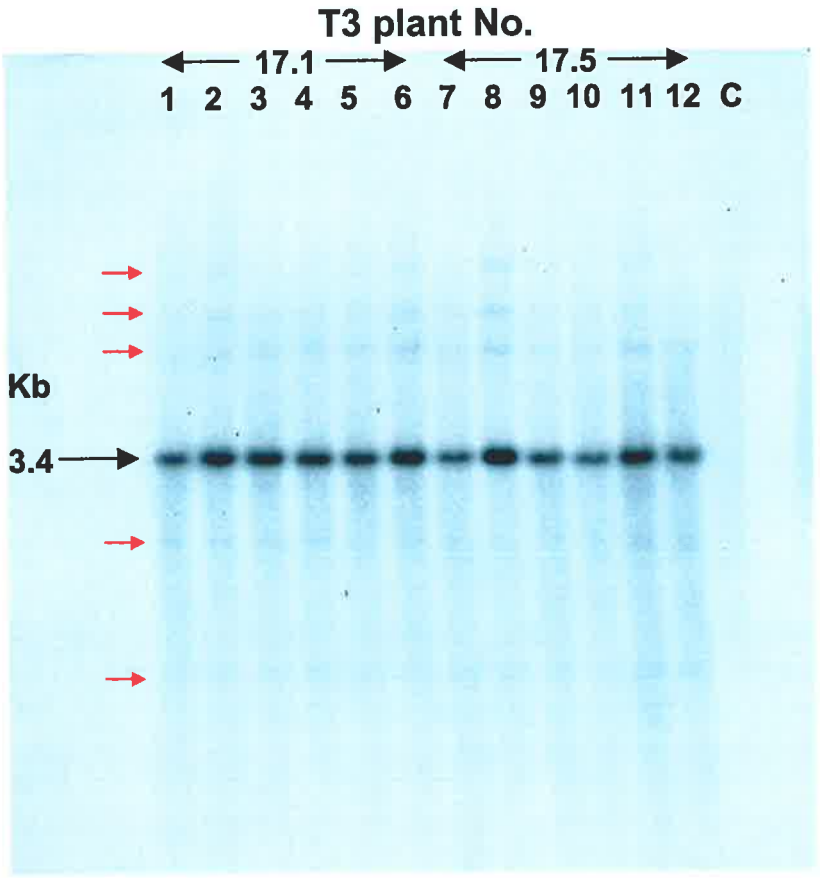
4.3.3.4 Southern blot analysis

The presence of the *bar* and the GUS genes in the T3 generation was confirmed by Southern blot analysis. As for T1 and T2 plants, genomic DNA isolated from the 12 T3 plants was digested with *Hin*dIII. All the T3 plants showed hybridization with the *bar* and *GUS* probes, indicating the presence of the *bar* and the *GUS* genes in the genome of these plants. These plants carried a single *bar* gene (approx. 3.4 Kb) and three "copies" of the *GUS* gene (approx. 6.0, 7.9 and 9.0 Kb), as identified in plant T1 no. 17 and T2 plants no. 17.1 and 17.5 (Figures 4.17A-B). As shown in Figure 4.17A, all plants had extra faint bands for the *bar* gene, and these extra bands were also observed in the primary transformant and T2 progeny.

Since all T3 plants analysed here possessed the same apparent copy numbers for both the *GUS* and the *bar* genes as the parental plants (T1 no. 17; T2 no. 17.1 and 17.5), it was concluded that this line was homozygous for the transgenes.

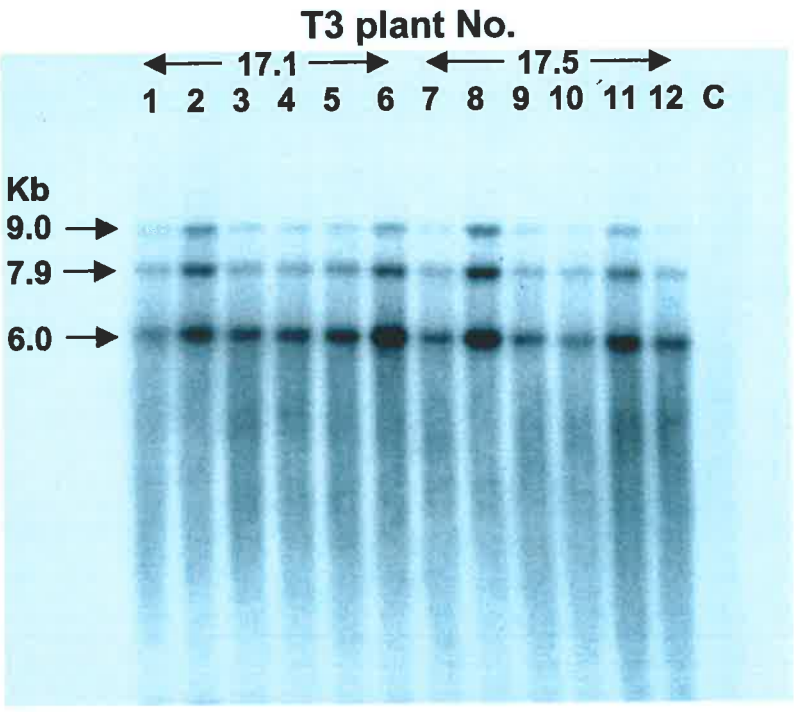
Results from the molecular analysis were subsequently compared with the results from histochemical GUS staining, the PAT assay and leaf dip tests. It was found that the results from the molecular analysis matched those from histochemical GUS staining, since all plants carried and stably expressed the *GUS* gene. However, although the results from the molecular analysis of the *bar* gene matched those from the PAT assay, some of the plants showed very low PAT activity and reduced tolerance to herbicide application (Table 4.6).

A



bar probe

B



GUS probe

Figure 4.17 Southern blot analysis of T3 progeny. Genomic DNA isolated from T3 plants (derived from T2 plants no. 17.1 and 17.5) was digested with *Hin*dIII. The digested DNA was hybridized with (A) *bar* probe, showing an intense hybridizing band (approx. 3.4 Kb) with several additional faint bands (indicated by red arrows); and (B) *GUS* probe, showing multiple bands (3 bands as observed for the parental T1 plant no. 17 and T2 plants no. 17.1 and 17.5). Abbreviations: C: wheat control; T3: third transgenic wheat generation; Kb: kilobase.

4.3.3.5 Appearance of T3 plants

All grains (12 grains) originating from self-pollinated T2 plants (no. 17.1 and 17.5) grew into mature plants. Visually, the majority of the T3 plants grew healthily (Figure 4.18). It was noticed that the vegetative growth of the T3 plants was in most cases similar to the T2 plants. The incidence of low fertility (low yield) was found in only one of the T3 plants (Table 4.7). Compared with control plants, the transgenic progeny showed retarded growth, lower yields and lighter grains. Phenotypic characteristics of the T3 plants and control plants are shown in Figure 4.19.


T3
T3

Figure 4.18 Mature, fertile transgenic wheat plants (*cv.* Frame) representative of T3 progeny.

Table 4.7 Phenotypic characteristics and yield of T3 progeny of *cv.* Frame

Plant code	Height (cm) (average)	Length of heads (cm)	Yield (No. of grains/plant)	Average weight per grain (mg)
T3.17.1				
1	58	4	327	37
2	49	4	171	31
3	51	4	156	31
4	42	4	147	30
5	50	4	310	32
6	49	4	246	34
T3.17.5				
7	48	4	339	30
8	42	4	211	32
9	51	4	200	31
10	27	3	23	24
11	47	4	218	35
12	54	4	244	30
Cont. 1	70	7	251	43
Cont. 2	74	8	273	44
Cont. 3	72	8	302	40

Notes:
Cont : control plants.
Total grains produced by T3 progeny: 2,592.
Average weight per grain of T3 progeny (mg) : 31.
Average yield of control plants : 275.
Average weight per grain of control plants (mg) : 42.

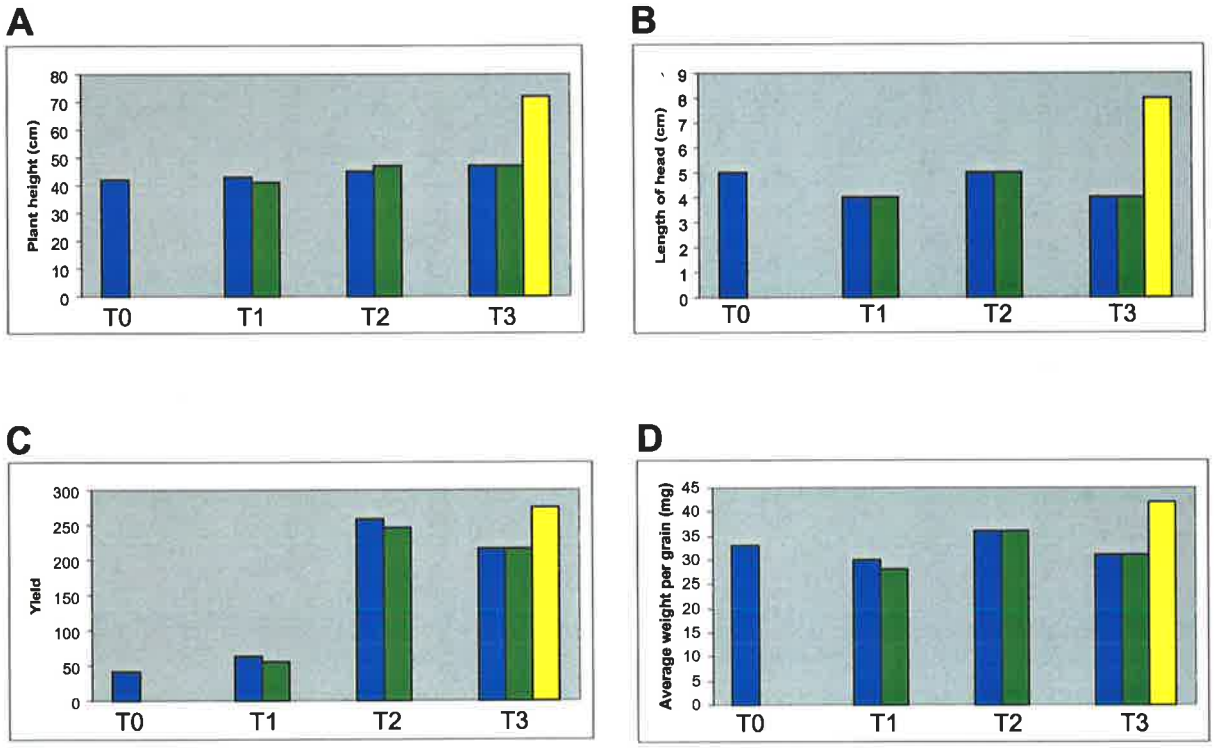


Figure 4.19 Phenotypic characteristics of transgenic plants (blue bars), T1, T2 and T3 population (green bars) and control plants (yellow bars). (A) height of plant; (B) length of head; (C) yield (no. of grains/plant) and (D) average weight per grain.

4.4 Discussion

The integration and expression of the *bar* and the *GUS* genes in the W2 primary transformant (T0 plant) have been described in Chapter 3. In the present Chapter, it is demonstrated that the transgenes were both inherited and in most cases expressed in successive generations up to and including the T3 generation.

The presence of the *bar* gene in T1 plants was confirmed by Southern blot hybridization. From 17 T1 plants analysed, 15 plants carried the *bar* gene (Figures 4.4A, 4.5A and 4.6A). Only one hybridizing band was found for the *bar* gene in digests of all restriction enzymes used, although in subsequent analysis of T2 and T3 plants, several additional weak bands were also detected (Figures 4.4A, 4.5A and 4.6A). The weak bands could be due to integration of a truncated fragment of the *bar* gene, or to incomplete DNA digestion. The presence of the *bar* gene in T1 plants implies that the *bar* gene earlier identified in the primary transformant (T0) was transmitted to T1 plants.

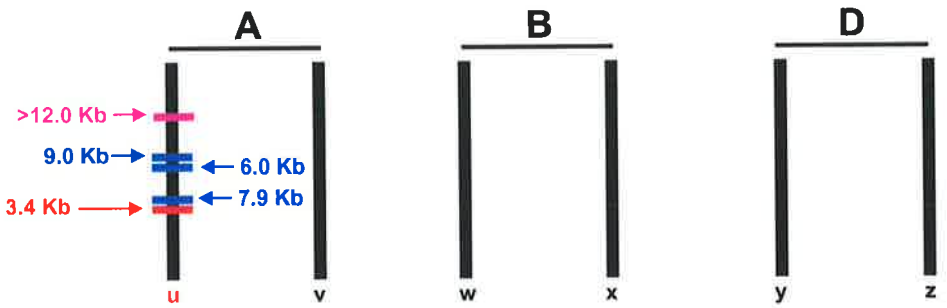
The 15 plants carrying the *bar* gene also had the *GUS* gene, whilst the two plants which were negative for the *bar* gene did not have the *GUS* gene (Figures 4.4B, 4.5B and 4.6B). Based on Southern blot analysis, it appears that the introduced *GUS* genes have been incorporated as multiple copies. This is indicated by the presence of multiple hybridizing bands (2-4 bands) of the *GUS* gene in *Hin*dIII digests and at least two different fragments in *Eco*RI digests (Figures 4.4B, 4.5B and 4.6B). However, a single band of predicted size, corresponding to the coding region the *GUS* gene, was found in *Bam*HI+*Sst*I digests, together with one extra, anomalous band (Figure 4.5B). The extra band could again result from partial hydrolysis of plant DNA. The *GUS* gene copies appear to occupy different sites in the genome, as indicated by the presence of

two hybridizing bands in *Nco*I digests (Figure 4.5B, see plants T1 17 and T2[17.3,4,7]).

Based on all molecular analyses presented here, it is clear that the transgenes integrated into the genome of the primary transformant (W2 plant) were transmitted to the T1 progeny. Since only a small number of plants were analysed during this study, it is difficult to deduce with surety the transgene inheritance pattern. With the availability of data collected in analysing T1 plants, it appeared that the transmission of transgenes from the primary transformant to T1 plants showed a Mendelian pattern for two unlinked insertions, e.g. a 15:1 ratio, which was close to the 15:2 ratio found here (Table 4.4). However, such a segregation pattern failed to explain the absence of segregants carrying one of the transgenes, especially the *GUS* gene.

Another possible pattern of transgene inheritance which would fit the segregation ratio found here was a Mendelian pattern for a single insertion, e.g a 3:1 ratio, which is still comparable to a segregation ratio of 15:2 (15:2 or 7.5:1 with χ^2 = 1.59; 0.2<P< 0.3, Appendix 4). Based on this segregation pattern, it is possible that the *bar* and the *GUS* transgenes were integrated into the same region of one chromosome, and as a consequnce the inheritance of the transgenes behaved as a single dominant trait (heterozygous genotype) (Figure 4.20). This possibility was subsequently strengthened by the observation that the transmission of transgenes from T1 to T2 plants, in this case from T1(11) to T2 (11.1-12), showed and fitted the expected Mendelian ratio of 3:1 (Table 4.4). It was noted that the nine T2 (11.1-12) plants carried the *bar* gene and multiple hybridizing bands (2-4 bands with sizes of approx. 6.0, 7.9, 9.0 and >12.0 Kb) of the *GUS* gene, as had been seen in the T1 plants (Figures 4.4A-B, 4.5A-B, 4.6A-

Model of transgene integration



Gamete cell

u+w+y		u+w+y
u+w+z		u+w+z
u+x+y	X	u+x+y
u+x+z		u+x+z
v+w+y		v+w+y
v+w+z		v+w+z
v+x+y		v+x+y
v+x+z		v+x+z

Notes:
u : chromosome carrying the *bar* (3.4 Kb) and *GUS* (7.9, 6.0, 9.0 with or without >12.0 Kb fragments) genes.
v, w, x, y, z : chromosomes carrying no transgene.

Gamete cell combination	u+w+y	u+w+z	u+x+y	u+x+z	v+w+y	v+w+z	v+x+y	v+x+z
u+w+y	u+w+y u+w+y	u+w+z u+w+y	u+x+y u+w+y	u+x+z u+w+y	v+w+y u+w+y	v+w+z u+w+y	v+x+y u+w+y	v+x+z u+w+y
u+w+z	u+w+y u+w+z	u+w+z u+w+z	u+x+y u+w+z	u+x+z u+w+z	v+w+y u+w+z	v+w+z u+w+z	v+x+y u+w+z	v+x+z u+w+z
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v+x+z	u+w+y v+x+z	u+w+z v+x+z	u+x+y v+x+z	u+x+z v+x+z	v+w+y v+x+z	v+w+z v+x+z	v+x+y v+x+z	v+x+z v+x+z

Figure 4.20 The proposed model of *GUS* and *bar* transgene integration into the genome of transformed wheat. Three copies (indicated by blue bars with sizes of 7.9, 6.0 and 9.0 Kb) or four copies (the three blue bars and one pink bar with sizes of >12.0 Kb) of the *GUS* gene and a single copy of the *bar* gene (3.4 Kb) were integrated into the same chromosome ("u"). According to this model, the pattern of transgene segregation is 3:1, and the chance of producing a homozygous genotype is 25%, a heterozygous genotype is 50%, and the chance of a non-transgenic segregant is 25%. In addition, it is unlikely that plants carrying either the *GUS* or the *bar* gene would be produced, because the *GUS* and *bar* genes sit in the same chromosome. Notes: A, B and D represent the three wheat genomes. Blue bars and a pink bar across chromosome "u" represent the copy numbers of the *GUS* gene and the red bar represent the *bar* gene which integrates into the genome. The positions of coloured bars indicate copies of the transgenes, but does not represent the actual integration distance in the plant genome.

Table 4.8 Comparison of expected and observed numbers of plant progeny carrying the *bar* and *GUS* transgenes

Segregation possibilities (size of *GUS* fragments, Kb)	Expected number in 64 possibilities (Figure 4.21)	Expected percentage (%)	T1		T2(11)	
			Observed number (n= 17)	Observed percentage (%)	Observed number (n= 12)	Observed percentage (%)
bar + *GUS*	48	75	15	88	9	75
None (no *bar* and no *GUS*)	16	25	2	12	3	25
Total	64	100	17	100	12	100
Ratio ([*bar*+*GUS*] : None)	3 : 1		7.5 : 1 (showing a 3 : 1 ratio; χ^2= 1,59; 0.2<P<0.3)		3 : 1	

B and 4.11A-B). In this analysis, several extra bands of the *bar* gene fragment could be detected (Figure 4.11A). The presence of additional bands here is not surprising because these had also been observed in analysing the primary transformant (Figure 3.6B). These bands could have resulted from the integration of fragments of the original pDM302 vector, from the rearrangement of the *bar* genes in the plant genome, or from partial hydrolysis of the plant DNA prior to Southern hybridization analysis.

Although the appearance of some of the *GUS* hybridizing bands could be formally attributed to incomplete DNA digestion prior to Southern hybridization analysis, it should be emphasized that multiple banding during hybridization with the *bar* gene probe was minimal. Thus, DNA digestion appeared complete for the *bar* gene analysis. It should be noted that in these experiments, the Southern analysis was initially performed with the *GUS* gene, after which the same membrane was stripped and re-probed with the *bar* gene.

Segregation of transgenes was also found in the progeny of T2 plants derived from T1 plant no. 15. Of six plants analysed, only two carried the transgenes, in a 1:2 ratio. Again, the anomalous ratio of inherited transgenes (1:2) might result from the small number of plants analysed. Both the *bar* gene and the two hybridizing bands of the *GUS* genes identified in the parental plant (T1 plant no. 15) were inherited by the two T2 plants (no. 15.2 and 15.5) (Figures 4.5A-B and 4.11A-B).

Unlike the pattern of transgenes observed in T2(11) and T2(15) progeny, all progeny (15 plants) of T2(17) analysed carried the *bar* gene and three hybridizing bands of the *GUS* gene, exactly as observed in the T1 parent (Figures 4.5A-B and 4.12A-B). This suggests that T1 plant no. 17 is homozygous for the transgenes.

To confirm this hypothesis, analysis was extended to the T3 progeny derived from this line. Again, all T3 progeny (12 plants) tested carried the *bar* and three hybridizing bands of the *GUS* gene, as identified in both the T1 and T2 parental generations (Figures 4.5A-B, 4.12A-B and 4.17A-B). The occurrence of a homozygous genotype, in this case in the T1 (17) plant and its progeny, was not surprising because it could be explained by simple segregation of a multiple insertion in one region of a single chromosome, which behaves similarly to a Mendelian inheritance for a single insertion, by self-pollination of transgenic plants (W2).

To illustrate potential integration sites of the transgenes, a model of initial transgene insertion is proposed (Figure 4.20). As identified from Southern blot hybridization, three (7.9, 6.0 and 9.0 Kb fragments) or four (7.9, 6.0, 9.0 and >12.0 Kb fragments) "copies" of the *GUS* gene and a single "copy" of the *bar* gene (3.4 Kb fragment) were possibly integrated into the same region of a single chromosome. If only three copies of the *GUS* gene copies had been integrated, the transgenic progeny plants should regularly have three hybridizing bands. Thus, to explain the presence of some plants showing two or four hybridizing bands, it can be postulated that there may be incomplete DNA digestion in some cases. When genomic DNA was completely digested, three hybridizing bands (6.0, 7.9 and 9.0 Kb) would occur (Figure 4.12B as an example). However, if the genomic DNA was not completely digested, four bands (6.0, 7.9, 9.0 and >12.0 Kb) are possible. The 7.9 Kb *GUS* gene (Figure 4.11B) was observed in all transgenic progeny, and this may indicate that this copy was digested more readily than the others. The 6.0 and 9.0 Kb copies of the *GUS* gene, however, might be digestion products of a larger, incompletely digested fragment (>12.0 Kb).

Therefore, complete digestion would give three bands and incomplete digestion could result in two or four bands, depending on whether the larger fragment was undigested (two bands) or partially digested (four bands). However, banding patterns of the *bar* gene suggested that digestion was complete.

If the *GUS* gene were integrated as four "copies", the possible outcomes are as follows. The four copies of the *GUS* gene were located in the same chromosome, but in three different sites (designated sites 1, 2 and 3). It is suggested that the 7.9 Kb *GUS* gene was integrated close to the single copy of the *bar* gene (3.4 Kb) in one site (site 1), whilst the other three *GUS* gene copies were integrated into two other sites. It is proposed that the 6.0 and 9.0 Kb *GUS* gene "copies" were integrated close together in one site (site 2), while the larger fragment (>12.0 Kb) was integrated in another site (site 3) (Figure 4.20). It appeared that the site where the 7.9 Kb *GUS* gene fragment and the *bar* gene were integrated (site 1) was stably inherited. It could be that this site was close to centromere and that low frequencies of recombination were therefore expected. However, the sites carrying the 6.0 and 9.0 Kb *GUS* gene "copies" (site 2) and the larger fragment (>12.0 Kb) (site 3) were not stably inherited. This might indicate that these two sites are far from the centromere and, as a result, the sites could undergo recombination during meiosis, and one of the two sites (site 2 [6.0 and 9.0 Kb] or site 3 [>12.0 Kb]) might have been deleted. Deletion of transgenes has also been reported in other transgenic plants (Spencer *et al.*, 1992; Srivastava *et al.*, 1996). In the current situation, the various possibilities of *GUS* gene copies observed in transgenic progeny would be four copies (all three sites present), three copies (sites 1 and 2 present) and two copies (sites 1 and 3 present).

Following transgene integration and segregation as proposed in this model (Figure 4.20), a single copy of the *bar* gene and multiple copies of the *GUS* gene occupied the same chromosome. In such situation, where the *bar* and *GUS* transgenes were integrated close to each other in one chromosome, it is unlikely that plants carrying one of the integrated transgenes (*bar* or *GUS*) would be produced (as opposed to two unlinked insertions). With this model (Figure 4.20), in which multiple copies of the transgene(s) were integrated into one chromosome of a heterozygous genotype, the segregation of transgenes in the progeny would be expected to show a Mendelian inheritance pattern, namely a 3:1 segregation ratio. In this model, a homozygous genotype for transgenes would be expected in 25% of progeny, heterozygous genotypes in 50% of progeny and a recessive genotype in 25%. Overall, the ratio of plants expected to carry and not to carry transgenes would be 3:1 (Table 4.8). The results observed here are similar to those reported by other research groups (Fromm *et al.*, 1990; Barcelo *et al.*, 1994; Becker *et al.*, 1994; Nehra *et al.*, 1994; Somers, *et al.*, 1994).

The expression of the *GUS* and *bar* transgenes in each generation was also investigated. Based on histochemical GUS staining results, it was found that GUS was functionally expressed in all plants carrying the *GUS* gene. GUS expression could be easily identified in spikelets, anthers, ovaries, grains and, in some cases, in leaves (Figures 4.3, 4.10 and 4.16). In general, the *GUS* gene was inherited and expressed in a Mendelian pattern, as found in the T1 (15:2 or 7.5:1 ratio with χ^2 = 1.59; 0.2<P< 0.3, Appendix 4) and the T2(11) (3:1 ratio) plants (Table 4.4). The segregation of transgenes in the progeny of T2(15), however, did not showed a 3:1 ratio, probably because of the small number of plants examined. As predicted from their homozygous genotype, all T2 and T3

plants derived from T1(17) showed GUS expression. The results are all in agreement with previous studies (Vasil *et al.*, 1993; Lörz *et al.*, 1998), which report that the segregation of transgenes in the progeny of transformed wheat follows a Mendelian inheritance pattern (as observed here in T1 plants and T2[11] progeny), and the occurrence of a homozygous phenotype for the transgenes (as observed in the progeny of T2[17] and T3[17.1 and 17.5]) in transgenic wheat generations.

Unlike the expression of the *GUS* gene, it seems that the expression of the *bar* gene has been silenced in some plants. This is indicated by the lack of PAT activity and herbicide tolerance, as identified in a number of T1, T2 and T3 plants (Tables 4.2, 4.4 and 4.6). A similar observation has been found by several research groups who reported the loss of expression of the *bar* gene but stable expression of the *GUS* gene over successive generations of transgenic wheat (Kluth *et al.*, 1998; Lörz *et al.*, 1998). In other work, however, the *bar* gene was found to be stably expressed in transgenic wheat (Witrzens *et al.*, 1998). In that work, the *bar* gene was driven by the *ubi1* promoter, whilst the *bar* gene used during this study was under the control of the rice *Actin1* promoter. However, Kumpatla and Hall (1998) reported that the *bar* gene, which was also driven by the *ubi1* promoter, did undergo inactivation in transgenic plants. Based on results accumulated here and on other published reports, it is clear that the cause of transgene inactivation does not solely result from the different promoters used.

Results from the present study also showed that T1 plant no. 9, which did not carry the *bar* gene, showed PAT activity (Figures 4.2, 4.4A and 4.6A). This result was similar to those from the primary transformants, where a number of plants showed PAT activity without the *bar* gene being present. As mentioned in

Chapter 3, such results could be attributed to problems that were experienced with the reliability of the PAT assay.

As mentioned above, the *bar* gene was apparently subjected to inactivation, whilst the *GUS* gene was stably expressed in all generations. The occurrence of transgene inactivation observed here might be explained by the methylation of the *bar* gene. It has been reported that DNA methylation in plants occurs at cytosine residues that are located within the target sequences CG and CnG, where n can be any base (McElroy and Brettell, 1994; Meyer, 1995[b]). Within the two plasmids used in these experiments, the *bar* and the *GUS* genes contain approx. 70% and 50% GC contents, respectively, so it is likely that the incidence of potential methylation sites in the *bar* gene sequences might be higher than in the *GUS* gene. A number of other research groups has reported *bar* gene methylation which resulted in its inactivation in transgenic wheat (Srivastava *et al.*, 1996; Karunaratne *et al.*, 1996). Work by Kumpatla and Hall (1998) clearly demonstrated that the incidence of *bar* gene inactivation in transgenic seedlings solely resulted from methylation. In that work, it was demonstrated that the methylated *bar* gene could be re-activated by growing the transgenic seedlings on a medium containing 5-azacytidine. Re-activation of the *bar* gene in the transgenic seedlings was indicated by resistance of the seedlings to herbicide application.

The transgene inactivation might also be explained in terms of the number of transgenes integrated into the plant genome. In this case, the presence of several *GUS* gene copies in the plant genome may help stabilize *GUS* expression in successive generations because inactivation of one or more copies could be compensated by expression of the other copies present. Several studies have

been directed to a comparison of transgene expression in relation to transgene copy number (Cooley *et al.*, 1995; Srivastava *et al.*, 1996). It is generally assumed that the presence of high numbers of transgene copies in the plant genome result in lower levels of expression. For instance, Hobbs *et al.* (1990) found that transgene silencing frequently occured in plants having high copy numbers of transgenes. In contrast to such observations, transgene inactivation also occurs in plants carrying a single copy of the transgene (Elmayan and Vaucheret, 1996; Kohli *et al.*, 1999). Work by Klein *et al.* (1989) and Spencer *et al.* (1990) showed stable expression of transgenes in plants having high or low copy numbers of the transgene. Therefore, the effects of transgene copy numbers on transgene expression remain unclear.

The incidence of transgene inactivation could also result from the physical location at which the *GUS* and the *bar* genes are integrated into the genome. In this instance the *GUS* gene may have a more favorable location for expression than the *bar* gene. Such "position effects" have been well-documented in the literature. Work by Pedersen *et al.* (1997) demonstrated that the majority of transgenes introduced by microprojectile bombardment were integrated into distal regions, which are the most decondensed parts of chromosomes and contain the bulk of actively transcribed genes. Transgenes integrated into actively transcribed regions are often stably expressed, while those integrated into transcriptionally-inactive regions or in highly condensed chromatin regions are often inactive (Pröls and Meyer, 1992; Meyer *et al.*, 1993; Stam *et al.*, 1997).

Another possible explanation is *trans* inactivation (co-suppression) of transgenes as described by Matzke *et al.* (1989). This may be related to the fact that the two plasmids (pDM302 and p*Act1*-D *GUS*) used in these experiments

contain the same promoter sequence (rice *Actin1* promoter). The presence of homologous regions in the two plasmids may have an undesired effect on transgene activity, which in this case leads to the loss of, or the decline in *bar* gene expression in successive generations, possibly through recombination events (Jorgensen, 1990; Matzke and Matzke, 1990; Meyer, 1995[a]).

The phenotypic characteristics and yields of transgenic wheat plants were also monitored. The majority of T1 plants, both transgenic progeny and non-transgenic segregants, showed poor vegetative growth and low yields, as observed with the primary transformant W2 (T0 plant) (Figure 4.8 and Tables 3.5 and 4.3). Substantial improvements in the phenotypic characteristics and yields were noted from the T1 to the T3 generations, but the appearance and yields of the transgenic progeny (T1 to T3) were always poorer than control plants (Figure 4.19). It was also noted that retarded growth and low yields were found not only in the transgenic progeny, but also in non-transgenic segregants (see Tables 4.3, 4.5 and 4.7). It is therefore likely that such effects were linked to the tissue culture procedures. Somaclonal variation could adversely affect agronomic performance, as could the transformation (bombardment and selection) procedures. Both transgenic and non-transgenic segregants originated from a single primary transformant (W2) which was regenerated from *in vitro* culture after the transformation and tissue culture procedures. Results found here were similar to work described by Bregitzer *et al.* (1998), who reported the occurrence of retardation in plant growth and reduction in yield of transgenic barley progeny. This research group emphasized the adverse effects of tissue culture and transformation procedures on agronomic traits, rather than the presence of the transgene itself. Based on results accumulated during this study and reported

elsewhere, it is obvious that the effects of somaclonal variation in transgenic plants are mostly undesirable. One of the possible strategies to alleviate such problems is to cross the transgenic plants with parental seed-grown plants. This should remove any deleterious mutations resulting from the tissue culture and/or transformation process.

Conclusions

All results accumulated during this study show that regeneration procedures developed for elite Australian wheat genotypes can be incorporated into a microprojectile bombardment-mediated transformation method, leading to the production of transgenic wheat carrying both the *bar* and the *GUS* genes. This is one of the first transgenic Australian wheat genotypes (*cv.* Frame) which shows stable expression and inheritance of the *GUS* gene (Murray *et al.*, 1998; Witrzens *et al.*, 1998). Although a model has been presented to describe possible transgene integration and inheritance patterns, the actual integration and inheritance patterns of the transgenes, especially the *GUS* gene, in the primary transformant and its progeny are still unclear. Therefore, it is necessary to analyse more samples of transgenic progeny in more detail, before integration and segregation patterns can be unequivocally described.

CHAPTER FIVE

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary of experiments described here

The main objective of work described in this thesis was to screen four Australian wheat genotypes (Hartog, Frame, Krichauff and Janz) for *in vitro* culture responsiveness, and to transform responsive genotypes with marker genes (*bar* and *GUS*) using the microprojectile bombardment technique. In early experiments, these genotypes were cultured on MS basal medium (Murashige and Skoog, 1962) containing different combinations and concentrations of 2,4-D and benzylaminopurine.

Using immature scutella as starting material, three of the four genotypes tested (*cvs.* Hartog, Frame and Krichauff) responded well in culture. Two genotypes (*cvs.* Hartog and Krichauff) required 2 mg/l 2,4-D for embryogenic callus induction while the third genotype (*cv.* Frame) required the addition of 0.1 mg/l benzylaminopurine as well. When cultured on the appropriate medium, isolated scutella of Hartog, Frame and Krichauff produced embryogenic callus capable of producing fertile plants.

In the second part of the study, using the regeneration systems developed for the three above-mentioned genotypes, a microprojectile bombardment strategy was used to achieve genetic transformation of these varieties. Isolated scutella or freshly isolated immature embryos were bombarded with a construct (pDM302) carrying the selectable marker *bar* gene with or without another construct (p*Act1*-D GUS) containing the reporter *GUS* gene. Four transgenic plants (cv. Frame) were produced from three separate experiments. The presence of transgenes in the four transgenic wheat plants was confirmed by Southern blot analysis. Based on the Southern blot analysis, the four transgenic plants each carried at least one intact copy of the introduced transgenes. The introduced transgene(s) were

expressed in the transgenic plants, as indicated by the presence of PAT activity, herbicide tolerance and GUS activity in both vegetative and reproductive organs, including leaves, spikelets, anthers and grains. The transformation frequency achieved in this study was approximately 0.7%, based on the number of explants bombarded in three successful experiments, or approximately 0.03%, based on the total number of explants bombarded during this study.

Toward the end of this study, the transmission and inheritance of the introduced genes was demonstrated up to the T3 progeny derived from the plant designated W2. A detailed analysis of progeny arising from the other 3 transgenic plants was not performed in this study owing to time constraints. The incidence of transgene inactivation was observed for the *bar* gene in several transgenic progeny, but this did not occur in the case of the *GUS* gene. The suppression or inactivation of the *GUS* gene was not observed, perhaps because the presence of multiple gene copies ensured that at least one copy was always functional.

The primary transformants were healthy but not as robust as the seed derived plants. However, subsequent generations showed a clear trend of phenotypic improvement.

5.2 Future directions

The application of transformation technology for wheat has widened during the past decade, but still there is not a routine method available for the majority of genotypes. There is no doubt that in some university laboratories like the University of Florida or the University of Hamburg and in some industrial laboratories such as Novartis or Monsanto, wheat transformation using biolistics is routinely achieving transformation frequencies of up to 40% (Sparks *et al.*, 1998; Qureshi, personal communication). Such success gives these laboratories the ability to transfer an array of genes which may be either potential commercial targets or used for functional analysis. Nevertheless, the varietal barrier is still present and success in wheat transformation is not yet achievable across the board. A case can be made that perhaps we do not need such an achievement in order to move forward, since transformed model varieties can be used as donors of newly introduced traits through backcrossing procedures. Perhaps a universal system for transforming all wheat genotypes will never be found, but it is not unreasonable to expand the technology to include as many elite varieties as possible or to those model varieties which share close relationships with new cultivars. The promise of biotechnology is in innovation and economy of time and labor. Hence, prolonged breeding efforts (many back-cross cycles) to move newly introduced characters from model varieties to elite varieties may render the technology less attractive. Therefore, expanding the technology to elite material remains important.

5.2.1 Donor material health and transformation efficiency

As observed from the work described in this thesis, the response of explants in culture appeared to be affected not only by plant genotypes and/or type and concentration of growth regulators used, but also by the condition of donor plants. Thus, one of the most important considerations in the future is the physiological condition of donor plants. It is fully understood that the successful outcome of transformation experiments is very much dependent upon the tissue culture response of the targets and upon the selection process. Thus, attention must be focused on these issues. Better donor plant management, careful tissue culture manipulations and the use of culture-friendly selectable markers will certainly help improve the overall efficiency of the method.

5.2.2 Transgene silencing

Transgene silencing is an important factor that adds to the unpredictability of the continued expression of the trait for which the line was produced. For the technology to be commercially viable, genetically modified lines must be stable and dependable. In the present study, the loss or silencing of the introduced *bar* gene expression was discovered in some transgenic wheat progeny. As illustrated in the literature, transgene silencing is a fairly common feature in transgenic plants. The loss of transgene activity may be caused by integration of transgenes into transcriptionally-inactive regions of the genome, by the insertion of multiple copy numbers of the transgenes, or by methylation of the transgenes. The microprojectile bombardment method is adirect and relatively primitive method of gene delivery. Through this method genes are inserted into the genome randomly and generally in high copy numbers (multiple integration sites

and multiple insertions per site). Also the choice of vectors where sometimes two genes are driven by the same promoter, can cause expression problems. To alleviate such problem, it is necessary to develop transformation strategies which could control the integration of transgenes into the plant genome. One approach is the targeted integration system (*Cre-lox* integration system) (Bayley *et al.*, 1992). The principle of this system is to target or to direct the integration of transgenes into a specific site. This system is described briefly as follows. Initially, plants are transformed with a construct containing 35S-*lox-Cre*. Transgenic plants carrying such genes are subsequently re-transformed with a plasmid carrying a promoterless *lox-hpt* construct, where *hpt* encodes hygromycin phosphotransferase. When the *lox-hpt* construct is present in the genomic DNA of transgenic plants carrying the 35S-*lox-Cre* and expressing the Cre enzyme, the Cre enzyme will direct the insertion of *lox-hpt* into the genomic 35S-*lox-Cre* site to produce a 35S-*lox-hpt-lox*-Cre linkage (Albert *et al.*, 1995). In this system the transgene, in this instance the *hpt* gene, is targeted to integrate into a specific region (35S-*lox-Cre*). Therefore, this system could avoid random and multiple transgene integration.

Another choice is to replace microprojectile bombardment with *Agrobacterium*-mediated transformation. Although this method is not yet readily available for wheat, efforts to develop this method will pay dividends in the future. The available evidence in the literature for *Agrobacterium*-mediated transformation of both dicots and monocots indicates that through this method more simple events are produced. For registration purposes as well, in the future, acceptance by consumers and regulatory bodies of transgenic crops generated

through biolistics may prove more difficult that those generated through *Agrobacterium*-based methods (Pawlowski and Somers, 1996).

5.2.3 Tissue culture effects

It was observed in this study that the transgenic plant (W2) and its progeny exhibited poor agronomic characteristics and low yields compared with seed-derived (control) plants. One of the possible causes of such problems is somaclonal variation (Bregitzer *et al.*, 1998). To minimise the effects of somaclonal variation on transgenic plants, it may be necessary to reduce the tissue culture phase or ideally, to entirely omit it. Some of the methods that can avoid tissue culture phase include the pipetting of *Agrobacterium* onto reproductive organs (Hess *et al.*, 1990) and the application of *Agrobacterium* onto apical meristem tissues (Gould *et al.*, 1991; Zhang *et al.*, 1999).

Another approach which could alleviate the effects of tissue culture is to backcross the transgenic plants with wild-type plants. The purpose of the crossing is to exclude any aberrant chromosomes or deleterious mutations, which could occur during tissue culture and/or transformation passage, from the genome of transgenic plants (Brettell and Murray, 1995).

5.2.4 Analysis of transgene integration patterns

In this study, it was shown that the *bar* and *GUS* transgenes had apparently been integrated into a single and multiple copies, respectively. All the *bar* and the *GUS* transgenes could have been integrated in one closely linked region as indicated by the pattern of inheritance showing a Mendelian pattern for a single insertion site.

To investigate the real copy numbers of the *GUS* gene, we could analyse more plants grown from T1, T2, T3 and T4 grains. The grains used for analysis should be mainly chosen from plants showing two (7.9 and >12.0 Kb fragments) and four (6.0, 7.9, 9.0 and >12.0 Kb fragments) hybridizing bands. In performing Southern blot analysis, it is important to guarantee that each sample of genomic DNA derived from these plants can be digested properly, and for these, several critical factors should be considered, including the purity of genomic DNA, the use of newly purchased restriction enzymes, and the time for incubation. The unequivocal determination of whether the plants have three or four *GUS* hybridizing bands will enable more accurate models for integration and inheritance of transgenes to be devised.

To determine the copy number of the *GUS* gene, crossing of wild-type wheat plants and transgenic wheat plants showing the three banding patterns namely plants having two (7.9 and >12.0 Kb fragments), three (6.0, 7.9 and 9.0 Kb fragments) and four (6.0, 7.9, 9.0 and >12.0 Kb fragments) hybridizing bands, could be performed. Genomic DNA from the progeny of the crossed plants would be analysed using Southern blot hybridization. With such analyses, the copy numbers of the *GUS* gene in each progeny could be determined. The results could ultimately be used to trace the actual copy numbers of the *GUS* gene in the genome of primary transformant (W2 plant).

Another approach is to map the location of the *bar* and the *GUS* genes. This could be performed using fluorescence *in situ* hybridization (FISH) (Pedersen *et al.*, 1997). With this method, the integration of transgenes in the plant genome, even in a single-copy insertions, can be localized. The mapping of transgenes could also be done by inverse PCR (IPCR) where the known transgene sequence

is used to design the primers. IPCR products can be sequenced and should contain parts of the DNA flanking the insertions. These can be compared with database sequences and any genes identified could subsequently be located on a genetic map.

5.2.5 Field trials and evaluation of transgenic plant products

As demonstrated here, transgenic wheat plants carrying herbicide resistance genes were successfully produced. Recently, the use of such genes for producing transgenic plants has raised public concerns (Chèvre, *et al.*, 1997; Daniell, 1999). One of the concerns relates to the potential transfer of the herbicide resistance genes to related plant species which might subsequently become resistant to the herbicide. With the availability of a considerable number of seeds collected during this study, field trials could be carried out to investigate the lateral transfer of transgenes from transgenic plants to neighbouring plant populations. These trials could also be used to study the effects of transgenes on phenotypic characteristics of transgenic plants grown under field conditions.

Other concerns arising in the community are the unforeseen impact of transgenic plant products on human and animal health (Kessler *et al.*, 1992). To overcome such concerns, long term studies must be undertaken to examine the safety of foods and feeds made from transgenic plants. Such studies will play a pivotal role in the public acceptance of this vital technology for human sustenance in the future.

5.2.6 Production of marker-free transgenic plants

With the increasing concerns relating to the presence of marker genes in transgenic plants (Käppeli and Auberson, 1998), transformation strategies must be in place to produce marker-free transgenics. One of the strategies is the use of co-transformation where the marker and gene of interest are carried on separate plasmids. If the co-introduced DNAs integrate in different locations, they would segregate independently in subsequent progeny, so that marker-free transgenic plants could be selected by repeated backcrossing experiments (Yoder and Goldsbrough, 1994). The success in using such strategy has been demonstrated by Komari *et al.* (1996) and Daley *et al.* (1998). In this work, two separate DNA fragments, one of which contains a selectable marker, were introduced into the plant genome using *Agrobacterium tumefaciens* and resulted in marker-free plants in the transgenic progeny. Using microprojectile methodology for transformation, however, the co-transformed genes usually integrate very close to each other and therefore create a potential problem for segregation (Pawlowski and Somers, 1996).

A potentially useful approach is to make use of site-specific recombination methods. The principle of these methods is as follows. Initially, plants are transformed with a selectable marker and a gene of interest. The selectable marker gene must be placed in a certain region, namely between two short, specific DNA sequences (i.e. *lox*), while the gene of interest is located outside this region (or in another vector). When transgenic plants are produced, in this case transgenic plants having the *lox* sequences, another gene (e.g. *Cre*) is introduced into the *lox* containing plants. If the *Cre* gene can also integrate and express in the transgenic plants, the Cre enzyme will catalyze recombination between two *lox*

sequences resulting in excision of internal sequences, in this case the selectable marker. As a result, the selectable marker will be removed from genomic DNA and eventually selectable marker-free transgenic plants will be produced (Russell *et al.*, 1992).

Another system for eliminating marker genes from transgenic plants is by performing transposition-based methods (Goldsbrough *et al.*, 1993). In this system, plants are transformed using a construct (or constructs) containing a gene of interest, the transposase gene (*Ac* element) and a marker gene which is placed within the *Ds* elements. If this construct(s) is integrated into the plant genome, the transposase will activate the excision of the *Ds* elements, in this case the region where the selectable marker sits. After excision, the *Ds* elements containing the selectable marker will be moved and re-inserted into another site in the plant genome. If the marker gene integrates into a new region which has sufficient genetic distance for recombination, this allows the segregation of marker gene and gene of interest in subsequent generations, so that marker-free transgenic progeny will be obtained. Furthermore, when the *Ds* element containing the selectable marker has been excised by transposase, but fails to reintegrate into the plant genome (Belzile *et al.*, 1989), the selectable marker will be lost, and as a result marker-free transgenic plants will be produced.

To avoid the difficulties associated with eliminating the selectable markers from transgenic plants, 'positive selection' strategies (Joersbo and Okkels, 1996), for example by the use of genes such as xylose isomerase (Haldrup *et al.*, 1998) and mannose-6-phosphate isomerase (Reed *et al.*, 1999) can be used, instead of herbicide resistance or antibiotic resistance-based selection. The use of such selection procedures precludes the use of selectable markers, so that these

strategies might be more acceptable and could tackle concerns relating to the presence of marker genes in transgenic plants.

5.2.7 Insertion of useful genes

In this study, regeneration systems and bombardment procedures have been successfully developed for an Australian wheat genotype, *cv.* Frame. With the availability of these regeneration and transformation protocols, it is now possible to introduce potentially useful genes into *cv.* Frame as the genotype of choice. Examples of available genes include those encoding barley limit dextrinase, limit dextrinase inhibitor, isoamylase, β-glucan synthases, (1→3)-β-glucanase, (1→3, 1→4)-β-glucanase and thaumatin-like proteins. These genes are now available in this laboratory (G.B. Fincher, personal communication).

APPENDICES

Appendix 1 Recycled soil composition

One cubic metre of used experimental soil was steam sterilized at 100 °C for 45 min, and cooled. Following this, 0.2 metre^3 of Eurotorf peatmoss, 1 kg blood meal, 0.4 kg agricultural lime, 0.4 kg potassium sulphate and 0.2 kg superphosphate were mixed with the soil. The soil was sieved through a 1 cm grid size sieve. The pH of the soil was about 6.5.

Source : SARDI, Plant Growth Service section.

Appendix 2 Culture media

INGREDIENTS	MS (mg/l)	L1 (mg/l)
MACROSALTS		
NH_4NO_3	1650	750
KNO_3	1900	1750
KH_2PO_4	170	200
$MgSO_4.7H_2O$	370	350
$CaCl_2.2H_2O$	440	450
MICROSALTS		
$MnSO_4.4H_2O$	22.3	15
H_3BO_4	6.2	5
$ZnSO_4.7H_2O$	8.6	7.5
KI	0.8	0.75
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
IRON SOLUTION		
Na_2EDTA	37.3	37
$FeSO_4.7H_2O$	27.8	28
VITAMINS		
Ascorbic acid	-	1
Biotin	-	0.005
Ca-panthothenate	-	0.5
Choline chloride	-	0.5
Folic acid	-	0.2
myo-Inositol	100	100
Nicotinic acid	0.5	1
p-Aminobenzoic acid	-	1
Pyridoxine-HCl	0.5	1
Riboflavin	-	0.1
Thiamine-HCl	0.1	10
Glycine	2	-
AMINO ACIDS		
Glutamine	-	750
L-Proline	-	150
Asparagine	-	100
SUGAR		
Sucrose	30000	-
Maltose	-	50000
OTHER		
2,4-D	2*	2
BAP	0.1*	-
pH	5.7	5.7

*: decided after preliminary experiment as described in Chapter 2 section 2.2.1.
Sources : MS ; Murashige and Skoog media (Murashige and Skoog, 1962).
L1 ; Lazzeri media (Lazzeri *et al.*, 1991).

Appendix 3 Horsham soil mix composition

Composted pine bark is mixed with the following fertilizers:

Composted pine bark	600 litres
Osmocote (high P)	1.8 kg
Ammonium nitrate	0.135 kg
Micro max (micro-nutrients)	0.135 kg
Iron sulphate	0.27 kg
Agricultural lime	1.33 kg

The mix is not heat treated and the pH is about 6.0 – 6.5

Source : SARDI, Plant Growth Service section.

Appendix 4 Calculation of χ^2 on T1 plants (based on molecular analysis for both the *bar* and the *GUS* genes, see Table 4.2)

Genotype	Observed number (O)	Expected number (E)	O-E	$(O-E)^2$	$(O-E)^2/E$
bar/*GUS* (+)	15	12.75	2.25	5.0625	0.40
bar/*GUS* (-)	2	4.25	-2.25	5.0625	1.19
Total	17		0.00		1.59
χ^2 = 1.59		dF = 1		0.2 < P < 0.3	

Notes :
χ^2 : *Chi-square.*
dF : Degree of freedom.
P : Probability.

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