CELL, TISSUE CULTURE AND TRANSFORMATION OF *TRITICUM TAUSCHII*

by

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A thesis submitted in fulfillment of the requirement

for the degree of Doctor of Philosophy

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March, 2000

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Acknowledgment

This thesis would not have been completed but for the support provided by others. I am grateful for their encouragement and assistance and especially I need to thank:

- my supervisors, Professor Geoffrey B. Fincher, Head of Department, Plant Science, University of Adelaide and Professor James F. Kollmorgen, Director, Joint Centre for Crop Improvement, Victorian Institute for Dryland Agriculture, Horsham for their guidance, support and also for putting up with my "Persian" English during writing this thesis
- Jodie Kemp, Bronwyn Clarke and Francis Ogbonnaya for their great technical support and friendship during the experimental work and for their patience and understanding to put up with my expectations
- Dr Ian Gordon from Melbourne University; Carole Wright from Victorian Institute for Dryland Agriculture, Horsham; Lyndon Brooks from Southern Cross University who have also helped me with statistical analysis
- Dr Tim Holton for his encouragement during writing and commenting on parts of this thesis
- Professor Robert Henry, Southern Cross University for the use of the Centre's resources to finish the writing up
- my family and friends for their support and encouragement
- Grains Research and Development Corporation for funding this project
- last, but not least, my son, Frank Sterle for his endless patience all along the way and understanding that this was something I had to do. I need also to thank him for his recent support helping in many ways so that I could spend more time writing up this thesis. In addition many thanks for checking the references.

Publications

The following publications have been arisen from work described in this thesis.

Afshar-Sterle, S., E.C.K. Pang, J.S. Brown and J.F. Kollmorgen. (1996). Embryogenic callus induction and plant regeneration from *Triticum tauschii*, the diploid D-genome donor for bread wheat (*Triticum aestivum*). *Aust. J. Bot.* 44: 489-497.

Afshar-Sterle, S., J.F. Kollmorgen and G. B. Fincher. (1999). Establishment of fine suspension cultures of *Triticum tauschii* ([Coss] Schmal.) which remain embryogenic for several years. *Aust. J. Bot.* 47: 611-622.

Afshar-Sterle, S., J.F. Kollmorgen and G. B. Fincher. Production of fertile regenerants from protoplasts of *Triticum tauschii*. *Aust. J. Bot.* In press.

Abbreviations

µE	microeinstein
2,4-D	2, 4-dichlorophenoxyacetic acid
ACT	actin
bp	base pair
Ci	curie
DIG	digoxigenin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GFP	Green Fluorescent Protein
GUS	β-glucuronidase
kb	kilo base
MES	2-[N-Morpholino]ethanesulfonic acid
mOsm	milliosmolar
MS	Murashige and Skoog
N:P:K	Nitrogen:Phosphate:Potassium
PAT	phosphinothricin acetyl transferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIG	Particle Inflow Gun
PPT	phosphinotricin
rpm	revolutions per minute
TBE	Tris-borate EDTA
T-DNA	transfer DNA
TE	Tris EDTA
Ti	tumour-inducing

Tris	Tris(hydroxymethyl)aminomethane
ubi	ubiquitin
v/v	volume/volume
vir	virulence
W	watt
w/v	weight/volume
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Abstract

Genetic engineering of *Triticum tauschii* is an alternative strategy for the genetic improvement of bread wheat, because transgenes introduced into *Triticum tauschii* could be easily transferred into elite bread wheat varieties by more conventional techniques. The aim of the present project was to develop efficient and reliable protocols for the production of embryogenic callus, suspension and protoplast cultures of *Triticum tauschii*, and to transform cells by direct uptake of DNA into protoplasts and by insertion of DNA using microprojectile bombardment.

Immature embryos of seven accessions of *Triticum tauschii* were used to produce embryogenic callus suitable for initiation of suspension cultures. Several modifications of the Murashige and Skoog (MS) medium were evaluated for callus induction from scutellar tissues of embryos. Nodular, embryogenic calli were induced from all accessions.

Using the protocol developed for the production of nodular, embryogenic callus from immature embryos, ten accessions of *Triticum tauschii* were used to produce embryogenic callus for initiation of suspension cultures. A three-step media change was the main feature of this protocol and was crucial for long-term maintenance of embryogenicity of these suspensions. Long-term embryogenic fine suspension cultures were established from two accessions (CPI 110813 and CPI 110649). Over 90% of plants regenerated from one-year-old embryogenic fine suspension cultures were fertile. Embryogenic suspension cultures retained their capacity to regenerate plants for more than three years.

Four suspension cell lines generated from two accessions of *Triticum tauschii* were used to develop an efficient protocol for producing fertile regenerants from protoplasts. Protoplasts were isolated from each cell line by incubating fine cell aggregates (< 500 μm in diameter) in a solution containing a mixture of hydrolytic enzymes. The first cell divisions of the protoplasts were observed after 5-7 days. Cell colonies were observed after 14 days and grew quickly into large clumps when transferred to half strength MS medium supplemented with 2,4-D, sucrose and solidified with Phytagel. The colonies produced somatic embryos within 21-28 days of transfer to this medium. The somatic embryos were

transferred to hormone-free MS medium for regeneration into plantlets. Although many regenerants produced shrivelled seeds, nine out of sixteen regenerants were fertile and produced normal seeds.

Two transformation methods, namely direct uptake of DNA into protoplasts and microprojectile bombardment, were evaluated for their suitability to transform *Triticum tauschii.* Initial experiments were aimed at achieving transient expression of the *GUS* reporter gene in protoplasts and cells. Transient expression of the *GUS* gene was observed in protoplasts from two *Triticum tauschii* accessions, but further studies are required if the protoplast method is to be of any practical use in transforming *Triticum tauschii.*

With the microprojectile method, delivery parameters including helium pressure to accelerate particles, microparticle density, and pre- and post-bombardment osmoticum conditioning were optimised for bombardment of immature scutellar tissue and of suspension cultures. A high level of transient expression of the *GUS* gene was obtained in both tissues. Pre- and post-bombardment osmoticum conditioning appeared to have a significant effect *GUS* activity in suspension cultures. It was important to reduce particle density to overcome tissue damage in scutellar tissues.

Stably transformed *Triticum tauschii* callus lines were obtained after bombardment of suspension cultures. The cultures were bombarded with a mixture of two plasmid constructs. One plasmid contained a selectable marker gene, *bar*, which encodes phosphinothricin acetyl transferase (PAT) and confers herbicide resistance on the transgenic plants, and the other plasmid contained a reporter gene (*GUS*). Bombarded cells were selected on a medium containing the herbicide bialaphos. Seven bialaphos-resistant lines expressed PAT. Integration of *bar* and *GUS* genes was confirmed by Southern hybridization analysis of all PAT positive callus lines. However, GUS activity was only detected in one herbicide-resistant callus line. Thus, the experiments described in this thesis confirm that *Triticum tauschii* can be transformed at the cell culture level, using microprojectile bombardment. However, no plants could be regenerated from the transformed cell lines.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

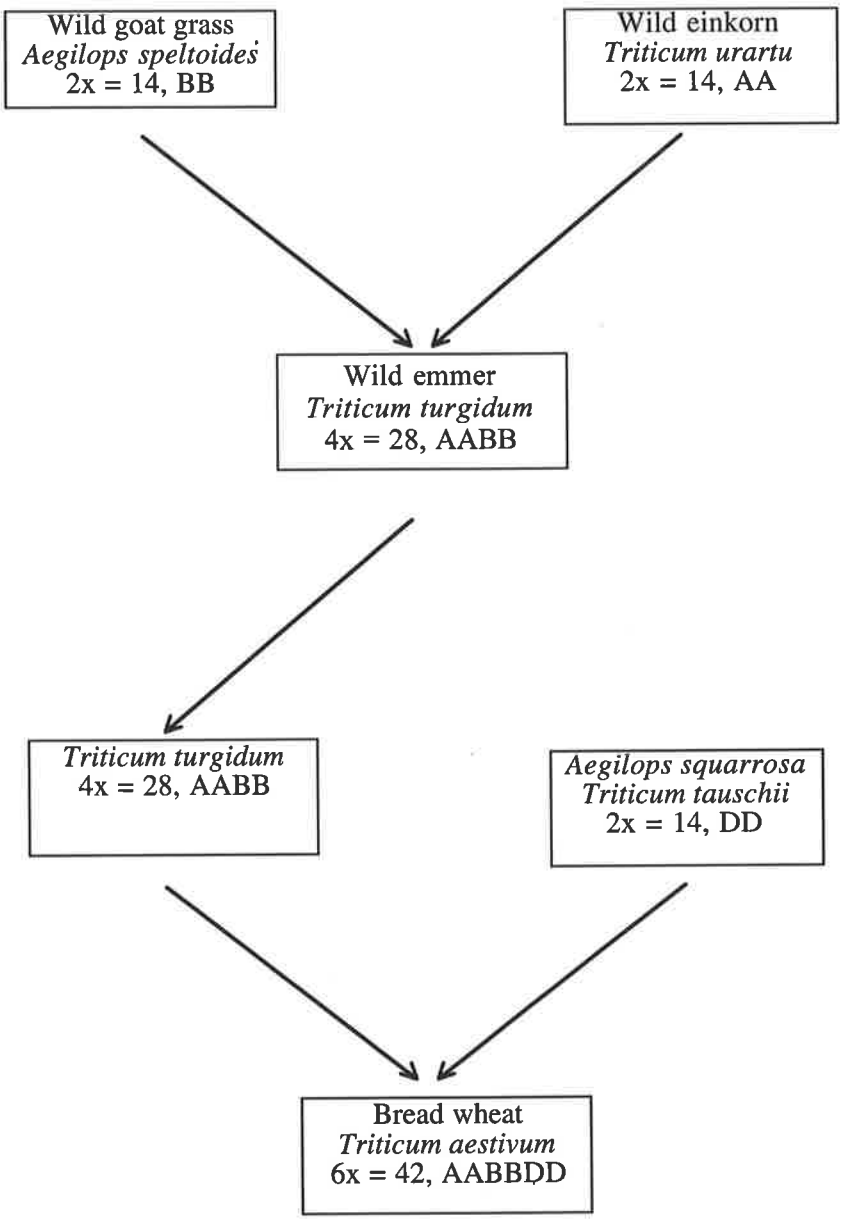
Wheat is the most important cereal crop in the world, in terms of area sown and production, and is a staple food for more than one third of the world's population (Cornell and Hoveling 1998). Wheat production in Australia was about 23.5 million tonnes in 1998-99 and about 70.2 per cent of this was traded on the international market (ABARE 1998). Further improvements in the yield and productivity of such a major source of food can be expected to have a significant impact on Australia and international economies.

Hexaploid wheat (bread wheat) *Triticum aestivum* L. Thell (2n = 6x = 42) consists of three related diploid genomes, designated A, B and D. It was one of the earliest plants cultivated by mankind and played an integral part in the development of ancient civilizations. The first steps towards its domestication took place approximately 8000 years ago and involved natural hybridization between two diploid species, 'wild einkorn' (*Triticum urartu* Thum.), donor of the A-genome, and *Aegilops speltoides*, donor of the B genome, to form the wild tetraploid Emmer (*Triticum turgidum* L.) Thell. Further hybridization of the tetraploid *Triticum turgidum* (2n = 4x = 28, genomes AB) with another diploid wheat *Triticum tauschii* (Coss.) Schmal. (2n = 2x = 14, *Aegilops squarrosa* L.), the donor of the D genome, resulted in the formation of hexaploid wheat (*Triticum aestivum* L., 2n = 6x = 42, genomes ABD) (Kihara 1944; McFadden and Sears 1946; Miller 1987; Lagudah *et al.* 1991; Fritz *et al.* 1995) (Figure 1.1).

The hybridization through which bread wheat arose presumably involved a very limited number of genotypes from each species and resulted in hexaploid wheats having a narrow genetic base relative to their wild progenitors (Lubbers *et al.* 1991; Fritz *et al.* 1995). For example, the low degree of genetic diversity of the D genome of hexaploid wheat (Lubbers *et al.* 1991) probably arose because only one or a few individuals of *Triticum tauschii* were involved in the original crosses that generated hexaploid wheat (Konarev *et al.* 1979; Lagudah and Halloran 1989).

Despite the low genetic diversity of the D genome in hexaploid bread wheats, the D genome progenitor species *Triticum tauschii* is a rich source of genetic variation and could

Figure 1.1 Pedigree of *Triticum aestivum* (bread wheat)



be used to introduce agronomically important traits into bread wheat by conventional hybridization (Appels and Lagudah 1990). The introduction of genetic material from *Triticum tauschii* into the common bread wheat genome has been used in the past to extend genetic variability and also provides great potential for future improvements of bread wheat (Gale and Miller 1987; Nkongolo *et al.* 1991; Cox *et al.* 1994).

Two methods have been used to introduce useful genes into hexaploid wheat from *Triticum tauschii* by conventional hybridization:

a) *Direct gene transfer from Triticum tauschii to hexaploid wheat*

Triticum tauschii can be crossed directly with hexaploid wheat, although hybrid grains often shrivel and die if left on the ear, because of failure of endosperm development. In these cases, the hybrid F_1 embryos can be rescued by removal and culturing on nutrient agar medium (Alonso and Kimber 1984; Gill and Raupp 1987; Gale and Miller 1987). When the F_1 plants are backcrossed to the hexaploid parent, the progeny are fertile and can be selected for meiotically stable 42 chromosome plants.

Direct transfer of genes from *Triticum tauschii* into hexaploid wheat has been used to develop stable hexaploid lines with genes conferring resistance to Hessian fly, greenbug and leaf rust (Gill and Raupp 1987; Cox *et al.* 1990), and to *Septoria tritici* and *Septoria nodorum* (Leath *et al.* 1994).

b) *Gene transfer from* Triticum tauschii *to hexaploid wheat via production of synthetic hexaploids*

The tetraploid wheat *Triticum turgidum* L. var. Durum can be used as a bridging species to introduce genes from *Triticum tauschii* by the formation of a synthetic hexaploid (McFadden and Sears 1946; Gill and Raupp 1987). In this approach, crosses between *Triticum tauschii* (DD) and *Triticum turgidum* (AABB) lead to production of triploid hybrids (2n = 3x = 21, ABD). These hybrids can be treated with colchicine at the seedling stage to initiate the formation of synthetic hexaploids (2n = 6x = 42, AABBDD) (Kerber

and Dyck 1969; Kerber 1987). The synthetic hexaploid is advantageous for crop improvement because it not only allows the desired *Triticum tauschii* gene(s) to be incorporated, but also exploits the genetic diversity of the A and B genomes of the particular durum wheat cultivars being used in such hybridizations (Mujeeb-Kazi *et al.* 1996).

Using the bridging technique, synthetic hexaploid wheats have been generated with introduced gene(s) from *Triticum tauschii* for resistance to greenbug C-biotype (Harvey *et al.* 1980), Hessian fly (Hatchett and Gill 1981), karnal bunt (Multani *et al.* 1988), cereal cyst nematode (Eastwood *et al.* 1991), Russian wheat aphid (Nkongolo *et al.* 1991), stripe rust (Ma *et al.* 1995), *Cochiobolus sativus* Ito and Kuribay (Mujeeb-Kazi *et al.* 1996). Furthermore, inheritance of genes introgressed into *Triticum aestivum* crossed with the synthetic wheat has been reported for leaf rust resistance (Dyck and Kerber 1970), greenbug resistance (Joppa *et al.* 1980), gliadin proteins and glume colour (Pshenichnikova and Maystrenko, 1995), and *Septoria tritici* blotch (STB) resistance (May and Lagudah 1992).

Apart from traditional hybridization, the development of molecular biology and genetic transformation techniques, combined with *in vitro* cell culture systems, has opened up new opportunities to introduce foreign genes for the improvement of quality, productivity and agronomic traits of commercially-important plants. These technologies can be used to introduce specific genes of interest into plants in a way which is potentially less time-consuming than conventional breeding methods. They can also be used to overcome the genetic barriers associated with incompatible varieties and species. Single genes can be moved into an elite genetic background, unlike direct hybridization techniques where large segments of the genome are transferred, or the synthetic hexaploid approach, where bread wheat varieties can not be used in the hybridization procedure.

Thus, the development of genetic engineering has the potential to significantly increase the available gene pool for crop improvement. It can overcome species barriers, and a vast array of genes can be made available to improve crop quality, to provide resistance to diseases, insects, and herbicides, or to increase tolerance to stress (Bowen 1993; Hinchee *et al.* 1994; Vasil 1994).

An essential component of genetic engineering technology is transformation, which encompasses the delivery, integration and expression of defined foreign genes in individual plant cells, which can subsequently be regenerated into fertile transgenic plants. Generally, the successful introduction of foreign genes into plant cells for the production of transgenic plants requires: 1) efficient *in vitro* cell culture systems, 2) reliable techniques for the delivery of genes into the plant genome and 3) efficient regeneration systems for producing normal, fertile plants from the individual transgenic cells (Vasil and Vasil 1992; Feher and Dudits 1994; Morrish *et al.* 1993; De Block 1993). *In planta* transformation techniques which do not require tissue culture techniques to produce transgenic plants are also available (Bechtold *et al.* 1993). However, in this chapter, attention is focused on transformation methods which have been used for cereals, all of which currently require tissue culture procedures. In following sections, methods for successful cell culture of cereals are described (section 1.2). Methods for DNA delivery are subsequently reviewed (section 1.3), and some of the most commonly used marker genes are discussed in section 1.4.

1.2 Plant tissue culture

The term "plant tissue culture" broadly refers to the *in vitro* cultivation of plant parts, whether they be single cells, tissues or organs. All living cells of a plant are potentially "totipotent". A totipotent cell is one that is capable of developing, by regeneration, into a whole plant. This capacity for totipotency can be used *in vitro* through the culture of organs, tissues, cells, or protoplasts and there is a wide range of cell types that have been used successfully for plant regeneration (Walden and Wingender 1995).

There are three major types of tissue culture techniques used in cereals: callus production, suspension culture and protoplast production. All have been used (Vasil and Vasil 1994; Walden and Wingender 1995; Maheshwari *et al.* 1995) to introduce genes into cereals cells or tissues.

1.2.1 Initiation of regenerable callus

Callus consists of a cluster of un-differentiated cells, which develops on a solidified nutrient medium. Callus can be induced by placing a sterile segment of excised plant tissue, known as an explant, onto a growth medium containing phytohormones such as cytokinins and auxins (Thorpe 1994). The totipotency of callus generated from different explants of cereal species has been investigated (Dudits *et al.* 1975; Chin and Scott 1977; Ahuja *et al.* 1982; Zamora and Scott 1983; Wernicke *et al.* 1986; Wernicke and Milkovits 1987; Barcelo *et al.* 1992; Mejza *et al.* 1993; Mordhorst and Lörz 1993). Immature developing embryos and tissue segments obtained from young inflorescences and from the bases of young leaves are reported to be suitable sources for initiation of regeneration callus in cereals. These explants are largely composed of meristematic and un-differentiated cells which are not yet committed to any specific developmental pathway. Callus derived from such cells is usually highly regenerable (Wernicke and Brettell 1980; Scott *et al.* 1990; Vasil and Vasil 1994; Maheshwari *et al.* 1995).

Immature embryos of cereals have now become the most common starting material for the establishment of embryogenic callus, which possesses the ability to develop somatic embryos (Scott *et al.* 1990; Vasil and Vasil 1994). The developmental stage at which immature embryo explants are isolated is important for the efficient induction of embryogenesis (Ozias -Akins and Vasil 1982; Sears and Deckard 1982; He *et al.* 1986; He *et al.* 1988). Two different components of immature embryos form embryogenic callus, namely the scutellum (Ozias-Akins and Vasil 1982; Hunsinger and Schauz 1987) and the epiblast, which is also known as "the shoot and root apical region" (Ozias-Akins and Vasil 1983; He *et al.* 1986).

Embryogenic callus contains small isodiametric cells which possess a high plant regeneration capacity, while non-embryogenic callus contains long tubular cells and infrequently produces plant regeneration. Because non-embryogenic cells generally grow much faster than embryogenic cells, selection of the embryogenic portion of the callus is required during subculturing if an embryogenic callus line is to be successfully generated

(Wernicke and Milkovits 1986). A study of induction frequencies of scutellar callus and epiblast callus from 35 cultivars of *Triticum aestivum* by He *et al.* (1988) indicated that genotype, culture medium composition and embryo age can significantly affect the induction frequencies of both scutellar callus and epiblast callus. Scutellar callus generally grew faster than epiblast callus and subsequently produced more plantlets.

A defined medium such as MS (Murashige and Skoog 1962), N6 (Chu *et al.* 1975) or B5 (Gamborg *et al.* 1968), supplemented with growth regulators is required for the production of regenerable callus in cereals. The auxin 2,4-dichlorophenoxyacetic acid (2,4-D), is routinely used for callus induction. Regeneration *via* somatic embryogenesis occurs upon withdrawal of 2,4-D from the medium. Considerable effort has been directed towards the improvement of efficiency of callus induction and plant regeneration from callus, by investigating the effect of other compounds in the callus induction medium. For example, addition of coconut milk to the callus-induction medium promoted plant regeneration in wheat (Maddock *et al.* 1983; Mathias and Simpson 1986). Papenfuss and Carman (1987) reported that the addition of kinetin (a cytokinin) and the auxin Dicamba (3,6-dichloro-O-anisic acid) to culture media enhanced shoot formation from callus.

L-Proline has been reported to promote embryogenesis in callus cultures of maize (Armstrong and Green 1985) and rice (Ozawa and Komamine 1989). In rice, addition of L-proline to the callus induction medium caused no significant difference in the frequency of callus formation but greatly enhanced the frequency of embryogenic callus formation (Chowdhry *et al.* 1993). Similar results have been reported in *Triticum tauschii* (Afshar-Sterle *et al.* 1996). Although its mechanism of action remains to be elucidated, it seems that L-proline plays an important role in both callus induction and plant regeneration.

Regardless of the method used to initiate callus, maintaining regenerability of cultures can still be a problem. In most cases, plant regeneration from embryogenic callus can be sustained for a limited period by regular transfer of the callus to fresh medium (Ozias-Akins and Vasil 1982; He *et al.* 1986; Wernicke and Milkovits 1986). However, regeneration capacity generally decreases with increasing age of the callus (Heyser *et al.* 1985).

Callus is not only used directly for plant regeneration but is also an essential source of material for initiation of suspension culture, which is discussed below.

1.2.2 Suspension culture and regeneration

Suspension cultures are clusters of undifferentiated cells growing in liquid medium and they can be initiated by placing segments of callus in vessels containing the liquid medium. The inoculated media are shaken mechanically, which causes the release of small clumps of cells from the callus into the medium. Suspension cultures usually require sub-culturing at more frequent intervals than callus cultures growing on solid medium (Gamborg and Shyluk 1981). Established suspension cultures are heterogeneous with respect to particle size, and consist of single cells and cell aggregates up to about 7 mm in diameter. Fine suspensions (< 500 μm) can be selected from these heterogeneous cultures by filtration. The resulted fine suspensions generally consist of small "cytoplasmic" cells, which lack large vacuoles but are rich in starch. If suspension cultures are embryogenic, then they are able to differentiate into somatic embryos.

Three important criteria for judging the usefulness of embryogenic suspension cultures in cereals (Jähne *et al.* 1991b; Yang *et al.* (1991) are:

- the time taken for their establishment (preferably less than 3 months)
- the ability of the suspension to regenerate fertile plants (preferably more than 50%)
- the retention of embryogenicity of the suspensions (preferably more than one year).

Several approaches have been taken to establish suspension cultures in which regeneration capacity remains for more than one year. The morphology of the callus type for the initiation of suspension cultures of hexaploid wheat has been studied by Redway *et al.* (1990a) who reported that aged (5-8 months) compact, nodular callus was the most suitable callus for the production of regenerable wheat suspension cultures. However, these workers were not able to produce regenerable suspension cultures from young (one month)

callus. In contrast, Wang and Nguyen (1990) and Yang *et al.* (1991), using primary callus, reported the production of long-term embryogenic suspension cultures of hexaploid wheat. Both groups used a simple medium (Basal MS medium without the addition of amino acids, vitamins or high concentrations of phytohormones) to produce suspension cultures. The Wang and Nguyen (1990) procedure was based on systematically selecting embryogenic cell clumps and discarding root-forming cell clumps from the suspension culture. The embryogenic capacity of these cultures was maintained for $2^1/_2$ years. Similarly, Yang *et al.* (1991) established fine embryogenic cultures (cell aggregates < 500 μm) by repeated selection of embryogenic cell clumps at each subculture. The regenerative potential of these cultures was maintained for 20 months. The time taken for the establishment of fine suspension cultures in the above studies was about one year (Wang and Nguyen 1990; Yang *et al.* 1991). Neither report indicated whether regenerated plants were fertile, although phenotypic variation amongst them was observed.

Embryogenic suspension cultures with the capacity for production of fertile plants were established in less than six months in wheat by Wang *et al.* (1990) and in barley by Jähne *et al.* (1991b). Both groups used relatively complex media supplemented with vitamin mixtures and amino acids. However, suspension cultures older than one year produced only albino plantlets (Jähne *et al.* 1991b).

The above studies indicate that a simple medium can be useful in maintaining the embryogenic capacity of suspension cultures, although the time taken in the establishment of fine suspension cultures is usually increased. A medium supplemented with vitamins and amino acids appears to reduce the time taken for the production of fine suspensions but, maintaining established cultures in a supplemented medium may result in the rapid decrease of their embryogenic capacity.

Embryogenic suspension cultures have now been generated for nearly all the cereals (Table 1.1) (Vasil and Vasil 1992). Embryogenic suspension cultures are not only useful for *in vitro* propagation and selection, they are also a suitable source for protoplast isolation.

Table 1.1 Cereal embryogenic suspension cultures obtained from callus.

Cereal	Source of explant	References
Maize	immature embryos	Rhodes *et al.* 1988a; Mórocz *et al.* 1990; Petersen *et al.* 1992
Rice	immature embryos	Abdulla *et al.* 1989; Ghosh-Biswas *et al.* 1994
	immature panicles	Utomo *et al.* 1996
	inflorescence	Ghosh-Biswas and Zapata 1990
	anther	Guiderdoni and Chaïr 1992
	mature seeds	Utomo *et al.* 1996
Wheat	anther	Harris *et al.* 1988
	immature embryos	Redway *et al* 1990b; Vasil *et al.* 1990; Wang *et al.* 1990; Wang and Nguyen 1990; Yang *et al.* 1991
Barley	immature embryos	Funatsuki *et al.* 1992; Singh *et al.* 1997
	anther	Jähne *et al.* 1991b
Sorghum	immature inflorescence	Wei and Xu 1990
Tritordeum	immature embryos	Barcelo *et al.* 1993

1.2.3 Protoplast production and regeneration

Protoplasts are plant cells from which the cell walls have been removed. They are isolated by treating tissues or cells with a mixture of cell wall degrading enzymes. The degree of success in isolating protoplasts depends upon a number of factors, including the physiological state of the tissues from which protoplasts were derived, and the particular enzyme mixture used to degrade the cell wall (Evans and Bravo 1983). Purified protoplasts are collected by filtration of protoplast suspensions through a series of sieves (Lazzeri *et al.* 1991; Ghosh-Biswas *et al.* 1994). Protoplasts can subsequently be cultured in suitable liquid media or can be embedded in solidified media containing growth regulators. Under appropriate environmental conditions of light and temperature, cell walls may regenerate and cell division may occur, resulting in the formation of minicolonies (~20 cells). Minicolonies can be transferred to fresh media for further growth and plants can subsequently be regenerated from embryogenic colonies. Nagata and Takebe (1971), using tobacco, were the first to regenerate plants from protoplasts.

Protoplasts can be isolated from various parts of the plant. Leaf mesophyll tissue is the most common and suitable source in many dicotyledonous species. In cereals, however, the successful isolation and culture of protoplasts from mesophyll cells has been limited to oats and rice. Leaf mesophyll protoplasts of oats are capable of sustained division (Hahne *et al.* 1989), and plants have been regenerated from protoplasts of rice mesophyll cells (Gupta and Pattanayak 1993).

Several other tissues have been used as a source of material for protoplast production in cereals. Thus, scutellar tissue from immature rice embryos has proved to be successful for the generation of protoplasts and for the subsequent production of fertile plants (Ghosh-Biswas *et al.* 1994). Similarly, the production of protoplasts from callus and cryopreserved callus has been reported in barley (Stöldt *et al.* 1996) and rice (Cornejo *et al.* 1995). However, suspension cultures are probably the most useful source of material for the production of protoplasts which have a capacity for sustained cell division for cereal species (Mórocz *et al.*1990; Datta *et al.* 1992; Utomo *et al.* 1995; Pauk *et al.* 1994; Golds *et al.*

1994; Singh *et al.* 1997).

Fertile plants have now been derived from protoplasts in several cereal species. The species include rice (Datta *et al.* 1990; Datta *et al.* 1992; Ghosh-Biswas *et al.* 1994), maize (Morocz *et al.* 1990), barley (Jähne *et al.* 1991a; Funatsuki *et al.* 1992; Golds *et al.* 1994; Singh *et al.* 1997) and wheat (Ahmed and Sagi 1993; Pauk *et al.* 1994). Amongst these important species, wheat (*Triticum aestivum*) has been one of the most recalcitrant for the establishment of totipotent suspension cultures and this may be due to instability in its chromosomal number during *in vitro* culture (Vasil *et al.* 1990).

Although some successes have been reported, many problems have been encountered in the production of plants from cereal protoplasts. Transformation of cereals by insertion of DNA directly into protoplasts has therefore lost favour in modern transformation technology. Problems include the formation of albino plantlets in wheat (Hayashi and Shimamoto 1988) and barley (Lührs and Lörz 1988; Mordhorst and Lörz 1992), incomplete growth of plantlets in wheat (Harris *et al.* 1988) and *Tritordeum* (Barcelo *et al.* 1993) and the production of sterile plants in wheat (Chang *et al.* 1991; He *et al.* 1992), barley (Wang and Lörz 1994), rice (Abdullah *et al.* 1986; Ghosh-Biswas and Zapata 1990) and maize (Rhodes *et al.* 1988a). Problems with morphological abnormalities and poor fertility of regenerated plants are believed to result from the nature of the donor suspension cell lines rather than from protoplast manipulation techniques (Jähne *et al.* 1991a; Wang and Lörz 1994; Utomo *et al.* 1995

1.3 Methods for delivery of DNA

Cereal transformation techniques that allow the delivery of foreign genes to the nucleus, without compromising the viability of the cell, must be developed for the production of transgenic cereals. Optimization of the survival and growth of the target cells after gene transfer is essential for efficient production of transformants and subsequent selection for the incorporated gene(s).

The first reported attempt at cereal transformation was that of Coe and Sarkar

(1966), in which crude nucleic acid extracts from a maize variety carrying several dominant marker genes were injected directly into the apices of seedlings carrying the recessive alleles of the genes. However, complementation of recessive mutations was not achieved by this treatment, and transformation was clearly not successful. Subsequently, many groups have tried various methods for transformation of cereals, including:

- microinjection, in which a needle was used to insert DNA directly into cells (Toyoda *et al.* 1990)
- macroinjection, in which a syringe was used to inject DNA into each tiller node of the plants (De la Pena *et al.* 1987)
- using cut-off pollen tubes to introduce DNA into the zygote (Luo and Wu 1988)
- laser treatments to create holes in cell walls or membranes (Weber *et al.* 1990)
- electrophoresis of DNA molecules into seed tissues (Ahokas 1989)
- silicon carbide fibre-mediated DNA delivery into intact plant cells (Kaeppler *et al.* 1990)
- tissue electroporation (D'Halluin *et al.* 1992; Laursen *et al.* 1994)
- direct DNA uptake by protoplasts (Krens *et al.* 1982)
- microprojectile bombardment (Gordon-Kamm *et al.* 1990)
- *Agrobacterium*-mediated transformation (Raineri *et al.* 1990; Chan *et al.* 1992).

Most of these techniques have not been successful for cereal transformation. However, methods which have some potential for cereal transformation include transformation of protoplasts using PEG and/or electroporation, tissue electroporation, silicon carbide fibre-mediated DNA delivery, *Agrobacterium*-mediated transformation and microprojectile bombardment (Table 1.2).

1.3.1 Direct DNA delivery into protoplasts

Both physical (electroporation) and chemical (polyethylene glycol) methods have been developed to deliver DNA directly into protoplasts (Krens *et al.* 1982; Potrykus *et al.* 1985; Fromm *et al.* 1985). Protoplasts have several key advantages over other acceptor

Table 1.2 Transgenic cereals obtained by the direct delivery of DNA into protoplasts (P), microprojectile bombardment (B), tissue electroporation (E) and *Agrobacterium*-mediated transformation (A).

Cereal	Method of transformation	References
Maize	P	Rhodes *et al.* 1988b; Golovkin *et al.* 1993; Bilgin *et al.* 1999; Wang *et al.* 2000
	B	Fromm *et al.* 1990; Gordon-Kamm *et al.* 1990; Walters *et al.* 1992; Koziel *et al.* 1993; Wan *et al.* 1995; Zhong *et al.* 1999; Frame *et al.* 2000
	A	Ishida *et al.* 1996
	E	D'Halluin *et al.* 1992; Laursen *et al.* 1994
Rice	P	Shimamoto *et al.* 1989; Hayashimoto *et al.* 1990; Datta *et al.* 1990, 1992; Terada *et al.* 1993; Rathore *et al.* 1993; Chaïr *et al.* 1996
	B	Christou *et al.* 1991; Cao *et al.* 1992; Li *et al.* 1993; Jain *et al.* 1996; Abedinia *et al.* 1997; Chen *et al.* 1998; Nandadeva *et al.* 1999; Tang *et al.* 2000
	E	Xu and Li 1994
	A	Hiei *et al.* 1994; Rashid *et al.* 1996; Hiei *et al.* 1997
Barley	P	Salmenkallio-Marttila *et al.* 1995; Funatsuki *et al.* 1995
	B	Wan and Lemaux 1994; Ritala *et al.* 1994; Hagio *et al.* 1995; Koprek *et al.* 1996; Leckband and Lörz 1998; Brinch-Pedersen *et al.* 1999; Harwood *et al.* 2000
	A	Tingay *et al.* 1997
Wheat	B	Vasil *et al.* 1993; Nehra *et al.* 1994; Becker *et al.* 1994; Altpeter *et al.* 1996; Ortiz *et al.* 1996; Leckband and Lörz 1998; Altpeter *et al.* 1999; Iser *et al.* 1999; Rasco-Gaunt *et al.* 1999; Uzé *et al.* 1999; Brinch-Pedersen *et al.* 2000; Liang *et al.* 2000; Zhang *et al.* 2000
	A	Cheng *et al.* 1997
Tritordeum	B	Barcelo *et al.* 1994
Rye	B	Castillo *et al.* 1994
Oat	B	Somers *et al.* 1992

cell systems for direct delivery of DNA (Potrykus 1991). The freely accessible plasma membrane enables DNA to reach and enter every protoplast in a given population, at DNA concentrations that can be regulated experimentally. Foreign genes can therefore reach every competent cell, thus increasing the chance of recovery of transgenic plants from a given population of cells.

Electroporation allows uptake of DNA into protoplasts by temporary permeabilisation of the plasma membrane. This is achieved by application of a high-voltage electric pulse to protoplasts that are suspended in buffer containing the DNA. DNA diffuses into protoplasts immediately after the electric field is applied and until the pores in the membrane reseal (Shillito *et al.* 1985; Fromm *et al.* 1988). Optimal DNA transfer is achieved by using the appropriate electric field strength which, in turn is dependent upon a number of parameters. These parameters include capacitor size (which is important in determining the pulse length), buffer composition and temperature, DNA concentration, protoplast density, protoplast size, addition of optimal concentrations of polyethylene glycol and the application of a heat shock (Hinchee *et al.* 1994).

Polyethylene glycol (PEG) is the most widely-used chemical treatment for facilitating DNA uptake into plant protoplasts. PEG-mediated transformation involves mixing freshly isolated protoplasts with DNA and immediately adding PEG dissolved in a buffer containing divalent cations. PEG treatment causes reversible permeabilisation of plasma membranes and thereby enables exogenous macromolecules to enter the cytoplasm (Krens *et al.* 1982). For this procedure, important factors for optimizing transformation frequency include: PEG concentration, salt composition and concentration, pH, DNA concentration and DNA size and form (e.g. linear or supercoiled) (Hinchee *et al.* 1994). The precise mechanisms of PEG-mediated membrane permeabilisation, transfer of DNA to the nucleus, and its incorporation into the genome are not understood (Hinchee *et al.* 1994; Songstad *et al.* 1995).

The first transgenic plants generated by the PEG procedure were reported by Paszkowski *et al.* (1984) where transfer and expression of *Agrobacterium tumefaciens*

T-DNA genes were demonstrated in tobacco protoplasts. Direct DNA-uptake has since been applied successfully for transformation of cereal protoplasts and would be an ideal experimental system for gene transfer to plants were it not for the problems experienced with plant regeneration from protoplasts (Marsan *et al.* 1993; Lazzeri *et al.* 1991). Initial efforts at DNA uptake by cereal protoplasts were successful in producing transformed callus lines but no transgenic plants were produced (Fromm *et al.* 1986; Rhodes *et al.* 1988a; Lazzeri *et al.* 1991). However, subsequent work using rice (Toriyama *et al.* 1988; Zhang and Wu 1988) and maize (Rhodes *et al.* 1988b) resulted in the production of the first transgenic cereal plants.

Although protoplast transformation has been successful in rice (Datta *et al.* 1990; Zhang *et al.* 1988; Terada *et al.* 1993 Chaïr *et al.* 1996) and maize (Golovkin *et al.* 1993; Omirulleh *et al.* 1993; Bilgin *et al.* 1999; Wang *et al.* 2000) and it has been possible to routinely obtain fertile transgenic plants in these species, it is not yet a reliable transformation approach for many other cereal species (e.g. wheat and barley). In wheat, protoplast transformation has resulted in the recovery of stably transformed callus lines (Zhu *et al.* 1993) and the production of infertile transgenic plants (He *et al.* 1994). Although transgenic barley plants have been obtained using protoplasts isolated from embryogenic callus (Salmenkallio-Marttila *et al.* 1995; Kihara *et al.* 1998) and embryogenic suspension cultures (Funatsuki *et al.* 1995), the production of fertile transgenic plants is not routine for this species. Difficulties in the production of fertile plants from protoplasts have limited the application of this transformation method for most cereal species.

1.3.2 Tissue electroporation for DNA delivery

Tissue electroporation, which uses a high-voltage electric pulse to introduce DNA into intact plant cells, rather than protoplasts, is an alternative transformation approach (Lindsey and Jones 1990). The production of transgenic plants by electroporation of suspension cultures and immature embryos has been reported in maize (D'Halluin *et al.* 1992; Laursen *et al.* 1994; Li *et al.* 2000) and wheat (Sorokin *et al.* 2000), but this method

is not widely used because of its low reproducibility.

1.3.3 Silicon carbide fibre-mediated DNA delivery

Silicon carbide fibre-mediated transformation has been investigated in attempts to develop a simple, rapid and inexpensive transformation method for both monocotyledonous and dicotyledonous plant species (Kaeppler *et al.* 1990). The method involves vortexing a microcentrifuge tube containing a mixture of DNA, silicon carbide fibres and plant explants. The silicon carbide fibres act as microinjection needles, which facilitate DNA delivery into the plant cells. Kaeppler *et al.* (1990) used this method to transform suspension cultured cells of maize and tobacco with the ß-glucuronidase (*GUS*) gene. Transient expression of GUS activity was demonstrated. Serik *et al.* (1996) used similar methods to deliver foreign DNA into mature embryos of wheat and GUS expression was demonstrated in leaf tissues derived from the germinating embryos and from one month-old callus derived from the embryos. Recently, transgenic plants have been obtained in four grass species (Dalton *et al.* 1998), maize (Petolino *et al.* 2000) and rice (Matsushita *et al.* 1999) using this procedure. The potential of this system for stable transformation is still under investigation. However, because the use of fibres and their potential carcinogenic effects, this transformation method has not been widely used (Kaeppler *et al.* 1990).

1.3.4 *Agrobacterium*-mediated DNA delivery

Agrobacterium spp. are commonly found in both cultivated and non-agricultural soils and can be readily isolated either from the soil itself (Burr and Katz 1983) or from the roots of infected plants (Bouzar and Moore 1987). *Agrobacterium tumefaciens* is the causal agent of crown gall disease of dicotyledonous plants. The "crown gall" is a tumourous growth which results from the expression of genes carried by a DNA segment of bacterial origin that is transferred and becomes stably integrated into the plant genome.

During infection, *Agrobacterium* has the ability to transfer a discrete portion of its large (approximately 200 kb) tumour-inducing (Ti) plasmid into plant chromosomes (Figure 1.2). There are two important regions on the Ti-plasmid: the oncogene-containing T-DNA

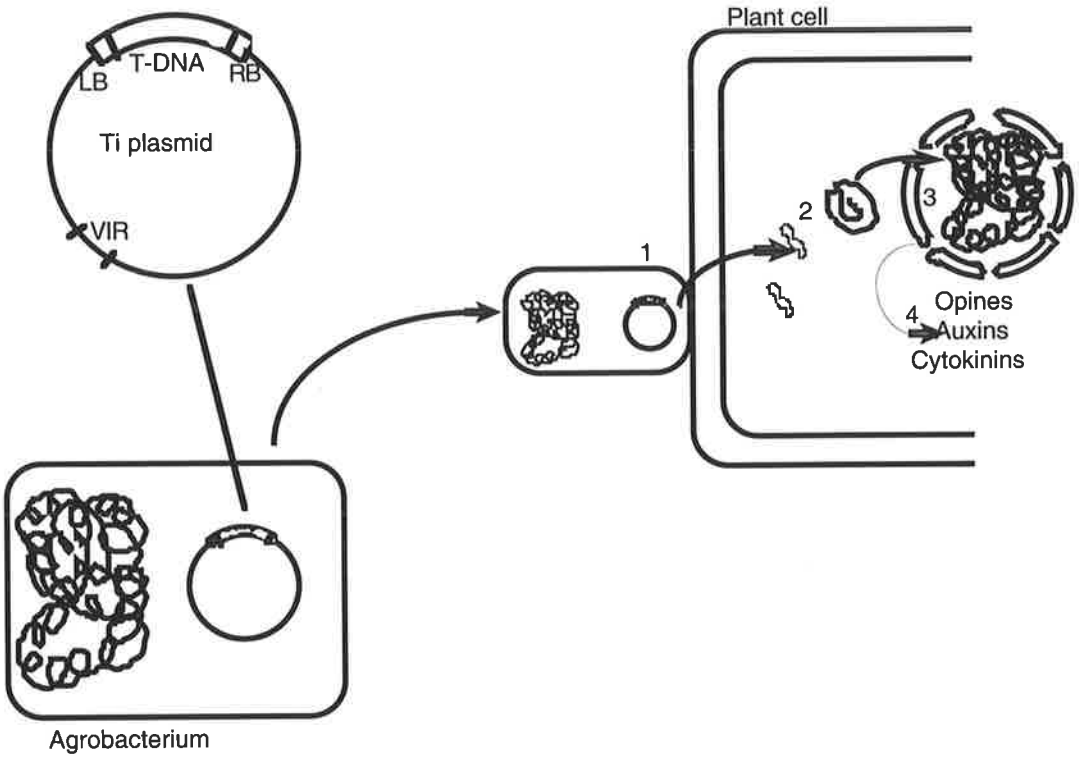


Figure 1.2 The basic steps in the transformation of plant cell *by Agrobacterium tumefaciens* (adapted from Lindsey 1992). LB – left border, T-DNA –transfer DNA, RB – right border, VIR – virulence gene and Ti – tumour inducing.

(Transfer DNA) and the virulence (*vir*) genes. The *vir* region contains seven operons, of which four (*vir*A, *vir*G, *vir*B, and *vir*D) encode a variety of proteins that are essential for excision and transfer of the T-DNA from bacterial to plant DNA (Binns and Thomashow1988; Zambryski 1992; Hinchee *et al.* 1994; Zupan and Zambryski 1995). The T-DNA is delimited by two 25 bp direct repeats, called the T-DNA borders. Any DNA located between these borders is transferred to the plant cell. T-DNA in the wild type oncogenic *Agrobacterium tumefaciens* strains contains genes which, when expressed in plant cells, cause over-production of the phytohormones auxin and cytokinin, and the production of these compounds in transformed plant cells results in uncontrolled cell division, and therefore in tumour formation. The T-DNA also encodes enzymes for the synthesis of novel amino acid derivatives called opines which are specifically metabolized by the bacterium and facilitate the establishment of bacterial infection. The Ti-plasmid encodes enzymes for opine catabolism (Zambryski 1992; Zupan and Zambryski 1995).

Molecular characterization of the DNA transfer process suggested some time ago that *Agrobacterium* might be used to deliver foreign genetic material into plant genomes (Lindsey 1992; Zupan and Zambryski 1995). Ti plasmid vectors were developed in the early 1980s based on the observations that the T-DNA of the Ti plasmid is stably transferred from *Agrobacterium tumefaciens* into the plant chromosome and that any foreign DNA sequences inserted within the T-DNA borders could be transferred. Because only the 25-bp border sequences located at each end of the T-DNA are required for the DNA transfer mechanism, "disarmed" Ti plasmid vectors were developed which allowed DNA transfer without causing tumour formation. Disarmed Ti plasmid vectors are created by replacing the oncogenic genes with genes of interest, using *in vivo* recombination (De Block 1993; An 1995).

Although the disarmed vectors have been used widely for transferring foreign genes into the plant chromosome, this system is not easy to use because the Ti plasmid is large and difficult to manipulate. As a result, much smaller and simpler 'binary' plasmid vectors were developed, based on the finding that the T-DNA region does not have to be physically

linked to the *vir* genes of the Ti plasmid (An 1995). In the binary vector system the *vir* genes are supplied by the resident disarmed Ti plasmid and the T-DNA is present on a separate plasmid vector which is capable of replicating in both *Agrobacterium* and *Escherichia coli*. The binary system is much easier to use because the vectors are smaller (about 10-15 kb) and do not require *in vivo* recombination with the Ti plasmid (Bevan 1984; An 1995).

Agrobacterium tumefaciens provided one of the first DNA delivery systems for plant transformation and it was used initially to produce transgenic tobacco (Fraley *et al.* 1983; Zambryski *et al.* 1983). For most dicotyledons, *Agrobacterium*-mediated transformation is now used routinely for the production of transgenic plants. In the past, cereals and most other monocotyledonous species were considered to be outside the natural host range of *Agrobacterium* (Potrykus 1990). However, modification of co-cultivation conditions can lead to successful gene transfer to species once thought to be beyond the host range of *Agrobacterium* (Godwin *et al.* 1992). Several efforts have been made to exploit this system to obtain transgenic cereals. Initial attempts were in rice (Raineri *et al.* 1990; Chan *et al.* 1992; Chan *et al.* 1993), maize (Gould *et al.* 1991) and wheat (Mooney *et al.* 1991). These reports provided no evidence for the stable integration of transgenes into the cereal genome, nor of their segregation pattern in the progeny. However, the results indicated that graminaceous species can at least be infected by *Agrobacterium*. Further evidence for the potential of *Agrobacterium*-mediated transformation of cereals was demonstrated by Hiei *et al.* (1994), who obtained transformation frequencies as high as that of dicots (between 12-29%) in Japonica rice and demonstrated Mendelian transmission of introduced DNA to progeny. Similar results have been reported in Basmati cultivars of rice by Rashid *et al.* (1996), and have now been extended to barley (Tingay *et al.* 1997) and wheat (Cheng *et al.* 1997). Thus, *Agrobacterium*-mediated gene transfer provides an attractive alternative to microprojectile bombardment for transformation of cereals.

One of the major advantages of this DNA delivery system is that a high percentage of transgenic plants have a single copy insertion of the transgene. For example, 35% of

wheat (Cheng *et al.* 1997), 32% of rice (Hiei *et al.* 1994) and 60-70% of maize (Ishida *et al.* 1996) transgenic plants contained single copies of the transgenes. Furthermore, there is no shearing of DNA during the transformation procedure.

1.3.5 Microprojectile bombardment for DNA delivery

Microprojectile bombardment is a method whereby small metal particles, normally tungsten or gold, are coated with DNA and accelerated into intact plant cells or tissues (Sanford *et al.* 1987). The transfer of DNA into plant cells is therefore a simple mechanical process, although details of the mechanism of subsequent incorporation of DNA into the genome are not well understood. Microprojectile bombardment has several advantages over *Agrobacterium*-mediated transformation, including:

- plasmid construction is simplified, because binary vector systems are not required
- there is no need to eliminate *Agrobacterium* by antibiotic treatment after DNA delivery
- no hypersensitive plant/pathogen response is encountered.

The first microprojectile device was based on the design of Sanford *et al.* (1987). This device used a gunpowder charge to propel microscopic tungsten particles on the face of a plastic cylinder, called a macrocarrier. The device proved successful for genetic transformation of diverse plant species in numerous laboratories (Sanford 1990). However, lack of control over the power of bombardment, as well as substantial damage to target cells resulted in low transformation frequencies. Nevertheless, microprojectile gun technology has been improved in recent years. Currently, the Biolistic® PDS-1000/He, which is marketed by Bio-Rad Laboratories, Richmond, California (Kikkert 1993), is the only commercially-available particle delivery system for plant transformation. The PDS-1000/He represents a significant technical improvement over the gunpowder device. It is powered by helium gas that builds up pressure behind a "rupture disk". When the pressure reaches a predetermined level, the disk ruptures and the burst of released helium gas accelerates a macrocarrier, upon which DNA-coated microprojectiles have been dried

(Figure 1.3). Rupture disks of different thickness allow the helium gas pressure to be varied. The macrocarrier hits a "stopping screen", but the DNA-coated microparticles continue and penetrate the plant material. The process is performed in a vacuum chamber (Figure 1.3). The vacuum reduces the drag on the particles and lessens tissue damage by dispersion of the helium gas prior to impact (Kikkert 1993). The PDS-1000/He is cleaner and safer than the gunpowder device. It allows better control over bombardment power, distributes microprojectiles more uniformly over target cells, is more gentle to target cells, is more consistent from bombardment to bombardment, and yields 4-300 fold higher transformation efficiencies in the species tested (Sanford *et al.* 1991).

A less expensive alternative to the PDS-1000/He microparticle gun is the "flowing helium gun" which accelerates particles directly in a stream of low-pressure helium (Takeuchi *et al.* 1992). The flowing helium gun was used as the basis for development of the Particle Inflow Gun (PIG). The PIG device uses compressed helium to propel the particles, a timer relay-driven solenoid to release the helium and a vacuum chamber to hold the target tissue (Finer *et al.* 1992; Vain *et al.* 1993b).

Other devices have been generated to accelerate DNA-coated particles into plant cells. These include an electric discharge particle accelerator (ACCELL™ technology), which accelerates DNA-coated gold particles to any desired velocity by varying the input voltage (Christou *et al.* 1988; McCabe *et al.* 1988; McCabe and Christou 1993) and an air gun device, which is used to propel DNA-coated gold or tungsten particles (Oard *et al.* 1990; Oard *et al.* 1993). The latter device delivers a high particle density to a small area and may therefore be better suited to the bombardment of targets such as meristems and embryogenic tissues (Sautter *et al* 1991; Sautter 1993). These devices have been developed with the same goals: increased simplicity, safety, accuracy, and lower cost for DNA delivery. Nevertheless, the Biolistic® PDS-1000/He and PIG devices remain the most commonly-used microprojectile guns for cereal transformation.

The choice of an appropriate target tissue is of major importance for cereal transformation by microprojectile bombardment. So far, tissues most often used have been

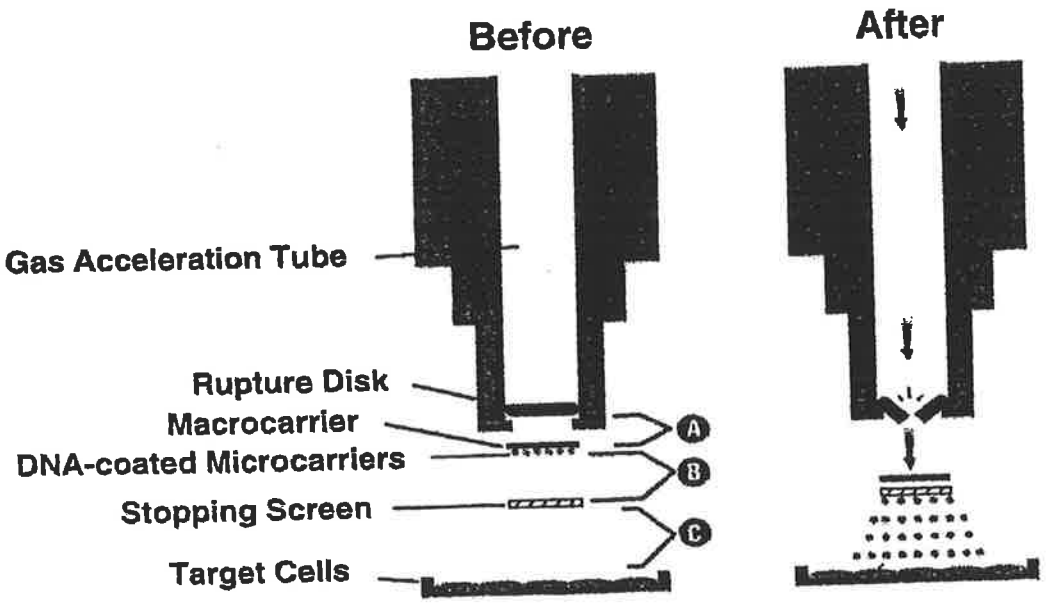


Figure 1.3 The Biolistic bombardment process (reproduced from Bio-Rad instruction manual).

suspension cultures, embryogenic callus and immature embryo explants. The first cereal transformation which resulted in the production of fertile transgenic plants was achieved by microprojectile bombardment of maize suspension cultures (Gordon-Kamm *et al.* 1990). Subsequently, transgenic rice and oat plants have been generated from suspension cultures (Cao *et al.* 1992; Somers *et al.* 1992). However, embryogenic callus is a preferred target tissue because the time for production of the cultures is short compared with suspension cultures. Transgenic plants have been produced from embryogenic callus of wheat (Vasil *et al.* 1992; Ortiz *et al.* 1996; Iser *et al.* 1999), barley (Wan and Lemaux, 1994), maize (Walters *et al.* 1992; Wan *et al.* 1995; Frame *et al.* 2000), rice (Abedinia *et al.* 1997; Tang *et al.* 2000), rye (Castillo *et al.* 1994) and oats (Somers *et al.* 1992). Transgenic plants have also been generated by microprojectile bombardment of scutellar tissues of rice (Christou *et al.* 1991; Jain *et al.* 1996), maize (Koziel *et al.* 1993; Zhong *et al.* 1999), barley (Ritala *et al.* 1994; Hagio *et al.* 1995; Koprek *et al.* 1996; Brinch-Pedersen *et al.* 1999; Harwood *et al.* 2000), wheat (Vasil *et al.* 1993; Nehra *et al.* 1994; Becker *et al.* 1994; Altpeter *et al.* 1996; Rasco-Gaunt *et al.* 1999; Brinch-Pedersen *et al.* 2000; Liang *et al.* 2000; Zhang *et al.* 2000) and *Tritordeum* (*Hordeum* chilense x *Triticum durum*) (Barcelo *et al.* 1994). Scutellar tissue of immature embryos is considered to be the best tissue for microprojectile bombardment transformation because it has a relatively high regeneration capacity and transformants can be produced in a short time (Jähne *et al.* 1995).

1.4 Marker genes

Marker genes are used to confirm DNA delivery during the development of transformation procedures (Bowen 1993). Marker genes can be subdivided into visual markers ("reporter" genes) and "selectable markers", which are used to select transgenic cells from a background of non-transformed cells.

1.4.1 Reporter genes

Reporter genes are exploited in transformation procedures because they can be easily

and directly detected in plant tissues. Such marker genes encode readily-detectable products, such as enzymes or antigens which are not usually present in the target plant cells. Delivery of DNA and its expression in plant cells can be evaluated 24-48 hours after transformation, through transient expression assays of the reporter genes. The detection of a reporter gene in transient assays does not require integration of the DNA into the host genome. Transient expression studies enable the number of "transformed" cells to be quantified and the location of cells where the reporter genes have been introduced to be defined. Thus, a reporter gene can be used in the establishment of a transformation system for the rapid optimization of the transformation protocol and also to evaluate the suitability of plasmid constructs (Bowen 1993; Vasil 1994; Brettell and Murray 1995).

The reporter genes which have been most commonly used in transformation studies are the *R-nj* gene of maize, which regulates anthocyanin biosynthesis and produces distinctive pigmentation in cells in which it is expressed (Ludwig *et al.* 1990; Bodeau and Walbot 1995), the luciferase (*luc*) gene of firefly (*Photinus pyralis*) (de Wet *et al.* 1987), for which expression can be detected by supplying appropriate luminogenic substrates, the β-glucuronidase (*GUS*) gene (Jefferson *et al.* 1987), which is encoded by the *uidA* locus from *Escherichia coli* and for which expression can be detected with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) substrate, and the green fluorescent protein (GFP) gene (Chiu *et al.* 1996) from *Aequorea victoria.*

The *GUS* gene has been most widely used in plant transformation. The advantage of the *GUS* gene over other reporter genes is the simplicity of the assay. In histochemical analysis 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) substrate is used to detect *GUS* activity in cells and tissue of transformed plants (Jefferson *et al.* 1987; Jefferson 1989). Although the X-gluc substrate is colourless, the GUS enzyme releases the blue compound 5-bromo-4-chloro-3-indole, which can be easily detected visually and indicates where the introduced reporter gene is being expressed. Expression of *GUS* can also be measured accurately in very small amounts of the transformed plant tissue using a fluorometric assay, where 4-methylumbelliferyl β-D-glucuronide (MUG) is used as substrate

(Jefferson *et al.* 1987). The major disadvantage of the *GUS* reporter gene system is that transformed tissue is destroyed during the assays.

More recently, a modified version of the green fluorescent protein gene (*GFP*) from the jellyfish *Aequorea victoria* has been developed (Chalfie *et al.* 1994). The modified GFP gene is expressed efficiently in plant cells and permits non-lethal fluorescence detection under specific excitation wavelengths of light (Chiu *et al.* 1996; Ahlandsberg *et al.* 1999; Chung *et al.* 2000). Thus, GFP has several significant advantages over other visual marker genes. Its expression can be detected in real time in living cells simply by its fluorescence. Detection of GFP does not require a substrate, unlike firefly luciferase (LUC) (Ow *et al.* 1986) and GUS (Jefferson 1987). A further advantage of GFP is that its detection is non-destructive. Thus, GFP allows ongoing monitoring of gene expression and protein localization at the sub-cellular, cellular and plant level.

1.4.2 Selectable marker genes

Selectable marker genes are generally used for the selection of transformed cells in the presence of native, untransformed cells. These markers, which include antibiotic resistance and herbicide resistance genes, allow plant cells, tissues or whole plants to grow in the presence of an appropriate selective agent. Antibiotic resistance genes have been used widely and successfully in transformation studies. The *hpt* gene from *Escherichia coli* (van den Elzen *et al.* 1985), which confers hygromycin resistance, and the *nptII* gene from a bacterial transposon *Tn5* (Herrera-Estrella *et al.* 1983), which confers kanamycin, G418 or geneticin resistance, have been used in the selection of transgenic cells during cereal transformation. Transgenic rice (Datta *et al.* 1990; Battraw and Hall 1992; Abedinia *et al.* 1997; Tang *et al.* 2000), wheat (Nehra *et al.* 1994; Ortiz *et al.* 1996), maize (Sukhapinda *et al.* 1993; D'Halluin *et al.* 1992) and barley (Salmenkallio-Marttila *et al.* 1995) have been selected with these markers. However, Vasil (1994) reported that the constitutive expression of antibiotic resistance genes has generated much public concern regarding the presence of these genes in food crops.

Attempts have therefore been made to use herbicide resistant genes as selectable markers, in preference to the antibiotic genes. The herbicide L-phosphinothricin (PPT or glufosinate) is an analogue of glutamate which inhibits glutamine synthetase (GS), an enzyme critical for the assimilation of ammonia and for general nitrogen metabolism in plants (De Block *et al.* 1987). Inhibition of GS causes accumulation of ammonia, which leads to cell death (Tachibana *et al.* 1986). PPT is chemically synthesized under the trade name Basta (Hoechst AG) or produced by *Streptomyces hygroscopicus* as a mixture known as bialaphos (Meiji Seika Ltd). The *bar* gene of *Streptomyces hygroscopicus* controls resistance to this herbicide. This gene encodes the enzyme phosphinothricin acetyltransferase (PAT) and provides resistance by acetylating the phosphinothricin herbicide (De Block *et al.* 1987). PPT or bialaphos selection has been successfully used in the transformation of major cereal species, including maize (Fromm *et al.* 1990; Gordon-Kamm *et al.* 1990) and wheat (Vasil *et al.* 1992; Brinch-Pedersen *et al.* 2000). However, Vasil (1994) recommended that in order to avoid the possibility of weeds becoming herbicide-resistant, great caution should be exercised in the introduction of herbicide resistant genes into cereal crops, especially oats (Somers *et al.* 1992) and sorghum (Casas *et al.* 1993), which can interbreed with weeds like wild oats and Johnson grass, respectively.

1.5 Transformation of agronomically useful genes into cereals

The success achieved with transformation techniques can be used to supplement breeding methods for the introduction of agronomically useful traits into cereal species. Some agronomically-useful genes have already been introduced into major cereal crops. For example, the *bar* gene from *Streptomyces hygroscopicus* which confers resistance to the herbicide glufosinate, has been successfully transferred into wheat (Becker *et al.* 1994; Nehra *et al.* 1994), rice (Christou *et al.* 1991), maize (Fromm *et al.* 1990) and barley (Wan and Lemaux 1994). The stilbene synthase gene *of Vitis vinifera* L. has been transferred into barley and wheat to increase fungal resistance (Leckband and Lörz 1998; Liang *et al.* 2000).

Transgenic rice with increased resistance to infection by the sheath blight pathogen has been achieved by transformation with a chitinase gene (Lin *et al.* 1995; Nishizawa *et al.* 1999). A gene to increase insect resistance (the truncated synthetic *cryIA(b)* gene from *Bacillus thuringiensis*) have been successfully transferred into maize (Koziel *et al.* 1993) and rice (Wünn *et al.* 1996; Alam *et al.* 1999; Shu *et al.* 2000). Insertion of the barley trypsin inhibitor CMe (BTI-CMe) into wheat has also increased insect resistance in transgenic wheat (Altpeter *et al.* 1999). A gene to improve dough quality (high-molecular-weight glutenin subunits, HMW-GS) has been successfully transferred into wheat (Blechl and Anderson 1996; Barro *et al.* 1997; Rooke *et al.* 1999). Furthermore, transgenic herbicide-resistant and insect-resistant (Bt) corn have been commercialized (James 1998).

1.6 Objectives

This study was initiated with the aim of establishing a system for genetic engineering of *Triticum tauschii* by:

- development of a reliable protocol for *in vitro* culture and regeneration of *Triticum tauschii*
- evaluation of two DNA delivery systems (protoplast transformation and microprojectile bombardment) and their adaptation to *Triticum tauschii*.

Triticum tauschii was chosen for this study because:

- there was little success in the transformation of *Triticum aestivum* when this study was commenced
- its chromosomes are relatively stable during *in vitro* manipulation (Winfield *et al.* 1992)
- synthetic hexaploid wheats can be generated relatively easily from *Triticum tauschii* through crosses with the tetraploid wheat *Triticum turgidum* (May and Lagudah 1992; Xiu-Jin *et al.* 1997)

- *Triticum tauschii* carries a number of potentially useful genes (Gill *et al.* 1986; Lagudah and Appels 1993) which could be introgressed into wheat varieties, together with the desirable transgenes.

Furthermore, at that time, there were no reports of regenerable *in vitro* culture and genetic transformation of *Triticum tauschii*. Several steps were therefore required to develop cell and tissue culture systems for this species. It was necessary firstly to produce protocols for the production of embryogenic callus. The development of these protocols is described in Chapter 2. Procedures were also developed for the establishment of long-term embryogenic suspension cultures (Chapter 3) and for the production of fertile protoplast-derived plants (Chapter 4). Finally, experiments were undertaken to evaluate two transformation systems, namely direct DNA transformation into protoplasts and microprojectile-mediated transformation (Chapter 5).

CHAPTER 2

EMBRYOGENIC CALLUS INDUCTION AND PLANT REGENERATION FROM *TRITICUM TAUSCHII*

2.1 Introduction

Triticum tauschii contains considerable genetic variation and therefore has potential for providing genes for the improvement of bread wheat (Gill *et al.* 1986; Lagudah and Appels 1993). To expand the repertoire of useful traits in *Triticum tauschii*, new or modified genes may be introduced into this species *via* genetic engineering. *Triticum tauschii* is also an attractive candidate for genetic transformation because of its chromosomal stability during *in vitro* manipulation (Winfield *et al.* 1992). Because *Triticum aestivum* has proved difficult to transform, it was proposed at the beginning of this project that *Triticum tasuchii* might be useful as a "shuttle" species for transferring genes into elite bread wheat varieties. Thus, if stable *Triticum tauschii* cultures could be more easily engineered with potentially useful genes, these genes, together with other desirable genetic variation in *Triticum tauschii* itsself, might then be transferred to *Triticum aestivum* L. either by direct crosses (Gill and Raupp 1987) or by synthesising an allohexaploid by hybridizing the diploid *Triticum tauschii* (DD) with the tetraploid *Triticum turgidum* (AABB) (Dyck and Kerber 1970; Joppa *et al.* 1980).

One essential pre-requisite for plant transformation *via* microprojectile bombardment or protoplast transformation is the effective production of embryogenic callus possessing long-term competence for regeneration. Embryogenic callus have been used to establish suspension cultures for genetic transformation from most cereal species (Jähne *et al.* 1995). In addition, embryogenic callus have been one of the most common sources of targeted material, after immature embryos, for the production of transgenic plants using microprojectile bombardment in cereals (Vasil *et al.* 1992; Wan and Lemaux 1994; Castillo *et al.* 1994). Furthermore, embryogenic callus have been used as a source for protoplast isolation and transformation (Zaghmout 1994; Stöldt *et al.* 1996; Cornejo *et al.* 1995).

In the genus *Triticum*, embryogenic callus have been derived from immature embryos of hexaploid wheat, *Triticum aestivum* L. (Ahloowalia 1982; Maddock *et al.* 1983; He *et al.* 1988; Redway *et al.* 1990a; Qiao *et al.* 1992; Ahmed and Sagi 1993), tetraploid wheat *Triticum turgidum* (Hagemann *et al.* 1988) and *Triticum durum* (Bennici 1986; Borrelli *et*

al. 1991). However, the production of embryogenic callus with long-term competence for regeneration had not been reported in *Triticum tauschii.* In this Chapter the development of a protocol for the induction of long-term embryogenic callus cultures from different genotypes of *Triticum tauschii* is described.

2.2 Materials and Methods

2.2.1 Source of *Triticum tauschii*

Seven accessions of *Triticum tauschii*, four from the Australian Winter Cereals Collection, Tamworth (AUS) and three from CSIRO Division of Plant Industry, Canberra (CPI) (Appendix 1) (Table 2.1) were tested for their capacities to produce callus in *in vitro* culture and for the production of embryogenic callus that retained their ability to regenerate fertile plants for lengthy periods.

2.2.2 Plant growth conditions

Grains were germinated at 22°C in Petri dishes on filter paper moistened with distilled water. After a week, seedlings were transplanted into 10 cm pots containing a pine bark and peat potting mix, fertilised with a 3 - 4 month slow release complete fertiliser (Osmocote®, N:P:K = 14:6.1:11.5), and grown in a glasshouse at 22 ± 4°C. Natural light was supplemented by 400W metal-halide lamps to provide a 16-hour photoperiod. Plants were vernalised at 4°C with a 9-hour photoperiod for 4 weeks, commencing 5 weeks after transplanting.

Immature grains were collected 12-15 days after anthesis and surface-sterilised by immersion in 70% ethanol for 1 min, followed by 1.5% (w/v) sodium hypochlorite for 20 min. The grains were rinsed three times in sterile, distilled water and the immature embryos (0.5-1.5 mm long) excised.

2.2.3 Callus induction from immature embryos

Excised embryos were cultured with the shoot-root axis in contact with the surface of a solid medium in 5.5-cm diameter plastic Petri dishes. The dishes were sealed with Parafilm and incubated in the dark at 22 ± 1°C.

Basal MS medium (Murashige and Skoog 1962) was supplemented with various hormones and amino-acids (Table 2.2) to determine their effect on callus formation. All

Table 2.1 Designation and origins of *Triticum tauschii* accessions used in this study.

Accession Number	Country of Origin	Sub-species
CPI 110718	Pakistan	*anathera*
CPI 110809	Iran	*meyeri/typica*
CPI 110810	Iran	*meyeri/typica*
CPI 110813	Iran	*meyeri/typica*
AUS 18912	Iran	*meyeri*
AUS 18913	Iran	*meyeri*
AUS 18914	Iran	*meyeri*

Table 2.2 Different growth additives evaluated for the induction of embryogenic callus using basal MS medium. All modified media were also supplemented with 30 g L^{-1} sucrose.

Growth additives	Medium (mg L^{-1})									
	A	B	C	D	E	F	G	H	I	J
2,4-D	2	2								
Dicamba®			5	10	20	24	30	24	24	24
Kinetin									0.2	0.2
Myo Inositol	100	100	100	100	100	100	100	100	250	250
L-Glutamine		750	750	750	750	750	750	750		400
L-Asparagine		100	100	100	100	100	100	100		150
L-Proline		150	150	150	150	150	150	725		
Casein hydrolysate									300	300

Modification after Wang *et al.* (1990).

media contained 3% sucrose, and the pH was adjusted to 5.8 with KOH or HCI. For solid media, double-strength nutrient solution was filter-sterilised using 0.22 μM Millipore filters and mixed with an equal volume of autoclaved Phytagel solution (Sigma, Catalogue No. P-8169) held at a temperature of 44 ± 2°C. The final concentration of Phytagel was 0.25% (w/v).

After 4 weeks, the number of embryos which had formed callus was recorded. Embryogenic callus were identified by microscopic examination and transferred either to media H, I or J (Table 2.2) for further subculturing.

2.2.4 Determination of regenerative capacity

Callus that was 4 weeks old and growing on medium H was subsequently placed on regeneration media. The regeneration media comprised basal MS supplemented with 2,4-D at 0 - 0.2 mg L^{-1}. The plates were incubated at 22°C under constant light (6o μE m^{-2} s^{-1}) for 6 weeks. The first shoots were visible after 2 weeks. Regenerated shoots (0.5-1 cm long) were excised from callus and transferred to 120 mL (105 X 42 mm) polystyrene screw-cap-jars containing hormone-free MS medium to enable further shoot elongation and root production.

Statistical analysis

Preliminary analysis of the data, using culture data as a random effect, and both medium (eight levels) and genotype (7 levels) as fixed effects, indicated in a generalised linear mixed model, that the data set contained more variation than was expected on strictly binomial assumptions (McCullagh and Nelder 1983). This was due mainly to the variation between different dates of embryo excision and culture. Consequently, a simpler model, the so-called "extra-variation" model described by McCullagh and Nelder (1983), was employed. For the binary data set derived from the experiment (i.e. absence or presence of an induced callus), the model is a natural extension of logistic regression, in which proportions are modeled as a function of explanatory variables (i.e. media and

genotype) with binomial errors. This model allowed for the following tests of significance:

1) whether there was an overall effect of genotype on callus formation, after removing the effect of media

2) whether there was an overall effect of media, after removing the effect of genotype

3) the significance of the overall interaction between media and genotype

4) estimates of the relative effects of the different media and genotypes, and tests of which pairwise differences were significant, using Tukey's method of adjusting for multiple comparisons (McCullagh and Nelder 1983).

2.3 Results

2.3.1 Callus production

The cultured embryos ranged in length from 0.5 to 1.5 mm (Figure 2.1) but callus formed most readily from embryos 0.5-1.0 mm long. In the present study, larger embryos generally germinated precociously. The scutella of immature embryos of all seven accessions of *Triticum tauschii* began to produce a callus-like growth on all the media tested within two weeks (Figure 2.2). Within 3-4 weeks, callus that developed from the cultured embryos were either (i) soft and watery type, (ii) solid yellowish or pale brown, or (iii) nodular (embryogenic) type with either a compact or a friable texture (Figure 2.3). In the following sections, a distinction is made between overall (total) callus induction, which includes all three types, and nodular, embryogenic callus induction, which refers to a type of callus subsequently found to possess long-term competence for regeneration.

2.3.2 Effect of medium composition and genotype on overall callus formation and growth

Medium composition had a marginally significant effect ($P = 0.03$) on overall callus formation. Pairwise comparisons revealed only one significant difference among the media, namely between media F and H. Generally, there were no statistical differences among the media (Table 2.3).

Callus was induced from immature embryos of all seven *Triticum tauschii* accessions on all media, but there was considerable variation in the frequency of induction (Table 2.3). Plant genotype had highly significant ($P < 0.0001$) effect on overall callus induction. Accessions CPI 110718, CPI 110810 and AUS 18913 were significantly more responsive to callus induction than were the other four accessions (Table 2.3), among which no significant differences in the percentage of callus induced was observed. There was no significant interaction ($P < 0.05$) between media and genotype.

Figure 2.1 An embryo dissected from an immature seed of a *Triticum tauschii* accession. Scale bar = 0.5 mm.

Figure 2.2 Production of scutella callus from immature embryo of *Triticum tauschii*, (2 weeks old). Scale bar = 0.5 mm.

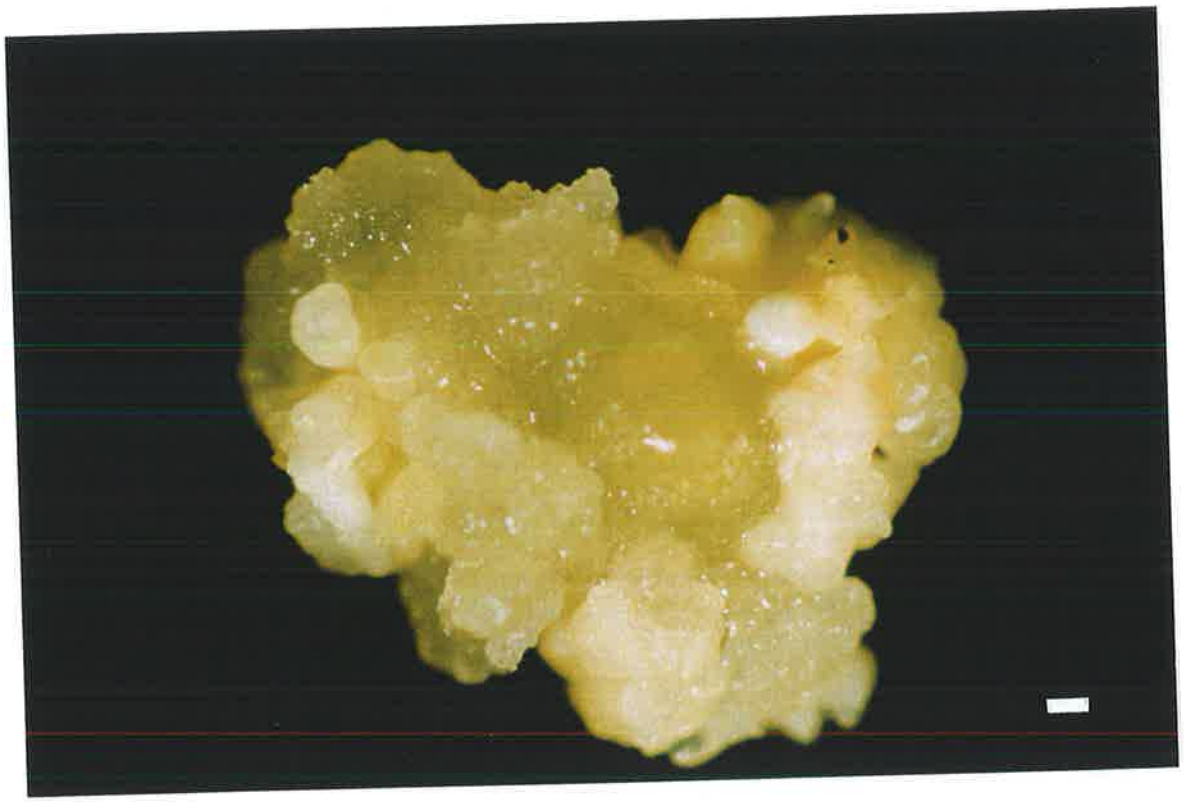


Figure 2.3 Nodular callus produced from scutella of *Triticum tauschii*, (4 weeks old). Scale bar = 0.5 mm.

Figure 2.4 Somatic embryoids produced from nodular callus, (6 weeks old). Scale bar = 0.5 mm.

Table 2.3 Percentage of embryos from seven accessions of *Triticum tauschii* forming callus on MS medium supplemented with different growth additives.

Accession	Medium								Mean	S.E.
	A	B	C	D	E	F	G	H		
CPI 110718	62.4	80.4	-	-	80.0	-	74.2	58.7	71.1^{d}	3.4
CPI 110809	36.4	27.5	28.6	20.0	22.8	6.4	6.3	26.6	21.8^{ab}	3.3
CPI 110810	47.1	43.5	21.2	16.5	45.8	37.5	52.0	62.4	40.8^{bc}	5.8
CPI 110813	7.6	10.5	11.5	14.7	14.7	-	18.9	38.1	16.6^{a}	3.5
AUS 18912	32.4	12.0	21.3	24.4	22.5	8.1	6.3	15.8	17.9^{a}	3.0
AUS 18913	54.5	38.0	22.0	48.5	31.4	14.4	44.7	60.9	39.3^{c}	4.7
AUS 18914	9.6	23.8	44.6	10.5	13.0	-	13.6	59.2	24.9^{ab}	6.8
Mean	35.7^{ab}	33.7^{ab}	24.9^{ab}	22.4^{ab}	32.9^{ab}	13.1^{a}	30.9^{ab}	46.0^{b}		
S.E.	7.4	8.4	3.8	4.7	8.2	3.3	9.2	6.7		

Means followed by the same superscript(s) are not significantly different at $P = 0.05$.

Table 2.4 Percentage of embryos from seven accessions of *Triticum tauschii* forming nodular callus on MS medium supplemented with different growth additives.

Accession	Medium								Mean	S.E.
	A	B	C	D	E	F	G	H		
CPI 110718	0	33.7	-	-	18.2	-	42.4	58.7	30.6^{d}	6.9
CPI 110809	0	11.8	5.0	19.2	15.4	5.4	5.3	26.6	11.1^{bc}	3.0
CPI 110810	0	24.2	19.2	16.5	31.3	30.7	12.0	62.4	24.5^{c}	6.4
CPI 110813	0	5.3	3.6	3.1	2.9	-	3.1	38.1	8.0^{a}	4.4
AUS 18912	0	4.0	12.4	24.4	22.5	8.1	0.6	15.8	11.0^{ab}	3.3
AUS 18913	0	12.0	12.0	40.9	25.5	14.4	30.9	60.9	24.6^{cd}	6.7
AUS 18914	0	11.1	13.6	10.5	7.4	-	9.1	59.2	15.8^{bc}	6.4
Mean	0^{a}	14.6^{ab}	11.0^{cd}	19.1^{cd}	17.6^{bc}	11.9^{abc}	14.8^{bc}	46.0^{d}		
S.E.	0	3.7	2.2	4.8	4.1	2.6	4.1	6.7		

Means followed by the same superscript(s) are not significantly different at $P = 0.05$.

2.3.3 Effect of medium composition and genotype on production of nodular, embryogenic callus

Media compositions had a highly significant effect ($P < 0.0001$) on the production of nodular embryogenic callus. Callus was induced from plated embryos of all accessions on all media, except medium A (2 mg L^{-1} 2,4-D), in which embryos from all accessions produced compact, non-embryogenic callus. However, when medium A was modified by the addition of L-glutamine, L-asparagine and L-proline (medium B, Table 2.2), nodular, embryogenic callus was induced at low, but statistically-significant frequencies, from plated embryos in all accessions (Table 2.4). Pairwise comparisons indicated that media containing increasing concentrations of Dicamba® (5-30 mg L^{-1}, media C-G) were usually not significantly different from medium B in their efficacy for embryogenic callus induction (Table 2.4). The effects of Dicamba® appeared to be equivocal, as medium D (10 mg L^{-1} Dicamba®) induced a significantly higher percentage of embryogenic callus than media containing 20, 24 and 30 mg L^{-1} Dicamba® (media E to G). However, the combination of both high levels of Dicamba® (24 mg L^{-1}) and L-proline (750 mg L^{-1}) in medium H resulted in the production of significantly higher numbers of nodular, embryogenic callus (46.0%) than media containing high levels of Dicamba® only, that is media F and G (11.9% and 14.8% respectively) (Table 2.4).

The production of nodular, embryogenic callus was significantly ($P < 0.0001$) affected by genotype. In general, the most responsive genotypes were the same as those in which the highest overall frequencies (all types) of callus formation were observed (Tables 2.3 and 2.4). This observation implied that media had a larger influence on the production of nodular, embryogenic callus, an inference supported by the larger F values for media (F = 17.5) over genotype (F = 14.8). There was no significant interaction between media and genotype for nodular, embryogenic callus production.

2.3.4 Effect of different concentrations of L-proline in nodular embryogenic callus

To further characterize the synergy between Dicamba® and L-proline in nodular, embryogenic callus induction in *Triticum tauschii*, experiments were employed in three *Triticum tauschii* accessions using medium H with four different concentrations of L-proline (Table 2.5). The concentration of L-proline had a significant effect ($P < 0.01$) on the production of nodular embryogenic callus. At 750 mg L^{-1} L-proline in the medium, a significantly higher number of nodular embryogenic callus was produced (Table 2.5).

Triticum tauschii genotypes had a highly significantly ($P < 0.0001$) effect on the production of nodular embryogenic callus. Accession CPI 110649 was significantly more responsive to nodular embryogenic callus induction than accessions CPI 110813 and CPI 110810 (Table 2.5). The interaction between medium and genotype was not significant ($P < 0.05$).

2.3.5 Somatic embryogenesis and regeneration

Callus induced on media containing Dicamba® was maintained by subculturing every three weeks on the same media. When these callus was transferred to the regeneration medium (hormone-free MS medium), numerous somatic embryoids were produced (Figure 2.4), and these further differentiated into shoots (Figure 2.5). The embryogenic capacity of callus was maintained for over 4 months by subculturing on medium H and I, whereas callus maintained on medium J remained embryogenic for more than a year.

Table 2.5 Percentage of embryos from three accessions of *Triticum tauschii* forming nodular callus on medium H supplemented with different concentrations of L-proline

Accessions	L-proline concentrations (mg L^{-1})				Mean	S.E.
	625	725	825	925		
CPI 110649	29.5	29.0	22.5	23.9	29.0^{b}	1.2
CPI 110810	15.0	30.4	27.8	23.6	21.0^{a}	1.4
CPI 110813	16.3	18.8	19.2	16.2	18.3^{a}	1.1
Mean	22.7^{a}	27.4^{b}	22.4^{a}	22.3^{a}		
S.E.	1.4	1.4	1.3	1.9		

Mean followed by the same superscript(s) are not significantly different at $P = 0.01$

Figure 2.5 Shoot development from embryoids. Scale bar = 0.5 mm.

2.4 Discussion

The induction of callus from *Triticum tauschii* embryos shared many features with those reported for tetraploid and hexaploid wheat. For example, MS medium, which was used effectively as a basal medium by other workers (Ozias-Akins and Vasil 1982; Maddock *et al.* 1983; He *et al.* 1988; Redway *et al.* 1990a) for the induction and maintenance of callus from wheat scutella, was found to serve effectively as a basal medium for the induction and maintenance of callus from *Triticum tauschii* embryos. In addition, the optimal size of *Triticum tauschii* embryos for callus induction (0.5-1.0 mm) was similar to that found for hexaploid wheat (Ozias-Akins and Vasil 1982; He *et al.* 1988; Redway *et al.* 1990a). Further, the three types of callus formed on various media in the present study were also observed during callus induction from immature embryos of *Triticum aestivum* L. (Heyser *et al.* 1985; He *et al.* 1988; Redway *et al.* 1990a).

By contrast to the findings of Hayashi and Shimamoto (1988), who reported that embryogenic callus was most frequently induced on media containing high levels of Dicamba® (32 mg L^{-1}), the results of the present study indicated that high levels of Dicamba® alone are not effective in inducing a higher level of embryogenic callus formation (Table 2.2 and 2.4). Papenfuss and Carman (1987) reported that Dicamba® was more effective than 2,4-D in terms of the induction of embryogenic callus formation from wheat embryos. However, the present study showed that 2,4-D was as equally effective as Dicamba® in inducing embryogenic callus from *Triticum tauschii* embryos (Table 2.3).

The effect of high L-proline concentrations on the frequency of overall (total) callus production was reported for maize (Armstrong and Green 1985) and hexaploid wheat (Vasil and Vasil 1986). In the present study, increasing the L-proline concentration from 150 mg L^{-1} to 750 mg L^{-1} resulted in only marginal improvement in overall callus production (Table 2.3). However, the combination of a high concentration of Dicamba® (24 mg L^{-1}) and increased L-proline concentration (750 mg L^{-1}, medium H) resulted in an enhanced frequency of nodular, embryogenic callus (Table 2.4). This effect was also observed in maize (Armstrong and Green 1985) and rice (Chowdhry *et al.* 1993). In the present

experiments, only two range of L-proline concentrations 150 mg L^{-1} and 750 mg L^{-1} (Table 2.4) and 625-925 mg L^{-1} (Table 2.5) were used in media containing high levels of Dicamba® (24 mg L^{-1}). To further characterize the synergy between Dicamba® and L-proline in nodular, embryogenic callus induction in *Triticum tauschii*, future experiments could employ media with a wider range of Dicamba® - L-proline combinations, especially those low in Dicamba® but high in L-proline.

Papenfuss and Carman (1987) experienced difficulty with precocious germination of embryoids and subsequently reduced vigour of regenerated plantlets from hexaploid wheat when they substituted Dicamba® for 2,4-D in MS medium. These problems were not observed with *Triticum tauschii* embryoids in the present study. The correlation between callus appearance (nodular) and long-term retention of embryogenic capacity observed in the present study is in accordance with previous results obtained for hexaploid (He *et al.* 1988; Redway *et al.* 1990a) and tetraploid wheat (Borrelli *et al.* 1991). This indicates possible commonalties between the three *Triticum* species in terms of the physiology of somatic embryogenesis.

In conclusion, the work described here was directed towards developing a protocol for the production of embryogenic callus with long-term competence for regeneration in *Triticum tauschii.* The present study showed that this may be achieved by culturing immature embryos (0.5-1.0 mm) on MS basal medium supplemented with 24 mg L^{-1} Dicamba®, 725 mg L^{-1} L-proline, 750 mg L^{-1} glutamine, and 100 mg L^{-1} asparagine. The successful establishment of fine suspension cultures from embryogenic callus grown under this protocol is described in the next chapter.

CHAPTER 3

ESTABLISHMENT OF FINE SUSPENSION CULTURES OF *TRITICUM TAUSCHII* WHICH REMAIN EMBRYOGENIC FOR SEVERAL YEARS

3.1 Introduction

The commercially valuable cereals rice, wheat, barley and maize remain central targets for crop improvement through genetic engineering and most of the current experimental protocols for their genetic transformation rely, to a large extent, on cell or tissue culture technologies. Immature embryos explants have been widely used as a source of tissue for transformation of wheat (Vasil *et al.* 1993; Becker *et al.* 1994; Altpeter *et al.* 1996) and barley (Ritala *et al.* 1994; Hagio *et al.* 1995; Tingay *et al.* 1997), but suspension-cultured cells from a range of tissues have also been used as direct targets for gene transfer using DNA delivery methods such as microprojectile bombardment (Klein *et al.* 1988; Cao *et al.* 1992; Chowdhury and Vasil 1992; Zhang *et al.* 1996), coated silicon carbide fibres (Kaeppler *et al.* 1990), and direct DNA uptake by protoplasts (Wang *et al.* 1992; Lazzeri *et al.* 1991; Funatsuki *et al.* 1995; Chaïr *et al.* 1996). Advantages that have been attributed to suspension cultures include the ease of maintaining them over long periods, the presence of large numbers of individual but small, cell aggregates in a relatively small volume of medium, the ease of generating large numbers of protoplasts from the suspension-cultured cell aggregates, and the efficiency of selection of transgenic cells for regeneration following transformation (Golovkin *et al.* 1993; Funatsuki *et al.* 1995; Chaïr *et al.* 1996). To realize the benefits of these advantages, it is necessary to generate suspension cultures in which relatively small cell aggregates, preferably less than 1 mm in diameter and containing about 200 cells (Yang *et al.* 1991; He *et al.* 1992), can be produced continuously.

Fine suspension cultures have been established for many monocotyledonous species including *Pennisetum americanum* (L.) K. Schum. (Vasil and Vasil 1982), *Triticum aestivum* L. (Maddock 1987; Redway *et al.* 1990b; Wang and Nguyen 1990; Yang *et al.* 1991; Qiao *et al.* 1992), *Oryza sativa* L. (Ozawa and Komamine 1989; Utomo *et al.* 1996) and *Hordeum vulgare* L. (Jähne *et al.* 1991b). Three desirable criteria for establishing fine, embryogenic suspension cultures are that a successful result can be achieved in a reasonable time frame, that the suspension-cultured cells can be readily induced to regenerate into fertile plants, and

that this embryogenicity is retained for as long a period as possible. Generally, 6 to 12 months is required to establish fine suspension cultures from the common cereals (Wang *et al.* 1990 and Jähne *et al.* 1991b). However, there have been many difficulties, including the progressive loss of embryogenicity over time (Maddock 1987), low recoveries or non-recovery of fertile plants (Wang and Nguyen 1990; Qiao *et al.* 1992), and the regeneration of albino plantlets once the suspension cultures are more than one year old (Jähne *et al.* 1991b).

In this Chapter, a protocol is described by which fine suspension cultures of *Triticum tauschii* have been established from immature embryos. A high proportion of plants regenerated from the cultures is fertile and embryogenicity is retained for more than three years.

3.2 Materials and Methods

3.2.1 Plant material

Five *Triticum tauschii* accessions from the Australian Winter Cereals Collection, (Tamworth NSW, Australia) and five from the CSIRO Division of Plant Industry (Canberra, ACT, Australia) carrying CPI designation were used in this study (Table 3.2). The origins and classifications of the accessions are shown in Appendix 6.1.

3.2.2 Initiation of suspension cultures

Plants of the ten *Triticum tauschii* accessions (Table 3.2) were grown in a glasshouse and immature embryos were dissected as described in Section 2.2. The immature embryos were cultured, scutellum face up, on MS (Murashige and Skoog 1962) medium supplemented with 24 mg L^{-1} Dicamba® (Serva Fine Biochemicals, Heidelberg Germany), 750 mg L^{-1} L-glutamine, 100 mg L^{-1}-asparagine, 725 mg L^{-1} L-proline, 3% (w/v) sucrose and solidified with 0.25% (w/v) Phytagel (Sigma, St Louis, MO, U.S.A.).

Embryogenic nodular callus (1-2 g) produced from the scutella was transferred to 10-15 mL medium A or B (Table 3.1) in plastic vessels (Greiner, volume approx. 190 mL). The cultures were shaken continuously in the dark on a rotary shaker at 120 rpm at 22°C. Each week necrotic or differentiated clumps with shoots and roots were discarded, and the medium was renewed. About 15 mL medium was used for 1-3 g cell clumps at sub-culturing.

In this Chapter, callus pieces less than about 1 mm in diameter and usually containing 2-200 cells are referred to as cell aggregates, while pieces larger than 1 mm are referred to as cell clumps.

3.2.3 Establishment of fine cell suspensions

Two to three months after initiation in media A or B, suspension cultures were

subcultured into two additional media (C and D, Table 3.1) to study the effect of different nutritional environments on the morphology and embryogenicity of the cell clumps. Cultures were sub-divided into three portions; one was subcultured in the original fresh medium (A or B), while the other two portions were cultured in either medium C or D (Table 3.1). The subculturing was performed at weekly intervals.

3.2.4 Maintenance of fine suspension cultures

Within 2-3 months of the transfer to media C and D during the establishment of fine cell aggregates, the cultures were filtered through a 500 μm polypropylene sieve. Cell aggregates passing through the sieve were collected, sub-divided and cultured in fresh medium C or D, and/or medium E (Table 3.1). The cultures were subcultured at weekly intervals.

3.2.5 Plant regeneration from fine suspension cultures

The regeneration capacity of cultures was tested every 3 months. About 150-200mg of fine suspension culture was transferred to medium E solidified with 0.25% (w/v) Phytagel and incubated in the dark at 22°C for three weeks for the production of somatic embryoids. Within 4-6 weeks, somatic embryoids and shoots developed on this medium and were transferred to hormone-free MS medium solidified with 0.25% (w/v) Phytagel for germination of somatic embryoids and further development of shoots. The cultures were incubated under fluorescent light (60 $\mu E\ m^{-2}\ s^{-1}$) at 22°C for six weeks. After 2-4 weeks, regenerated shoots (0.5-1 cm long) were selected and transferred to plastic screw-cap vessels (volume approx. 120 ml) containing hormone-free MS medium, to enable further growth and the development of roots. Plantlets longer than 10 cm, and with a developed root system, were subsequently transplanted to 10 cm pots containing a pine bark and peat potting mix and a 3-4 months slow release complete fertilizer (Osmocote®, N:P:K = 14:6.1:11.5). Pots were placed in propagation trays with plastic covers, for two weeks prior to their transfer to the glasshouse. The glasshouse was maintained at 22 ± 4°C. Natural light was supplemented by 400 watt metal-halide lamps to provide a 16-hour

Table 3.1 Components of five media evaluated for establishment of fine suspension cultures. The pH was adjusted at 5.7 for all the media.

Component (mg L^{-1})	Medium				
	A[a]	B[b]	C[b]	D[b]	E[c]
Inorganic salts	MS	L1	L1	L5	MS
Myo-inositol	250	100	100	100	100
Vitamin	MS	L1	L1	L1	MS
L-glutamine	200	750	750	1500	
L-asparagine	150	100	100	200	
L-proline		150	150	300	
Casein hydrolysate	300			1000	
Dicamba®	12	12	6	6	
2,4-D					1.1
$AgNO_3$	6				
Sucrose	20000				30000
Sorbitol	20000				
Maltose		50000	50000	50000	

[a] modification of MS (Murashige and Skoog 1962) medium after Wang *et al.* (1990).
[b] modification of L1 (Lazzeri *et al.* 1991).
[c] modification of MS medium after Yang *et al.*(1991).

photoperiod. Established plantlets were vernalized at 4°C with a 9 hours photo-period for 4 weeks.

3.3 Results

3.3.1 Initiation of suspension cultures

Embryogenic callus (1-3 months old) originating from immature embryos of ten accessions of *Triticum tauschii* grown on solid MS medium was transferred to liquid media A and B (Table 3.1). Considerable differences in growth were observed for different genotypes in the liquid culture (Table 3.2). Callus of accessions AUS 18911 and AUS 18914 grew only in medium A, accession CPI 110809 and CPI 110649 only in medium B, and CPI 110810 and CPI 110813 grew in both media. Callus of accessions AUS 18913, AUS 21714 and CPI 110909 failed to grow in either medium.

During the first 2 to 3 months of culture, three morphologically distinct types of cell clumps were identified and these were designated Type I, Type II and Type III. Type I was a mixture of white, friable and compact cell clumps surrounded by transparent soft tissues (Figure 3.1). The clumps produced single embryoids, which were released into the culture medium. Type II cell clumps were white and compact (Figure 3.2). The liquid culture of Type II cultures became very viscous during subculturing, probably as a result of polysaccharide secretion. Type III clumps were compact and yellow to brown in colour. They failed to grow, eventually became necrotic and were therefore discarded.

To evaluate whether it would be possible to regenerate plants from Type I and II *Triticum tauschii* cell clumps, and therefore to decide whether or not to proceed with attempts to establish fine suspension cultures, both types were transferred onto regeneration medium. About 95% of Type I cell clumps produced somatic embryoids and formed shoots, while Type II clumps mainly differentiated into roots, with the occasional formation of green spots or green root (data not shown). On the basis of the promising behaviour of the two types of cell clumps during this preliminary assessment of regeneration capacity, both types were subsequently used in attempts to establish fine cultures.

Table 3.2 Response and initiation of suspension cultures for ten *Triticum tauschii* accessions.

Accession	Media		Type of cell clumps
	A	B	
CPI 110809	-	+	II
CPI 110810	+	+	II
CPI 110813	+	+	I and II
AUS 18911	+	-	II
AUS 18912	+	+	II
AUS 18913	-	-	III
AUS 18914	+	-	II
CPI 110649	-	+	I
AUS 21714	-	-	III
CPI 110909	-	-	III

- failed to grow, + continued to grow, I white friable and compact with translucent tissue, II white solid and compact, III yellow solid

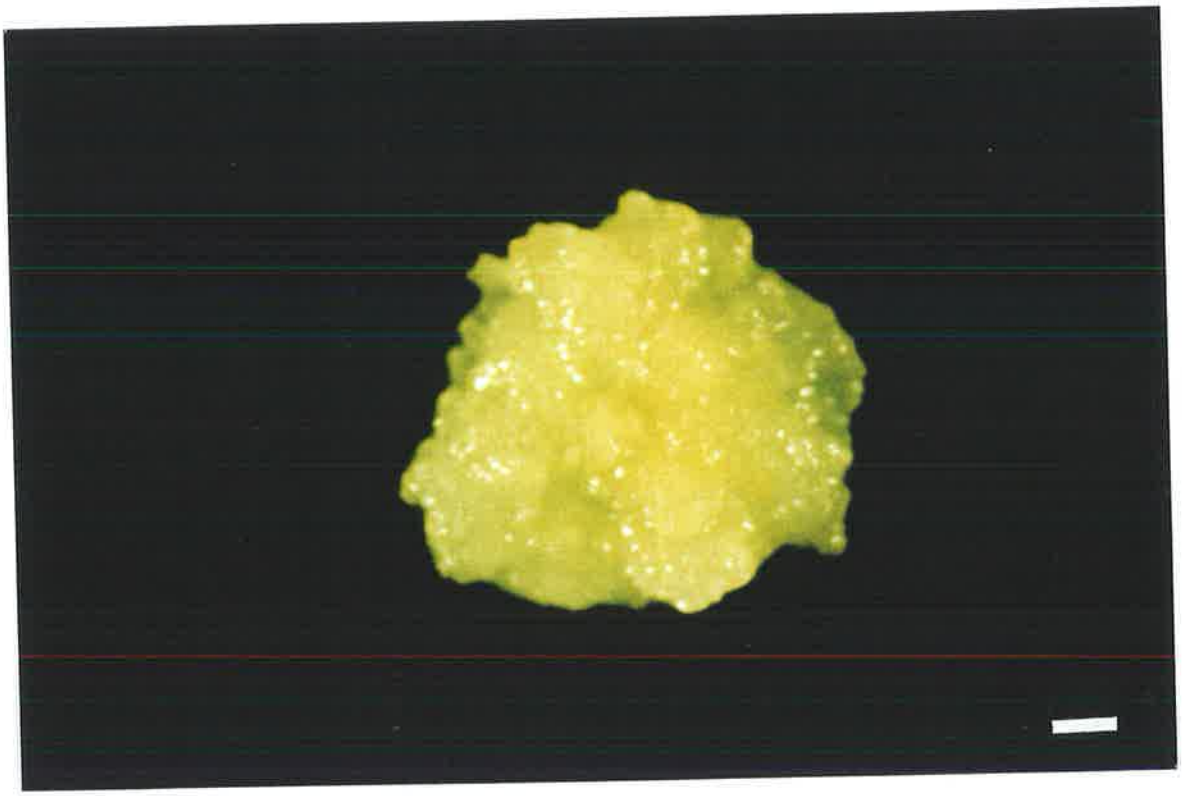


Figure 3.1 Type I cell clumps developed during initiation of suspension cultures. Scale bar = 1 mm.

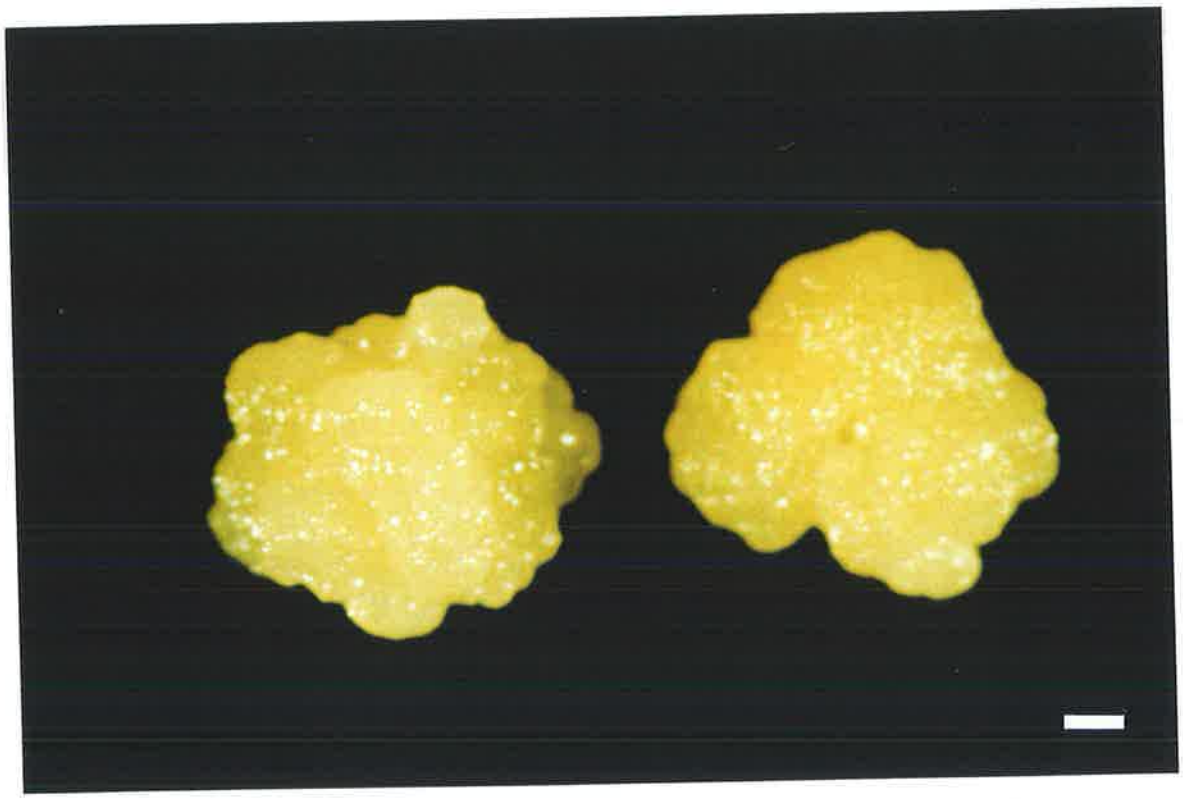


Figure 3.2 Type II cell clumps produced during initiation period. Scale bar = 1mm.

3.3.2 Establishment of fine suspension cultures

In addition to media A and B, two other media designated as C and D were included in attempts to establish fine embryogenic suspension cultures of *Triticum tauschii*. The cell clumps described in the previous section and shown in Figure 3.1 and 3.2 were transferred to medium C or D, or retained on the original A or B medium.

Type I cells. When Type I cultures were maintained in initiation medium A or B, actively dividing and friable cell clumps were produced. These eventually became more uniform in size and appearance. They appeared pale yellow in colour. When Type I cell clumps were transferred to medium C they continued to produce meristematic tissue mixed with translucent tissue, and the cultures were whitish colour (Figure 3.3). This tissue occasionally produced embryoids but empty elongated cells were also released from the translucent tissue (Figure 3.5).

Type I cell clumps which were transferred to medium D continued to produce friable meristematic cell clumps similar to those established in medium A or B (Figure 3.4). However, when fine cell aggregates from Type I cell aggregates established in either medium C or D were collected and subcultured into medium, they rapidly (within 1-2 weeks) reverted into large cell clumps. These cell clumps were similar to Type I cell clumps which were observed at the initiation stage.

Type II cells. When the culture of Type II cell clumps was continued in medium A or B, the clumps remained compact and the medium again became highly viscous (Figure 3.4). After transfer to medium C, Type II cultures retained their characteristic compact morphology but showed very slow growth. These clumps gradually became yellowish in colour over a period of 4-6 weeks in culture and stopped releasing material, which was presumably polysaccharide, into the medium.

When Type II cell clumps were transferred to medium D, they continued to release material into the medium, as suggested by the high viscosity of the culture medium. However, friable and relatively small cell aggregates were visible within 1-3 months of transferring to this medium. When these small friable aggregates were selected and

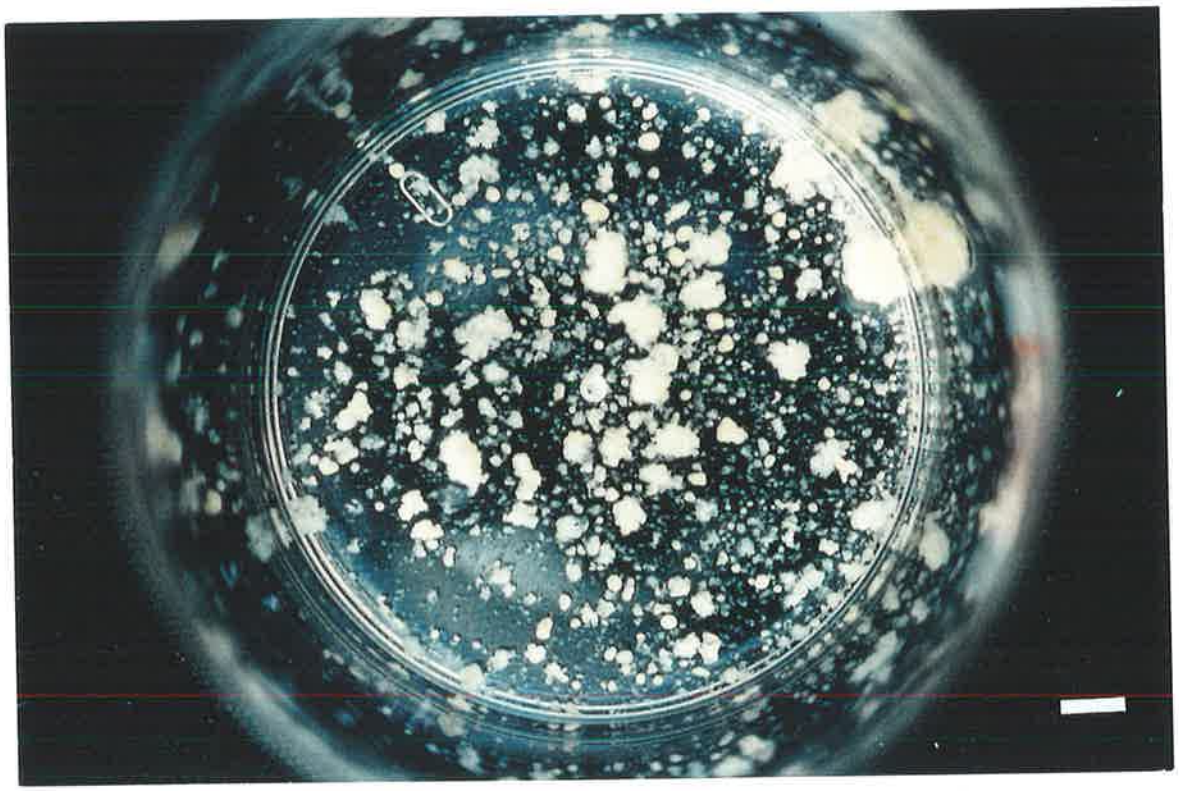


Figure 3.3 Suspension cell clumps produced from type I cell clumps in medium C. Scale bar = 5 mm.

Figure 3.4 An example of suspension cell clumps produced from type II cell clumps in medium A, B and D. Scale bar = 5 mm.

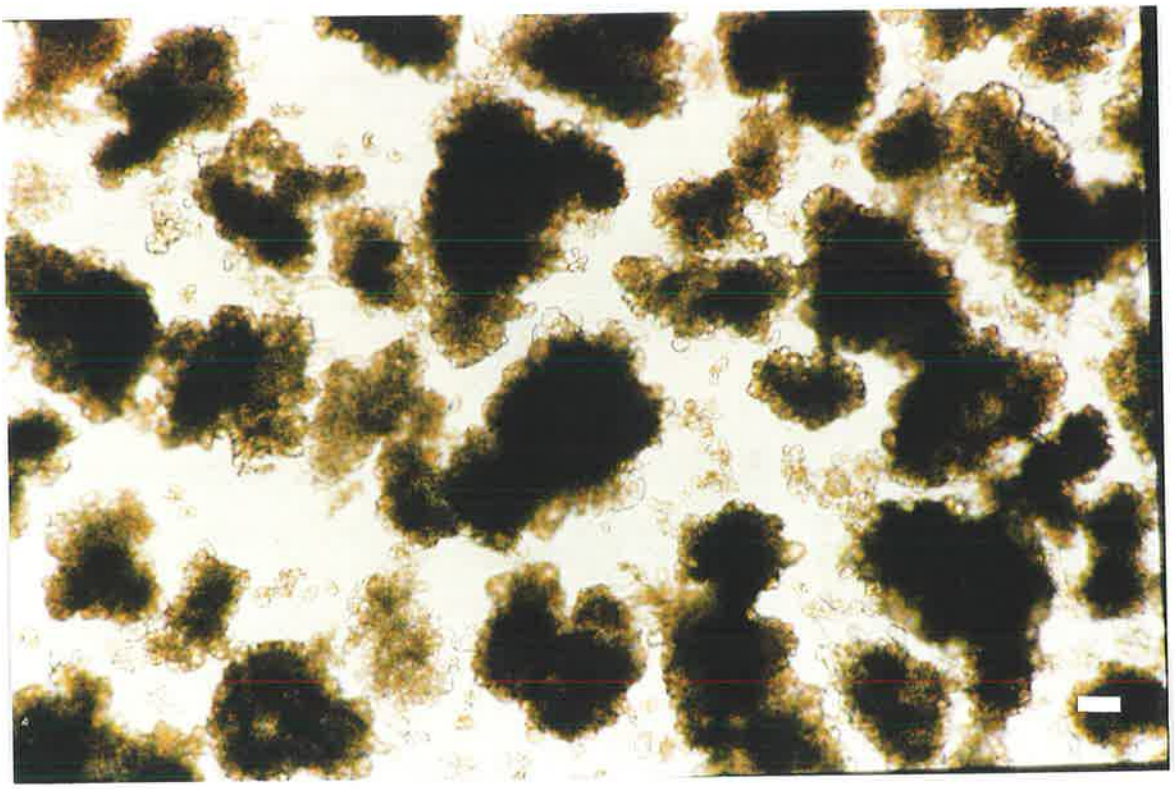


Figure 3.5 Suspension cell aggregates produced from type I in medium C. Scale bar = 0.2 mm.

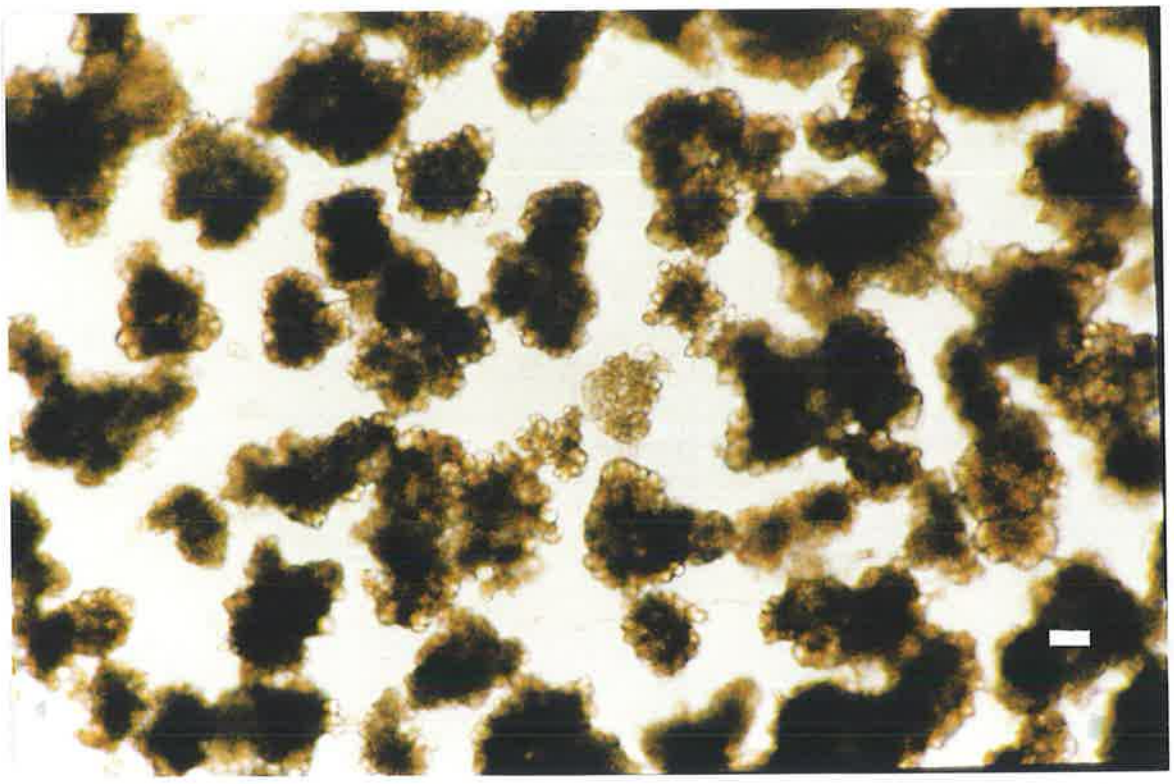


Figure 3.6 Suspension cell aggregates produced from type II in medium D. Scale bar = 0.3 mm.

transferred to fresh medium, the release of viscous material ceased. The aggregates contained densely cytoplasmic cells, and had a similar appearance to Type I cell aggregates that had been established in medium D (Figure 3.6). The growth rate of the Type II cultures in medium D was high; the volume of cell aggregates doubled with each subculture. Although these fast-growing and homogeneous fine cultures were not embryogenic, they could be useful as an ongoing source of cells for protoplast isolation, for transformation or for transient expression studies.

Subculturing did not affect the production of fine cell aggregates. During 2-3 months of culture the Type II cell clumps gradually produced smaller fragments and eventually fine cell aggregates less than 500 μm were observed. Unlike the Type I cells, the fine cell aggregates produced from Type II cell clumps did not revert to large cell clumps when transferred into fresh medium.

3.3.3 Maintenance of fine suspension cultures

The established cultures from Type I cell clumps which were maintained in medium C or D, gradually became more and more homogeneous with respect to aggregates size when subcultured at weekly intervals (Figure 3.6). However, they rapidly lost competence to form shoots when placed on regeneration medium (Table 3.3). In contrast, the embryogenic competence of fine suspension aggregates derived from Type I clumps obtained in medium C, but subsequently transferred to medium E, was maintained at a high level. For example, after one year in medium E, regeneration frequencies of 85% were obtained (Table 3.3). The cultures maintained in medium E contained a mixture of large and small aggregates and it was necessary to filter them during subculture; only aggregates less than 500 μm in diameter were subcultured (Figure 3.7). The Type I cultures obtained through this protocol readily differentiated into shoots and roots, and at each subculture it was necessary to discard all differentiated shoots and roots. There was also an increase in the number of embryoids produced in these cultures and the number of empty elongated cells remained high.

Table 3.3 Percentage of shoot forming fine cell aggregates in established cultures, growing in different media.

Media	Competence to produce shoots/(months)				
	3	6	9	12	>15
C	90-100	75-85	30-40	0	0
D	50-60	30-40	0		
E via C	90-100	90-100	87-95	80-85	75-80
E via D	50-65	50-65	40-50	35-45	25-30

Table 3.4 Percentage of suspension cultures forming embryogenic and non-embryogenic fine suspension from ten *Triticum tauschii* accessions.

Accession number	Total of initiated cultured lines	Type of cell clumps[A]	Embryogenic lines	Non-embryogenic line
CPI 110809	14	II	0	2 (14%)
CPI 110810	19	II	0	8 (44%)
CPI 110813	7	I and II	1 (14%)	2 (28.5%)
AUS 18911	5	II	0	1 (20%)
AUS 18912	10	II	0	2 (20%)
AUS 18913	10	III	0	0
AUS 18914	10	II	0	2 (20%)
CPI 110649	6	I	3 (50%)	0
AUS 21714	8	III	0	0
CPI 110909	6	III	0	0

[A] I - white friable and compact with translucent tissue; II - white solid and compact; III - yellow and solid.

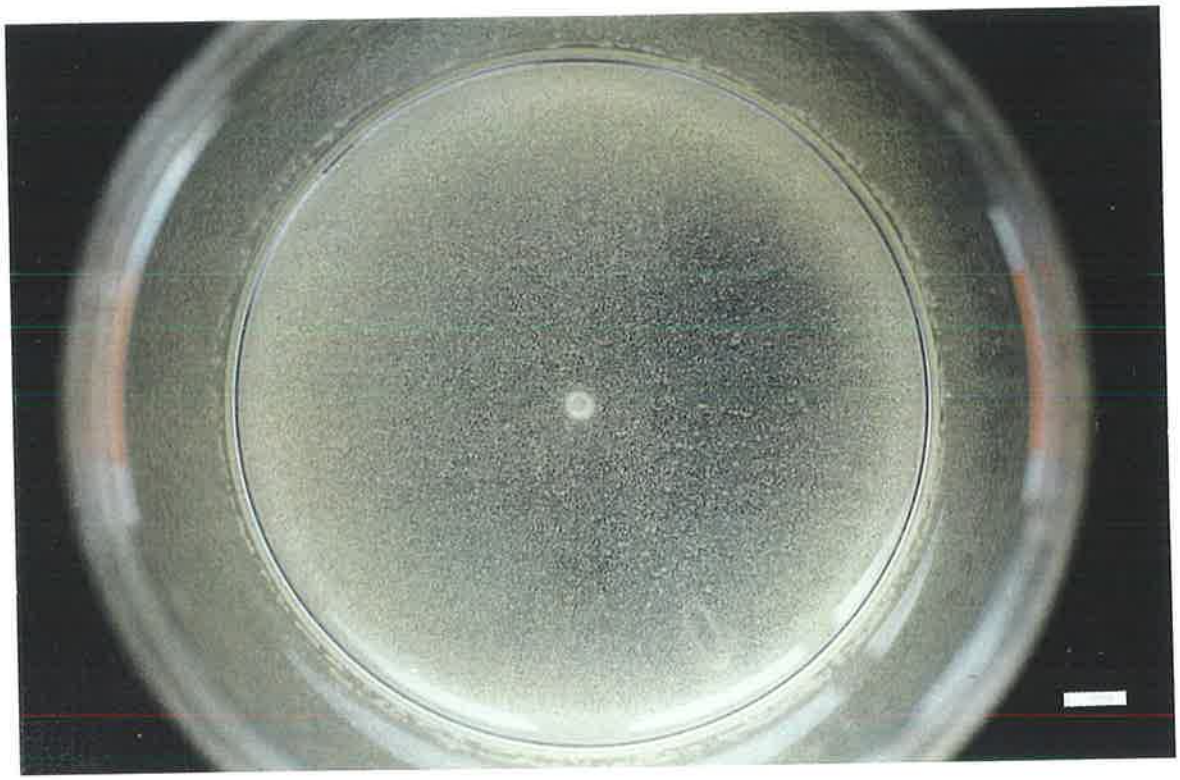


Figure 3.7 Fine cell aggregates less than 500 μm in diameter produced from type I cell clumps in medium E. Scale bar = 0.5 mm.

Figure 3.8 A shoot regenerated from ageing fine suspension cultures. Scale bar = 1 mm.

The morphology of fine suspension cell aggregates derived from Type I cell clumps produced in medium D was maintained following transfer to medium E but the embryogenic capacity of the cultures was relatively low; a regeneration frequency of 45% was observed in one year-old cultures (Table 3.3).

3.3.4 Plant regeneration from suspension cultures

The composition of maintenance medium greatly influenced the subsequent regenerative capacity of suspension cultures (Table 3.3). While suspension cultures maintained in medium C or D lost their regenerative capacity within nine months, those transferred to and maintained on medium E retained their regeneration capacity for more than three years. In the present work, the production of long-term embryogenic fine suspension was strongly affected by genotype (Table 3.4). The most responsive accessions were CPI 110813 and CPI 110649, which produced Type I cell clumps from which a total of four embryogenic lines were produced. Over 100 plantlets from one-year-old suspension cultures of three independent lines of accession CPI 110649 and one line of accession CPI 110813 were regenerated and transferred to the glasshouse where they all grew vigorously (Figure 3.9). Over 90% of the plants were fertile, based on their ability to produce viable seed. Five accessions produced Type II cell clumps, but these resulted in non-embryogenic lines.

3.3.5 Overall strategy for the production of embryogenic fine suspensions

Embryogenic fine suspension cultures were achieved most effectively when one to three month-old embryogenic callus was cultured in medium A or B and subcultured weekly for 2-3 months. Type I cell clumps produced in these cultures were transferred to medium C where they were maintained for a further 2-3 months. During this period, fine heterogeneous cell aggregates containing both meristematic tissue and translucent tissue developed and were transferred and maintained in medium E. On the bases of these results, we conclude that the steps summarized in Figure 3.10 are necessary for the successful

Figure 3.9 A fertile plant regenerated from aging (18 months) fine suspension cultures.

production of long-term embryogenic cell suspension cultures of *Triticum tauschii*.

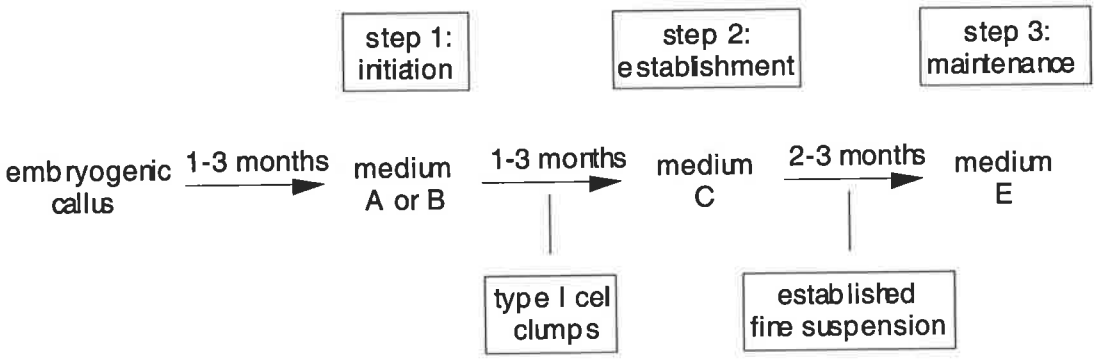


Figure 3.10 Three steps required for production of embryogenic fine suspension cultures from *Triticum tauschii.*

3.4 Discussion

The identification of three different cell types, designated Type I, Type II and Type III, (Figure 3.1 and 3.2) was crucial for the establishment of embryogenic suspension culture in this study. Yang *et al.* (1991) also reported variation in morphology and growth characteristics of cell clumps in liquid cultures of *Triticum aestivum* and identified six different types of clumps in the early stages of initiation of cell suspension cultures. The highly embryogenic cell clumps described by Yang *et al.* (1991) probably correspond to Type I cultures in the present study (Figure 3.1). However, the *Triticum tauschii* and *Triticum aestivum* cell lines differed in texture and appearance, and this could reflect genotypic differences. Yang *et al.* (1991) also reported observations similar to these noted in this study, in that their embryogenic cell aggregates reverted into large cell clumps after the cell aggregates were selected and subcultured into fresh medium. The considerable genotype dependency in initiation of suspension cultures observed in this study (Table 3.2) is consistent with genotype effects reported in barley (Jähne *et al.* (1991b) and rice (Utomo *et al.* 1995).

Apart from the type of cell clumps and genotype, media composition also has an effect on the establishment of embryogenic fine suspensions. Four complex media (medium A, B, C and D) were tested for the initiation and establishment of fine embryogenic suspension cultures of *Triticum tauschii* (Table 3.1). The results indicate that supplementation of the initiation medium is important for rapid growth of embryogenic cell aggregates (Figure 3.10). Jähne *et al.* (1991b) and Wang *et al.* (1990) also used media that were supplemented with complex vitamin mixtures and amino acids to initiate and establish barley and wheat suspension cultures, respectively, within a time frame similar to that achieved here. However, Jähne *et al.* (1991b) were only able to regenerate fertile barley plants from cultures that were less than 6 months old and the frequency of sterility in the regenerated plants increased as the suspension aged. In contrast, fertile *Triticum tauschii* plants were obtained after eighteen months of cell suspension culture in the present study (Figure 3.9). This is probably attributable to the use of a complex medium, containing three

amino acids and eight vitamins in the initial suspensions, and the subsequent change to a simple medium that lacked both amino acids and vitamins.

The production of long-term, fine suspension cultures of hexaploid wheat has also been reported by Wang and Nguyen (1990) and by Yang *et al.* (1991). In these reports, basal MS medium without the addition of amino acids, vitamins or high concentrations of phytohormones was used to initiate, establish and maintain the suspension cultures. Yang *et al.* (1991) observed fine cell aggregates of less than 1 mm in diameter after one year, but Wang and Nguyen (1990) did not report the time taken for production of fine cell aggregates. Although phenotypic variation amongst the regenerated plantlets was reported, neither group presented data on the production of fertile plants. These findings may indicate that a simple medium contributes to the maintenance of embryogenic capacity of suspension cultures, although time required for the production of fine suspension cultures is extended. The supplemented medium used here (Table 3.1) appeared to alleviate this problem and led to decrease in the time taken to produce fine suspension cultures.

In conclusion, the protocol described in the chapter enabled the establishment from immature embryo explants of fine suspension cultures of *Triticum tauschii* cells that remained embryogenic over several years. These cell cultures were therefore used for the production of protoplasts, as outlined in the next chapter.

CHAPTER 4

THE PRODUCTION OF FERTILE REGENERANT FROM PROTOPLASTS OF *TRITICUM TAUSCHII*

4.1 Introduction

Fertile plants have now been derived from protoplasts in several cereal species, including rice (Datta *et al.* 1990; Datta *et al.* 1992), maize (Mórocz et al. 1990), barley (Jähne *et al.* 1991a; Funatsuki *et al.* 1992; Golds *et al.* 1994) and wheat (Ahmed and Sagi 1993; Pauk *et al.* 1994). Amongst these important species, wheat (*Triticum aestivum*) has been one of the most recalcitrant cereal species with respect to the capacity for the establishment of regenerable suspension cultures and hence for production of regenerable protoplasts. Considerable efforts in attempting to obtain plant regeneration from wheat protoplast culture initially resulted in green plantlets that grew in culture (Harris *et al.* 1988). Subsequent work in *Triticum aestivum* by Chang *et al.* (1991) and He *et al.* (1992) produced plants, but they were sterile. Other workers (Qiao *et al.* 1992; Vasil *et al.* 1992) transferred green plantlets to the glasshouse, but it is not known if fertile plants developed. Fertile plants from protoplasts of *Triticum aestivum* were eventually obtained by Ahmed and Sagi (1993) (one fertile plant), and Pauk *et al.* (1994) (three fertile plants). Considerable chromosome loss from the normal wheat complement (2n=6x=42) has been reported in suspension cultures and in dividing protoplasts, together with the loss of chromosome arms and chromosome segments (Karp *et al.* 1987). This is a major cause of somaclonal variation in cereals, such as wheat (Karp *et al.* 1987). In addition, Winfield *et al.* (1992) found that chromosome number of hexaploid wheat was less stable than diploid and tetraploid wheats in cell culture. For this reason, *Triticum tauschii* was considered potentially useful for the production of protoplasts and for subsequent regeneration of plants (Chapter 1).

Despite some success in the regeneration of plants from protoplasts isolated from the scutellar tissue of immature embryos of rice (Ghosh-Biswas *et al.* 1994), from primary callus of barley (Stöldt *et al.* 1996) and from cryopreserved callus of rice (Cornejo *et al.* 1995), suspension cultures which exhibit sustained division in culture have been the most common source of cereal protoplasts. Establishment of embryogenic suspension cultures suitable for protoplast isolation is therefore an important prerequisite for the production of regenerants from

protoplasts in cereals. However, the establishment of such cultures from cereals is often difficult and labour-intensive (Chapter 3).

The production of fertile plants from protoplasts had not been reported in *Triticum tauschii*. The main objectives of this study were therefore to develop an efficient method for the large-scale isolation of protoplasts of *Triticum tauschii* from suspension cultures, and to produce fertile plants from those protoplasts *via* somatic embryogenesis.

4.2 Materials and methods

Two accessions of *Triticum tauschii* (CPI 110813 and CPI 110649) obtained from CSIRO Division of Plant Industry (CPI) were used (Appendix 1). A previous study showed that these two accessions were amenable to the production of fine cell suspension cultures possessing long-term regeneration capacity (Chapter 3).

4.2.1 Suspension cultures

In initial experiments, four 12-month-old cell suspension lines, one from accession CPI 110813 and three from accession CPI 110649, were generated as described earlier (Section 3.2). A repeat experiment involved re-initiation of cultures of accession CPI 110813 and its use, after 10 months culture, for protoplast isolation and regeneration. The cultures were maintained in liquid MS medium (Murashige and Skoog 1962) containing 1.1 mg L^{-1} 2,4-D and subcultured at weekly intervals (Section 3.2).

4.2.2 Protoplast isolation and culture

Fine cell aggregates (< 500 µm in diameter) were collected by filtering suspensions through 500 µm diameter sieves, 2-5 days after subculture. Aggregates (0.7-1 g) were mixed with 10 mL filter-sterilized enzyme solution. The enzyme solution consisted of 3% (w/v) Cellulase "Onozuka" RS (Yakult Pharmaceutical Industry Co. Ltd. Japan), 0.5% (w/v) Macerozyme R10 (Serva Feinbiochemica GmbH and Co., Heidelberg, Germany) and 0.2% (w/v) Pectolyase Y23 (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) dissolved in 20 mM $CaCl_2$ and 0.6 M mannitol. The aggregate-enzyme mix was incubated on a rotary shaker at 40 rpm at 26°C in the dark for 3-4 h. The resulting protoplast suspension was purified by filtering through a series of 100 µm, 50 µm, and 30 µm nylon mesh sieves. Protoplasts that passed through the three sieves were collected by centrifuging at 50 x g for 5 mins and the pellet was rinsed three times with 20 mM $CaCl_2$ and 0.6 M mannitol. Protoplast yield was determined

using a haemocytometer.

For generation of cell walls and initial cell division, the protoplasts were re-suspended in half-strength MS medium supplemented with 1.1 mg L^{-1} 2,4-D, 0.6 M glucose and 1.2% (w/v) Sea-Plaque agarose at around 40°C at densities in the range of 0.75 x 10^6 to 1-1.5 x 10^6 protoplasts mL^{-1}. The osmolarity of the medium was adjusted to 750 ± 50 mOsm per kg H_2O using an Osmometer. The suspended protoplasts were plated in Petri dishes (3.5 cm in diameter) and incubated at 22°C in the dark. After 7 days, the agarose in each Petri dish containing microcolonies was cut into 8 segments and transferred to 5.5-cm Petri dishes containing 1 mL of liquid protoplast culture medium. The liquid medium was removed and replaced with fresh medium every week for 14-21 days.

In attempts to obtain cell division from protoplasts using cell lines of accession CPI 110649, various media including MS basal medium supplemented with different concentrations of 2,4-D (1.1, 2.2, 4.4 mg L^{-1}) and L1 and L5 basal medium (Lazzeri *et al.* 1991) supplemented with different concentrations of Dicamba® (Serva Fine Biochemicals, Heideberg, Germany) (3, 6, 12, 24 mg L^{-1}) were used. All of these media contained 0.6 M glucose and 1.2% (w/v) Sea-Plaque agarose.

A 12-month-old, fast growing non-embryogenic suspension culture from accession CPI 110810 was used to provide feeder cells. For feeder cultures, a modification of the mixed nurse method described by Funatsuki *et al.* (1992) was employed in which medium containing embedded protoplasts was cut into eight pieces. The separated pieces were placed in 5.5-cm Petri dishes containing 3 mL liquid protoplast culture medium with 100 mg of feeder cells and incubated on a rotary shaker at 24 rev min^{-1} at 22°C in the dark. The liquid medium was removed and replaced with fresh medium every three days. After 14 days, feeder cells were removed by washing the agarose segments with liquid protoplast medium. The agarose segments were placed in 5.5-cm Petri dishes containing 1 mL protoplast culture medium and incubated for 7 days.

Developing minicalli (1-2 mm diameter) were transferred to MS medium supplemented

with 1.1 mg L^{-1} 2,4-D and 30 g L^{-1} sucrose and solidified with 0.25% (w/v) Phytagel (Sigma, St. Louis, Mo. USA) after 21-28 days of culture. This experiment was repeated on three occasions.

4.2.3 Plant regeneration

Somatic embryoids which formed on callus cultured in the medium described above were transferred to Petri dishes (7 cm in diameter) containing hormone-free MS medium for germination. The plates were incubated at 22°C under cool white fluorescent lights (60 μE m^{-2} s^{-1}). When the plantlets were 0.5-1 cm long, they were transferred to polystyrene screw-cap vessels (105 x 42 mm) containing hormone-free MS medium to enable further shoot elongation and root production. Plants with leaves longer than 10 cm and with well-developed root systems were transferred to 10-cm pots containing a pine bark / peat potting mix and a 3-4 month slow release complete fertiliser (Osmocote®, N : P : K = 14 : 6.1 : 11.5). Pots were placed in propagation trays with plastic covers for two weeks prior to their transfer to the glasshouse. The glasshouse was maintained at 22 ± 4°C. Natural light was supplemented by 400-W metal-halide lamps to provide a 16-h photoperiod. Resulting plantlets were vernalised at 4°C with a 9-h photoperiod for 28 days.

4.3 Results

4.3.1 Suspension cultures

Embryogenic cell lines contained a mixture of large and small friable, compact aggregates and empty elongated cells (Figure 4.1). To increase the concentration of fine and highly cytoplasmid cell aggregates, the heterogeneous cultures were sieved (using 850 μm sieve) to remove the large aggregates. Several washing steps were subsequently required to remove the empty elongated cells. A 500 μm sieve was finally used to collect fine cell aggregates.

Non-embryogenic suspension lines were more homogeneous, containing only small friable and compact aggregates. Fine cell aggregates were isolated from them by passing the cultures through a 500 μm sieve.

4.3.2 Protoplast isolation and culture

Preliminary work on optimising enzyme concentrations showed that a combination of 3% Onozuka, RS, 0.5% Macerozyme and 0.2% Pectolyase Y23 was the most effective in producing high yields of purified protoplasts from the fine, highly cytoplasmic cell aggregates (Figure 4.2 and Figure 4.3).

In general, cell lines from CPI 110649 yielded fewer protoplasts per gram of cell suspension aggregates than the single line from CPI 110813 (Table 4.1). It was not possible however, to determine whether these differences were statistically significant. Highest protoplast yields were obtained from all cell lines after 4-5 h incubation at the enzyme concentrations specified above.

Cell division occurred when the protoplasts were cultured in agarose-medium but not in the liquid protoplast culture medium. The optimal concentration of Sea Plaque agarose was 1.2% (w/v). Protoplasts derived from CPI 110813 regenerated cell walls and about 20-25% of them divided within 5-7 days (Figure 4.4, Table 4.1), when cultured at a density of 1-1.5 x 10^6

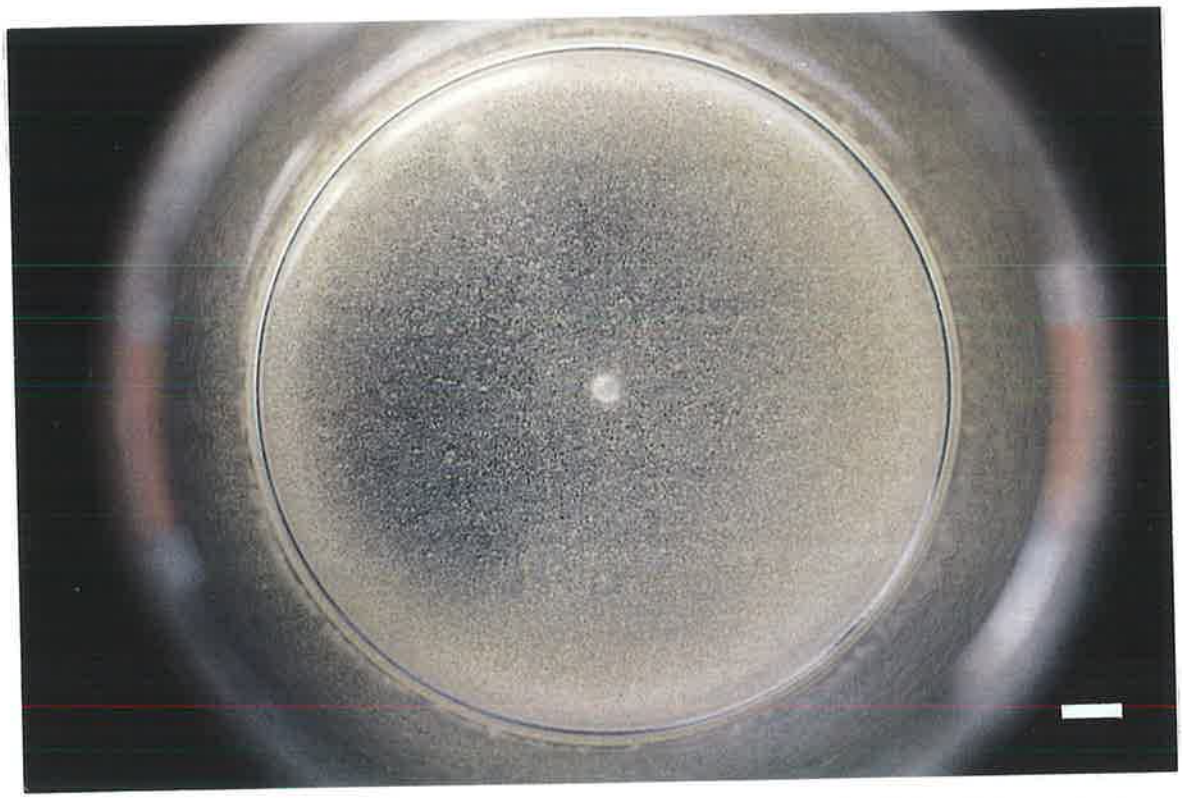


Figure 4.1. Suspension culture containing cell aggregates (< 500 μm) for protoplast isolation. Scale bar = 5 mm.

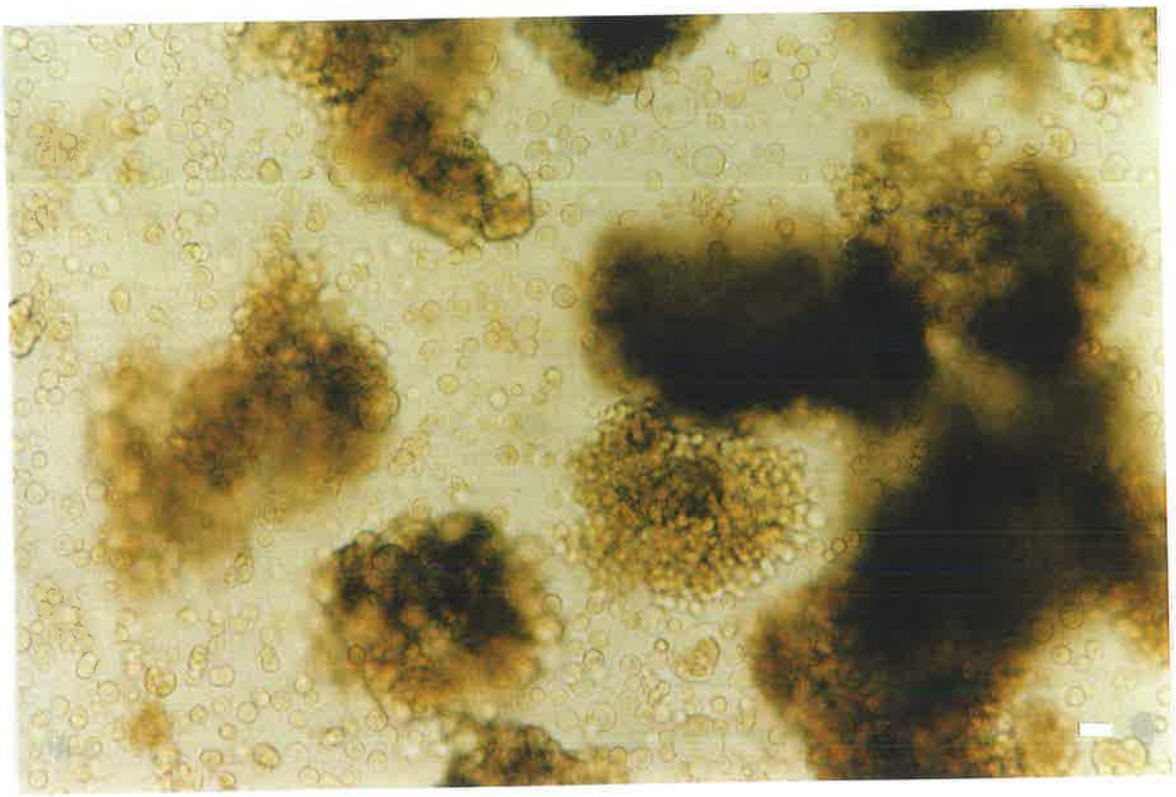


Figure 4.2. The release of protoplasts from cell aggregates in present of enzyme solution. Scale bar = 20 μm.

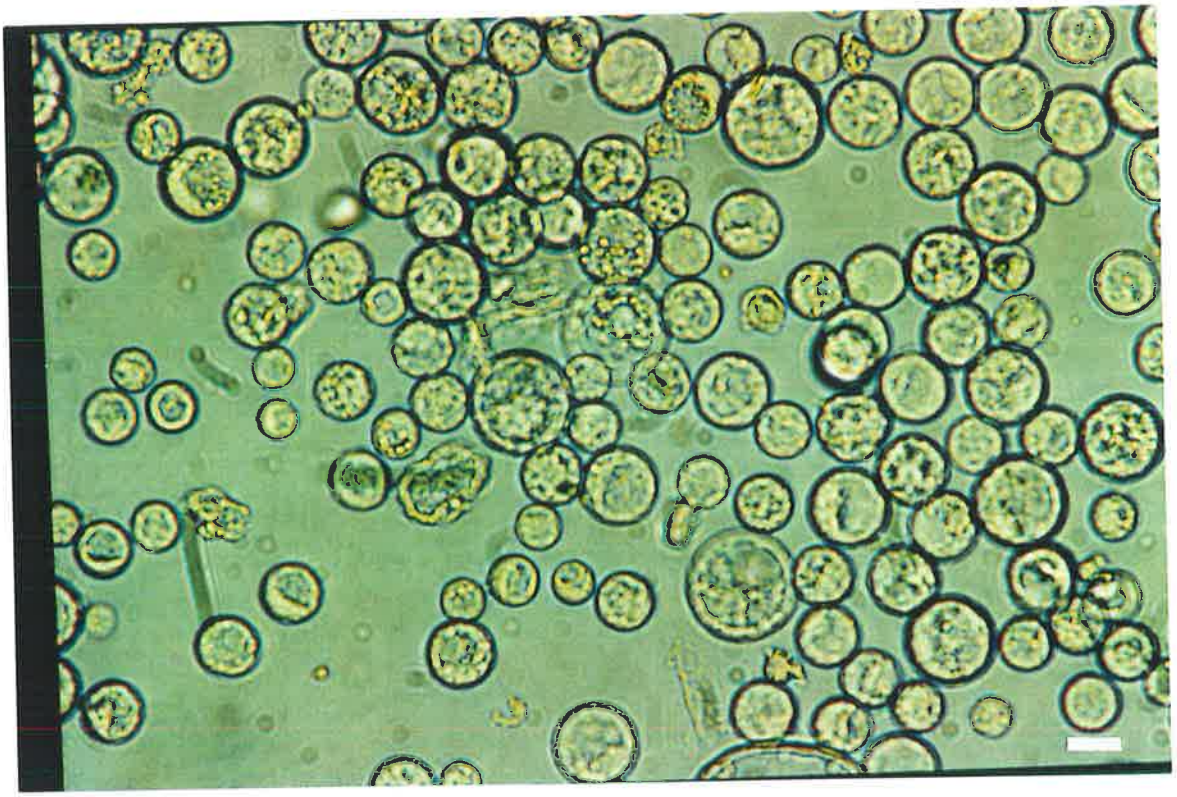


Figure 4.3. Protoplasts isolated from suspension cell aggregates of accession CPI 110813. Scale bar = 10 μm.

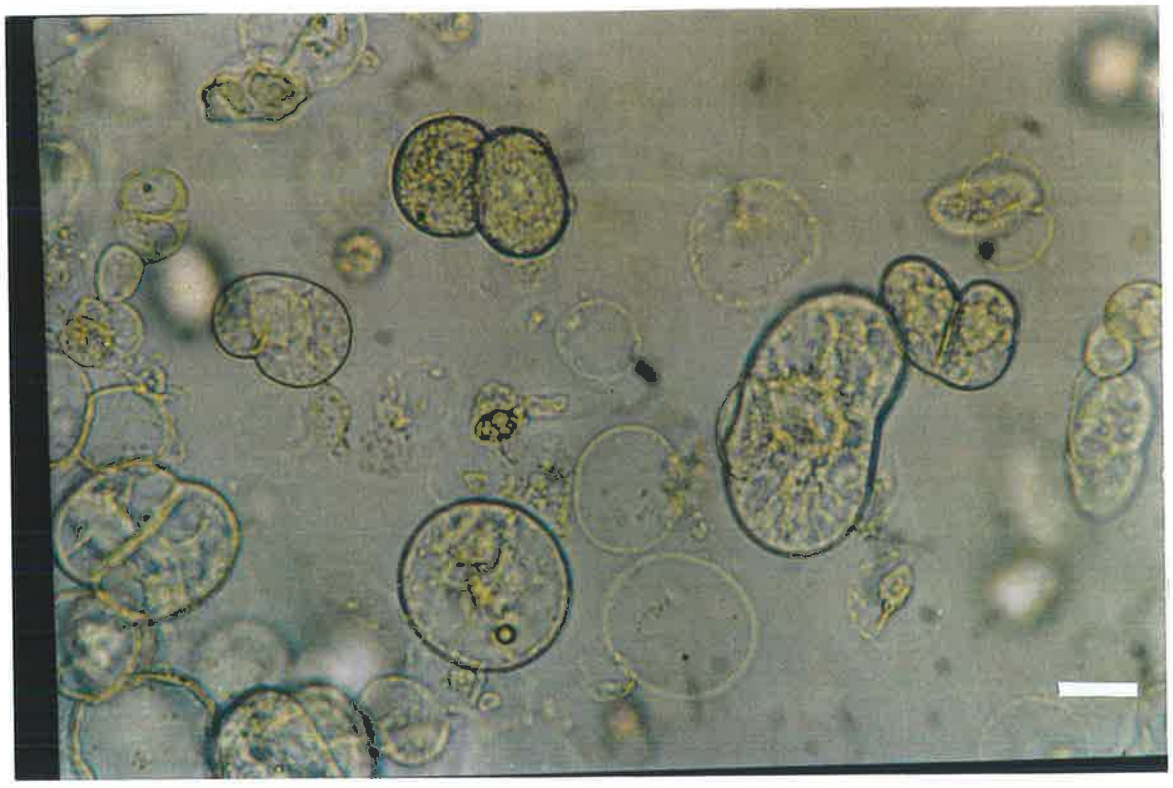


Figure 4.4. First cell division of *Triticum tauschii* protoplasts - 5 days after isolation. Scale bar = 10 μm.

Table 4.1 Protoplast yield and cell division efficiency of four cell lines of *Triticum tauschii* in 1.2% agarose medium.

Accession (line)	Protoplast yield per gram cell suspension (10^6)	Cell division frequency (%)
CPI 110813	7.6 - 9	20 to 25
CPI 110649 (1)	1.6 - 4.5	0
CPI 110649 (2)	3 - 7.5	0
CPI 110649 (3)	2 - 4.5	0

protoplasts mL^{-1}. Cell division did not occur with the CPI 110813 cell lines when protoplasts were cultured at a density of 0.75 x 10^6 protoplasts mL^{-1}. However, with feeder cells, a cell division frequency of 16.6% was obtained. Protoplasts from the three suspension lines of CPI 110649 did not regenerate cell walls, despite repeated attempts to culture them using various modifications of the protocols described above.

Protoplasts from line CPI 110813 developed cell colonies after 14 days (Figure 4.5). After 21-28 days of culture, colonies were 1-3 mm in diameter and visible to the naked eye. They were transferred to half-strength MS medium containing 1.1 mg L^{-1} 2,4-D and solidified with 0.25% (w/v) Phytagel. The morphology of the colonies was similar to the embryogenic callus from which suspension cultures were initiated (Figure 4.6).

4.3.3 Plant regeneration

Protoplast-derived colonies of CPI 110813 grew into large clusters when they were transferred to half-strength MS medium with 1.1 mg L^{-1} 2,4-D. They resembled the Type I cell clusters observed during the initiation of the donor suspensions (Section 3.2), in that they were friable and compact, and surrounded by soft transparent tissue. The colonies produced somatic embryoids after 21-28 days on medium containing 1.1 mg L^{-1} 2,4-D (Figure 4.6).

Somatic embryoids from the clusters germinated upon transfer to hormone-free MS medium (Figure 4.7). The frequency of plant regeneration from protoplast-derived callus of CPI 110813 was 85%, which is similar to that obtained from the donor suspension. In all, 150 fully developed plantlets (Figure 4.8) were transferred to the glasshouse (Figure 4.9).

All plants grew to maturity, set flowers and were morphologically similar to plants derived from grains. However, none of the protoplast-derived plants produced seeds. Another cell line from accession CPI 110813 therefore was established using the same protocols. Protoplasts were isolated from a 10-month-old suspension culture and 16 regenerants were transferred to the glasshouse. Root tip chromosome counts showed that all the regenerants had 14 chromosomes, the normal diploid number for *Triticum tauschii*. Nine plants (Figure 4.10)

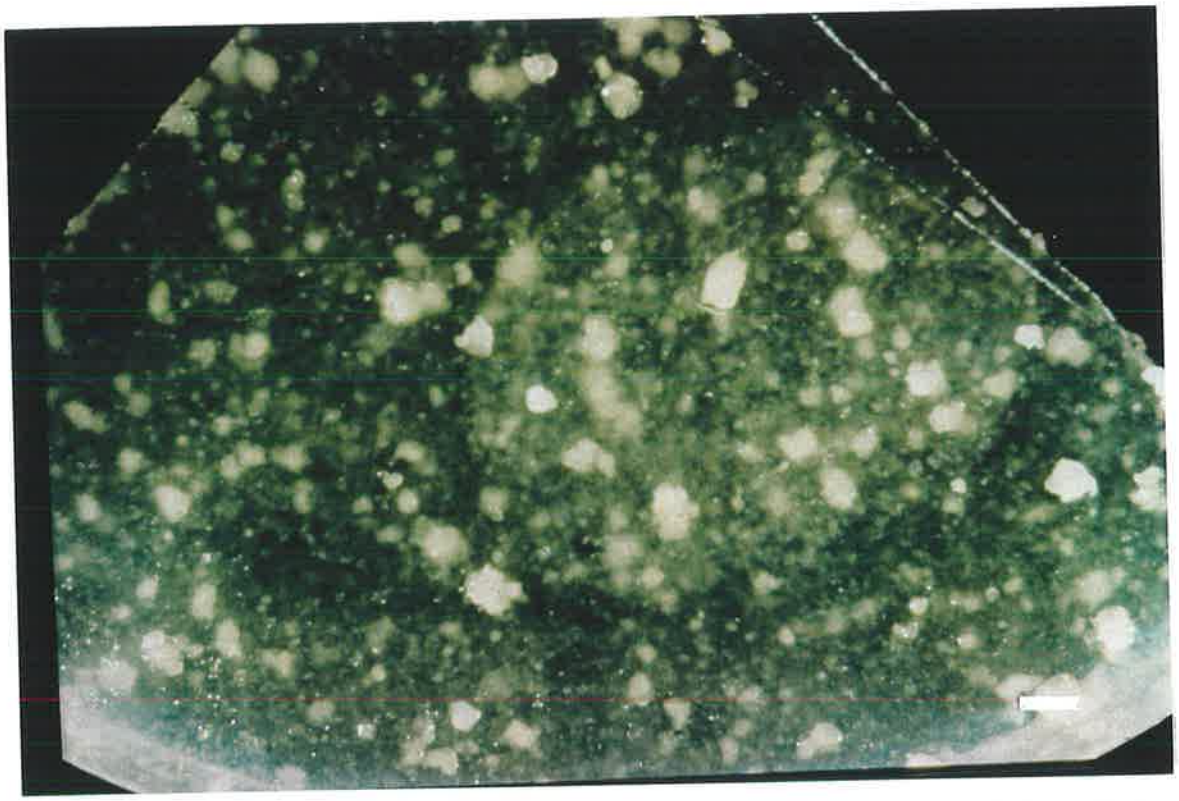


Figure 4.5. Minicolonies formed from protoplasts - after 14 days of incubation. Scale bar = 0.5 mm.

Figure 4.6 Embryogenic callus developed from a protoplast. Scale bar = 0.5 mm.

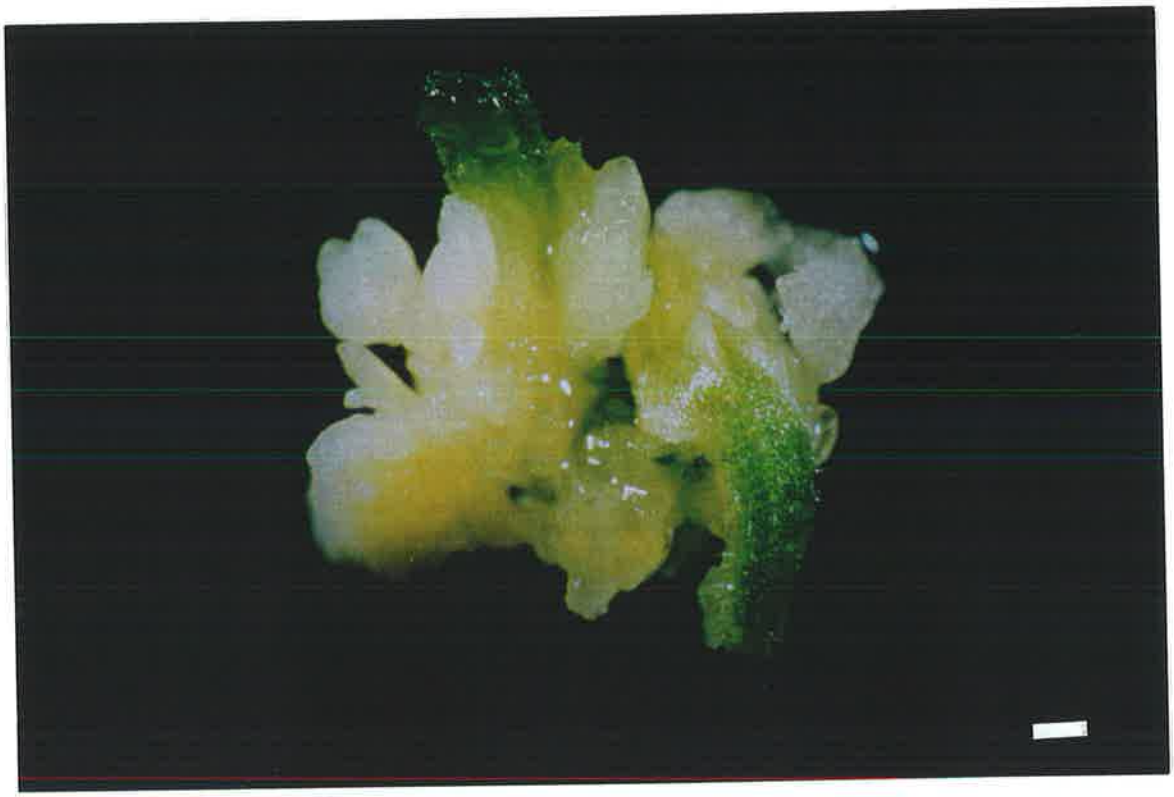


Figure 4.7. Shoots emerging from somatic embyroids developed from protoplast callus. Scale bar = 0.5 mm.

Figure 4.8. A fully developed plantlet derived from a protoplast.

Figure 4.9. Protoplast-derived plants in the glasshouse.

Figure 4.10 A fertile plant derived from a protoplast.

produced well-filled grains but shriveled grains were produced by the other seven plants (Figure 4.11).

Figure 4.11 Well-filled seeds (right) compared to shrivelled seeds (left) produced from protoplast-derived plants.

4.4 Discussion

In the present study, a method has been developed for the regeneration of fertile plants from protoplasts of *Triticum tauschii*. The method depends on the establishment of a specific type of embryogenic suspension culture (Chapter 3) and the selection of small (< 500 µm in diameter) cytoplasmic cell aggregates for protoplast isolation (Figure 4.1).

Fertile plants were produced from protoplasts of the accession CPI 110813 (Figure 4.10). A protoplast plating density of 1-1.5 x 10^6 protoplasts / ml was required for a high frequency of cell division and for the subsequent development of colonies. Feeder cells of non-embryogenic cell aggregates of *Triticum tauschii* were required when lower plating densities were used. The benefit of feeder cells in increasing cell division has also been reported in barley (Wang and Lörz 1994).

Despite several attempts, protoplasts derived from the three cell lines of CPI 110649 did not divide (Table 4.1). Therefore, the present study indicates that genotype is as important as other factors known to affect cell division, such as the size of cell aggregates of donor suspension cultures (Yang *et al.* 1994), protoplast plating densities (Jähne *et al.* 1991a), the use of feeder cells (Funatsuki *et al.* 1992; Gupta and Puttanayak 1993) and the age of the donor suspension cultures (Guiderdoni and Chaïr 1992).

In this study, cell division was not observed in protoplasts cultured in liquid medium. This is in contrast to the findings of Chang *et al.* (1991), He *et al.* (1992), Qiao *et al.* (1992) and Ahmed and Sagi (1993) in *Triticum aestivum*, and Yang *et al.* (1993) in *Triticum durum*, who reported high cell division frequencies in both liquid and agarose-solidified media. The absence of cell division in *Triticum tauschii* protoplasts may be related to the fact that protoplasts of the *Triticum tauschii* cell lines aggregated strongly in the liquid medium used.

Plantlets derived from CPI 110813 protoplasts were sufficiency well developed (Figure 4.8) to enable them to be transferred to soil 10-12 weeks after protoplast isolation. This time frame is similar to that achieved by Vasil *et al.* (1990) for hexaploid wheat, but is considerably

shorter than that observed by Jähne *et al.* (1991a) in barley and He *et al.* (1992) and Ahmed and Sagi (1993) in hexaploid wheat, where protoplast colonies required several subculture steps to initiate somatic embryoids and shoot formation.

The decreased fertility together with small grain size (shriveled grains) observed in the initial regenerants in this study may be due to somaclonal variation (Karp *et al.* 1987). However, fertile plants were successfully generated from protoplasts of *Triticum tauschii* (Figure 4.10 and 4.11). While it might be expected that the primary use of the protocol in this Chapter would be in the genetic transformation of *Triticum tauschii* as outlined in the next chapter and the subsequent transfer of introduced genes to hexaploid wheat, it could also be used for *in vitro* selection of somaclones if the methods were subsequently shown to be useful in generating stable genetic variation.

CHAPTER 5

EVALUATION OF TWO TRANSFORMATION METHODS FOR *TRITICUM TAUSCHII*

5.1. Introduction

Genetic transformation of wheat, combined with conventional breeding, has the potential to accelerate improvements in both the yield and quality of wheat. However, transformation of common bread, *Triticum aestivum*, is still inefficient; the highest reported transformation frequency being 3.6% (Uzé *et al.* 1999) with the selectable marker, *bar* gene, and 5.5% (Ortiz *et al.* 1996) with the *hpt* gene. Furthermore, successful transformation has been restricted to just a few varieties of wheat.

The significance of *Triticum tauschii* as a wheat progenitor and its potential for use as a vehicle for production of transgenic bread wheat has been described in Chapter 1. In Chapters 2, 3 and 4, the potential of *Triticum tauschii* for producing transgenic hexaploid wheat was confirmed because protocols for *in vitro* culture of *Triticum tauschii*, including the regeneration of fertile plants from callus, suspension cultures and protoplasts, were successfully developed. The most widely used transformation methods for cereals have also been described in Chapter 1.

In this chapter, attempts to transform *Triticum tauschii* using two different methods are described. Protoplast transformation was initially evaluated because this method has been successful in rice (Zhang *et al.* 1988; Datta *et al.* 1990; Terada *et al.* 1993) and maize (Golovkin *et al.* 1993; Omirulleh *et al.* 1993). However, protoplast transformation is a complicated and difficult method for the production of transgenic cereals. It relies on the availability of embryogenic suspension cultures which produce high yields of protoplasts with a high capacity for cell division and from which fertile plants can be regenerated. Although the production of such embryogenic suspension cultures and fertile protoplast-derived plants is achievable, the procedures are laborious and time-consuming. In view of successes (Fromm *et al.* 1990; Cao *et al.* 1992; Somers *et al.* 1992; Vasil *et al.* 1992) obtained using bombardment of intact plant cells with DNA-coated microprojectiles, we decided to evaluate both the direct insertion of DNA into protoplasts, and the use of microprojectile bombardment for transformation of *Triticum tauschii*.

For protoplast transformation, the polyethylene glycol (PEG) (Krens *et al.* 1982)

method was used, because of its simplicity compared to electroporation-mediated protoplast transformation (Shillito *et al.* 1985; Fromm *et al.* 1986). Several factors have been shown to affect the efficiency of gene transfer and expression using PEG-mediated protoplast transformation. These include protoplast source, the procedures used to isolate protoplasts (Krautwig *et al.* 1994), the plasmid construct and the form of plasmid (Armstrong *et al.* 1990; Chaïr *et al.* 1996), plating methods and the concentration of agarose (Armstrong *et al.* 1990), plant species (Hayashimoto *et al.* 1990; Zaghmout and Trolinder 1993) and plant genotypes (Zaghmout 1994). In the present study, the conditions used for other cereal species were adopted as a starting point to develop transformation methods for *Triticum tauschii*. Conditions affecting protoplast transformation were optimised using transient expression of the *GUS* reporter gene.

Microprojectile bombardment is a more attractive method for cereal transformation because it enables delivery of foreign DNA into regenerable cells, tissues or organs, thereby eliminating the need for protoplast isolation and culture. Furthermore, somaclonal variation is minimised because DNA can be delivered into explants from which plants can be regenerated either directly or without a prolonged tissue culture phase (Sanford *et al.* 1987; Sanford *et al.* 1993). The microprojectile method has been the most successful DNA delivery method for the production of transgenic cereal plants including rice, wheat, barley and maize (see Table 1.2).

Efficient production of transgenic plants by particle bombardment requires the optimisation of conditions not only for DNA delivery into cells, but also for the selection and regeneration of transformed cells. Transient gene expression assays are often used to optimise DNA delivery conditions. Several factors influencing microprojectile transformation have been investigated in cereals. They include helium pressure (Casas *et al.* 1993; Becker *et al.* 1994; Brettschneider *et al.* 1997; Rasco-Gaunt *et al.* 1999), amount of particles (Brettschneider *et al.* 1997; Rasco-Gaunt *et al.* 1999; Harwood *et al.* 2000), particle size (Rasco-Gaunt *et al.* 1999; Frame *et al.* 2000), amount of plasmid DNA (Nandadeva *et al.* 1999; Rasco-Gaunt *et al.* 1999; Harwood *et al.* 2000), form of plasmid DNA (Nandadeva *et al.* 1999), pre- and post-bombardment osmotic treatments (Jain *et al.*

1996; Brettschneider *et al.* 1997; Nandadeva *et al.* 1999), and the distance between target tissues and the launch point of the microparticles (Jain *et al.* 1996). Physical parameters such as particle amount, helium pressure and bombardment distance are also the major cause of tissue damage (Perl *et al.* 1992; Taylor and Vasil 1991) and therefore must be carefully calibrated to minimise tissue damage.

One method of minimising the damage from bombardment is to plasmolyze the target tissue by treatment with an osmoticum. Higher transformation rates have been obtained when cells were subcultured for several hours on a medium with a high osmolarity before and after particle bombardment (Armaleo *et al.* 1990; Vain *et al.* 1993a). Plasmolyzed cells are less likely to extrude their protoplasm following penetration of the cell by particles (Armaleo *et al.* 1990). However, in some cases, the physical parameters and osmotic treatment which have been optimised for transient gene expression have not provided a similar effect on stable transformation (Becker *et al.* 1994; Brettschneider *et al.* 1997; Nandadeva *et al.* 1999).

There are no general conditions or rules that are optimal for microprojectile transformation of all species, or all cell cultures, tissues or organs within a species (Jähne *et al.* 1995, Walden and Wingender 1995). Thus, it is necessary to optimise the variables for each species and for each target tissue individually. The scutellar tissue of immature embryos, embryogenic callus and suspension cultures have been widely used as target tissue for microprojectile transformation in cereals and were used in the present study. Furthermore, fertile transgenic plants have been produced from these tissues in rice, wheat, barley and maize (see Chapter 1.3.3).

Experiments to transform *Triticum tauschii* by direct uptake of DNA into protoplasts and by insertion of DNA into tissues by microprojectile bombardment are described in this Chapter.

5.2. Materials and Methods

5.2.1. Protoplast transformation

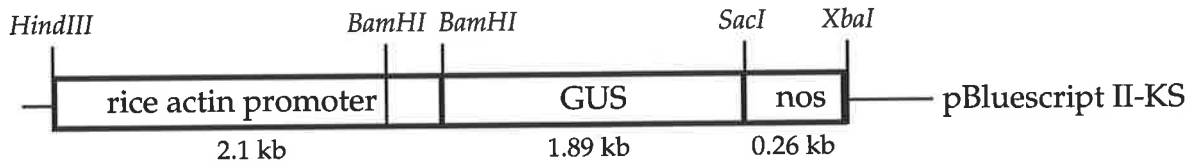
Suspension and protoplast cultures: Two accessions of *Triticum tauschii*, CPI 110649 and CPI 110813 obtained from the CSIRO Division of Plant Industry, Canberra (CPI) (Appendix 1). The embryogenic suspension lines were initiated and established as described in Section 3.2 and maintained in liquid MS medium containing 1.1-2.2 mg L^{-1} 2,4-D. Protoplasts were isolated and purified as described previously (Section 4.2) using enzyme solution supplemented with or without Macerozyme R 10 and suspended in 1 mL of washing solution (20 mM $CaCl_2$ and 0.6 M mannitol).

Plasmid construct: Plasmid pAHC25 (Chritensen and Quail 1996) was provided by Drs Alan Christensen and Peter Quail (Plant Gene Expression Centre, USDA/ARS, CA, USA). It contained the *ubi*1 promoter linked to the *GUS* reporter gene (Jefferson *et al.* 1987) and the *bar* selectable marker gene (Figure 5.1). The *bar* gene confers resistance to phosphinothricin (PPT), the active ingredient of the herbicide Basta® (De Block *et al.* 1987).

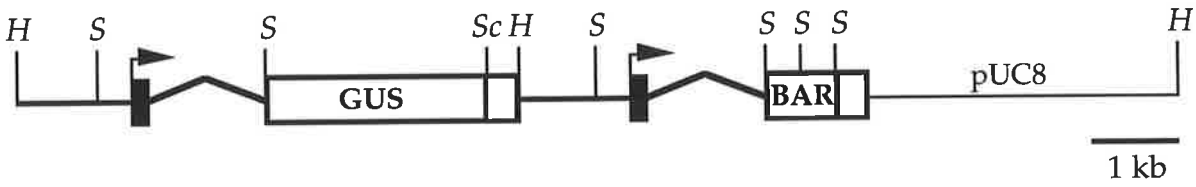
The plasmids were grown in *Escherichia coli* and purified using Qiagen columns (as described by the manufacturer). Plasmid DNA was dissolved in sterile TE buffer and both the DNA concentration and quality were determined spectrophotometrically and by agarose gel electrophoresis (Sambrook *et al.* 1989).

Transformation: Protoplasts were treated using the protocol originally developed by Krens *et al.* (1982) for tobacco and later modified for barley by Lazzeri *et al.* (1992). The suspended protoplasts were placed into 50 mL-centrifuge tubes to give 2-4 x 10^6 protoplasts per tube, and centrifuged at 50 g for 5 mins. The supernatant was poured off and the pellet was dispersed in the remaining solution. To each tube containing dispersed protoplasts, 250 µL C100M solution (15 g L^{-1} $CaCl_2 . 2H_2O$, 1 g L^{-1} MES, and 70 g L^{-1} mannitol, pH 5.7), plasmid DNA (50 to 100 µg μL^{-1}), and 600 µl PEG solution [40% w/v PEG (Merck -

P*ACT*1-D



pAHC25



pDM302

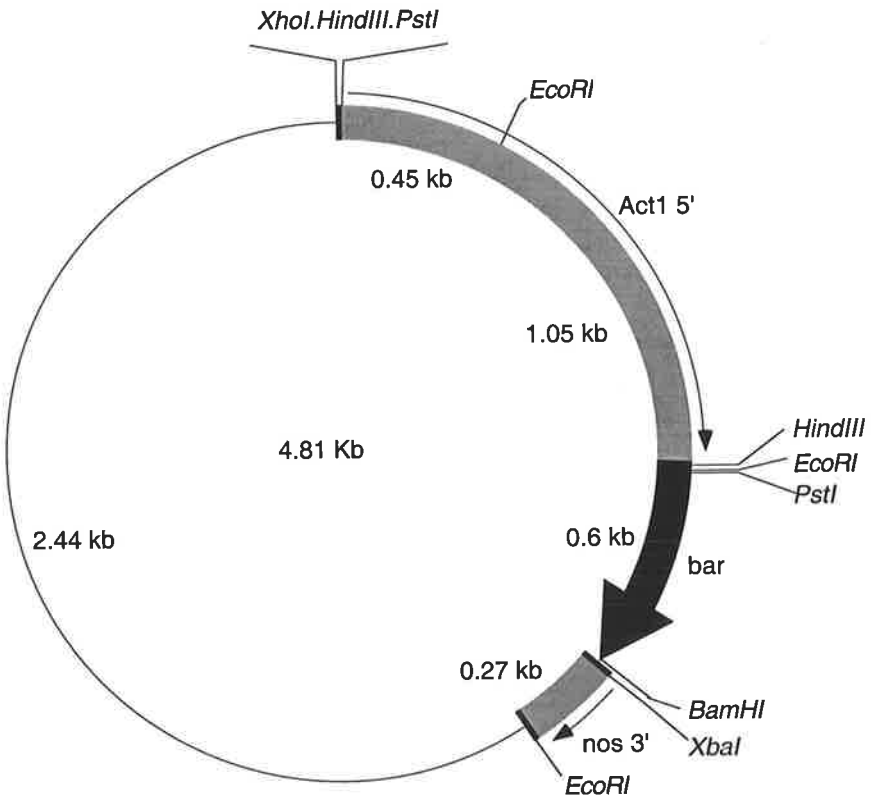


Figure 5.1 Maps of plasmid pAHC25, pDM302 and p*ACT*1-D.

Schuchadt) 1500, dissolved in C100M solution, pH 7.0] was added. In the control tube, DNA solution was replaced by an equivalent volume of sterilised distilled water. The suspensions were inverted gently three times every 2-3 min for a period of 15 mins. Washing solution (15 mL) was added and protoplasts were pelleted by centrifugation of 50 g for 10 mins, washed with 1 mL washing solution and centrifuged three more times. Protoplasts were resuspended in half strength MS medium supplemented with 1.1 mg L^{-1} 2,4-D, 0.6 M glucose and 1.2% (w/v) Sea Plaque agarose at about 40°C, at a density of 1-1.5 x 10^6 protoplasts/mL, and plated in 2.5 cm diameter Petri dishes.

Transient GUS-assays: Transient GUS activity was determined histochemically according to the protocol of Jefferson (1987). The GUS assay buffer contained 0.2 M sodium phosphate buffer, pH 7.0, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.05 % (w/v) X-gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide) and 0.06% (v/v) Triton X-100. The solution was sterilised by passing it through a 0.22 µM micro-filter. A quarter portion of the protoplasts embedded in agarose was transferred to a Petri dish, overlayed with X-gluc solution and incubated at 37°C for 48 h. The number of cells expressing *GUS* (blue cells) was counted under a microscope.

5.2.2 Microprojectile transformation

All experiments were conducted with a Du Pont Biolistics® PDS - 1000/He, (Richmond, Ca., USA) Biolistic delivery system. The method used for coating plasmid DNA onto gold particles was developed by Sanford *et al.* (1993) and is described in the instruction manual for the biolistics apparatus. Gold particles (Heraeus, Karlsruhe, Germany) (60 mg) with an average size of 0.4-1.2 µM were placed in a 1.5 mL Eppendorf tube and washed once with 70% ethanol and three times in sterile distilled water by centrifugation and resuspension. The washed particles were suspended in 1 mL 50% (v/v) glycerol. To study the effect of particle concentration, a portion of the suspended particles was taken and diluted by adding 50% (v/v) glycerol to obtain the desired particle concentration. Fifty microlitres of particle suspension were placed in a 1.5 mL Eppendorf

tube and while vortexing, 5 µL plasmid DNA (1 µg μL^{-1}), 20 µL spermidine (0.1 M) and 50 µL $CaCl_2$ (2.5 M) were added. The mixture was vortexed for 3-5 min and centrifuged in a microfuge (6.2 x 10^3 r min^{-1}) for 5 sec. The supernatant was removed and the pellet washed briefly and carefully with 70% and 100% ethanol. DNA-coated particles were suspended in 60 µL 100% ethanol and 10 µL suspension was pipetted onto each macrocarrier. Optimal conditions for particle bombardment were determined using transient assays of the β-glucuronidase (*GUS*) reporter gene. Particle acceleration parameters were a vacuum strength of 27 inch Hg, and a distance of 9.5 cm from the rupture disk to the target (2 cm from rupture disk to macrocarrier; a distance of 1.5 cm from macrocarrier to stopping screen and 6 cm from stopping screen to target tissues).

The influence of osmotic treatment on transient expression was tested by incorporating 30 g L^{-1} each of mannitol and sorbitol in the callus induction medium (Vain *et al.* 1993a) for immature embryos and in the maintenance medium for suspension cultures. The osmotic treatments consisted of a 4-6 h pre-bombardment treatment and 16-20 h post-bombardment treatment.

Sixteen to twenty hours after bombardment, the target tissues under osmotic treatment were transferred to the original medium and incubated in the dark at 22±2°C.

5.2.2.1 Optimisation of bombardment parameters

Immature embryos: The methods used to grow *Triticum tauschii* plants, and dissect and sterilise immature embryos were as previously described (Section 2.2). Immature embryos were cultured on callus induction medium H (Chapter 2, Table 2.2) for 5-7 days. Twenty pre-cultured embryos were arranged with their scutella facing upwards, in a circle of about 1.5cm diameter in the centre of a 5.5-cm diameter Petri dish containing medium H.

Bombardment conditions for scutellar tissues from accession CPI 110649 were analysed by transient gene expression using plasmid pAHC25, which contains both the *GUS* reporter gene and the *bar* selectable marker gene (Figure 5.1). Bombarded tissues were stained histochemically two days after bombardment and the number of blue spots

determined. The amount of gold particles used ranged from 125 to 500 μg per bombardment. To investigate the effects of wounding caused by the gold particles, two different amounts of particles (120 and 60 μg per bombardment) were tested. After bombardment, the embryos were cultured on callus induction medium for 2 weeks. The number of undamaged tissues, based on callus forming scutella, was counted.

Statistic analysis: A two-factor (accession by concentration) logistic regression model (Hosmer and Lemeshow 1989) was fitted to the data to analyse the effect of particle amount on the number of damaged scutellar fragments. Dummy coding was employed for the independent variables, with an amount of 60 μg/bombardment and the accession CPI 110649 as base categories. In the presence of a significant interaction effect, the approach taken for further analysis was to test the effects of particle amounts on each accession and at each particle amount. Because there are two tests in each of these two 'families', each test within each family is tested at $\alpha = 0.05/2 = 0.025$, according to the Bonferroni procedure for planned comparisons (Harris 1985).

Suspension culture preparations: Eight-month-old fine embryogenic suspension cultures of accession CPI 110649 were obtained as previously described (Chapter 3.2) and maintained in medium E (Table 3.2). The suspension aggregates, which had been subcultured 2-4 days before, were filtered through an 800 μm polypropylene sieve and fine aggregates passing through the sieve were collected. Around 200 mg fine suspension aggregates were spread over a circular area (2 cm in diameter) in the centre of a 4 cm Whatman 1 filter paper, which had been placed on a 5.5-cm Petri dish containing medium E solidified with 0.25% (w/v) Phytagel.

Histochemical GUS assay: Expression of *GUS* in targeted tissues was determined histochemically two days after bombardment, using X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) as described by Jefferson (1987). Transient GUS activity was assayed by incubating targeted tissue in the dark for 24 h at 37°C.

5.2.2.2 Stable transformation

Plasmid: For stable transformation experiments, two plasmids pDM302 and p*ACT*1-D were provided by Professor R. Wu (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York, USA). Plasmid pDM302 contains the coding region of the *Streptomyces hygrocopicus* phosphinothricin acetyl transferase (PAT) gene (*bar*) under control of the 5' region of the rice actin 1 promoter (*Act1*) (Figure 5.1) (McElroy *et al.* 1991; Rathore *et al.* 1993). Plasmid p*ACT1-D* contains the gene coding β-glucuronidase (*gus*A) driven by the rice *actin*1 promoter (Figure 5.1) (McElroy *et al.* 1990).

Suspension cultures: Eight-month-old suspension cultures of two accessions (CPI 110810 and CPI110649) of *Triticum tauschii* were used. Six plates containing fine cell aggregates on filter paper were prepared from each accession, as described before. One plate from each accession (control) was not bombarded.

A helium pressure of 1300 psi and a gold particle amount of 125 μg per bombardment were used. Osmotic treatments were applied 4-6 h before bombardment and 16-20 h after bombardment. Two days after bombardment, cells on one bombarded filter from each accession were stained histochemically and the remaining filters were maintained on the same medium for an additional four days.

Selection of transgenic callus: The herbicide bialaphos (Meiji Seika, Yokohama, Japan) was used as the selective agent in these experiments (De Block *et al.* 1987). It was dissolved in water (1mg mL^{-1}) and purified by filtration through a 0.22 μM sterile microfilter.

Selection for herbicide resistant lines commenced 4 days after bombardment by placing the filter carrying the cell aggregates on solidified E medium containing 1 mg L^{-1} bialaphos. Filters were transferred to fresh herbicide medium every week. The concentration of herbicide was increased to 2 mg L^{-1} in the second week, 3 mg L^{-1} in the third week, and was maintained at 3 mg L^{-1} for the following 12 weeks. After 4-6 weeks, the healthy cell clumps growing on filter papers were transferred to medium containing herbicide. Six to eight weeks after bombardment, callus with developed embryoids was

transferred to half-strength hormone-free MS medium to induce shoot initiation.

Expression of GUS activity was determined by testing a small amount of tissue from each transformed callus line.

PAT assay: All bialaphos-resistant cell lines, as well as a non-bombarded cell clump, were analysed for PAT activity 10-12 weeks after bombardment. The assays were performed using Basta®, a commercial herbicide formulation containing chemically synthesised PPT.

PAT activity was analysed using thin layer chromatography (Spencer *et al.* (1990). Crude protein extracts were made from putatively transformed and non-transformed callus. Approximately 100 mg fresh callus tissue was placed in 1.5 mL Eppendorf tubes and 50 µl extraction buffer (50 mM Tris-HCl buffer, pH 7.5, 2 mM EDTA, 0.15 mg L^{-1} bovine serum albumin and 30 mg L^{-1} dithiothreitol) was added. Tissues were squashed using a pestle and centrifuged for 10 min at 14000 rpm at 4°C. The supernatant was transferred to a 1.5 mL Eppendorf tube, centrifuged and transferred in another Eppendorf tube, and protein was quantitated using the method of Bradford (1976). To an Eppendorf tube containing 25 µg protein in 20 µL buffer, 2 µL Basta® (Hoechst, FRG) (diluted 1:10 in extraction buffer) and 3 µL ^{14}C-acetyl Coenzyme A (48.1 mCi / mmol, NEN) were added. The solution was incubated at 37°C for 30 min, in water at 100° C for 5 min and was centrifuged at 14000 rpm for 5 min. The supernatant was transferred to an Eppendorf tube and lyophilised in a Speed-Vac for 10-15 min. The dried extract was resuspended in 3 µL extraction buffer and spotted onto silica gel thin-layer chromatography plates, and chromatographed in a 3:2 mixture of 1-propanol and NH_4OH. ^{14}C-Acetylated PPT was detected by autoradiography.

DNA isolation: Genomic DNA was extracted from callus lines using a method based on that described by Weining and Langridge (1991). Callus (0.5-0.7 g) two weeks after being subcultured was ground to a fine powder in liquid nitrogen and suspended in 1 mL extraction buffer (0.1 M Tris-HCl buffer, pH 8.0, 10mM EDTA, 4% sarkosyl) in a 2 mL microfuge tube. An equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was added and the slurry was incubated on ice for 20 min. The aqueous phase was recovered

after centrifugation and the DNA precipitated in ethanol.

PCR labelling of probe DNA: A 0.87 kb fragment of plasmid pDM302 and a 1.89 kb fragment of plasmid p*ACT*1-D were labelled with Digoxigenin (DIG) by PCR using a PCR DIG Probe Synthesis Kit as described by manufacturers for DNA labelling. The PCR assay was carried out in a reaction volume of 50 µL. The complete PCR mixture contained 100 pg template DNA, pDM302 or p*ACT1*-D, 5 µL 10 x PCR amplification buffer, 5 µL 10 x DIG-nucleotide mixture, 0.75 µL (3.5 units μL^{-1}) Taq-polymerase, 2.5 µL (20 pmol μL^{-1}) each primer, to give a final concentration of 1 µM of each two specific primers. The primers used were:

GUS-1: 5' - GGT TGG GCA GGC CAG CGT ATC –3',

GUS-2: 5' - CCA ATG CCT AAA GAG AGG TTA – 3',

for labelling of the GUS gene from plasmid p*ACT*1-D and

PAT-1: 5' - ACC ATC GTC AAC CAC TAC ATC G –3',

PAT-2: 5' - GGC AGG CTG AAG TCC AGC TGC – 3',

for labelling of the *bar* gene from plasmid pDM302.

Amplifications were carried out on a Gene Amp 9600 PCR system and cycling parameters for the reaction were as follows: an initial incubation (denaturation) at 95°C for 2 min; 10 cycles at 95°C for 10 s (denaturation), 60°C for 30 s (annealing), 72°C for 2 min (elongation); 20 cycles at 95°C for 10 s (denaturation), 60°C for 30 s (annealing), 72°C for 2 s (elongation), and cycle elongation of 20 s for each cycle; 72°C for 7 min for the final elongation step.

Southern hybridisation analysis: For Southern hybridisation blots, 5-10 µg genomic DNA of transformed callus lines, 5-10 µg DNA from an untransformed callus line (negative control) and 5 and 20 pg plasmid DNA (positive control) were digested with *Eco*RI. The pDM302 plasmid (4.81 kb) is 10 times smaller than the *Triticum tauschii* genome (5 x 10^6 kb). Therefore, 5 and 20 pg plasmid DNA corresponds one and four copies of plasmid per genome copy in 5 µg *Triticum tauschii* DNA. The digested DNA samples were electrophoresed through 0.8% (w/v) agarose gel using 1 x TBE buffer at 110 V for 4 hrs and

transferred to positively charged (BM) Nylon Membrane (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. Filters were hybridised with a DIG-labelled *bar* or *GUS* probe. The labelled probes were detected using a DIG Luminescent Detection Kit (Boehringer Mannheim, Biochemica) as described by the manufacturer.

5.3. Results

5.3.1 Protoplast transformation

The histochemical assay showed that up to 0.005% of protoplasts which survived the PEG treatment expressed the *GUS* gene after 48 h (Table 5.1 and Figure 5.2). The number of cells showing transient GUS activity was not affected ($P < 0.1$) by DNA concentration.

Protoplast counts before and after PEG transformation showed that 19–27 % of protoplasts survived the PEG transformation (Table 5.2). There was no apparent genotype effect on the number of protoplasts that survived the PEG procedure when the two accessions, CPI 110813 and CPI 110649, were used.

The influence of the enzyme solution with or without the addition of Macerozyme, on protoplasts (from accession CPI 110649) undergoing PEG transformation and its effect on transient expression were studied. The results show that omitting Macerozyme from the enzyme solution increased ($P < 0.1$) the percentage of protoplasts that survived the transformation procedure (Table 5.3). The histochemical assay for *GUS* expression also show that isolation of protoplasts without Macerozyme resulted in a higher ($P < 0.1$) percentage of protoplasts expressing the *GUS* gene (Table 5.3).

Protoplast aggregation was observed after transformation and the aggregated protoplasts did not disperse during washing. Aggregated protoplasts burst during the first three days after embedding in agarose medium.

5.3.2 Microprojectile transformation

5.3.2.1 Optimisation of bombardment parameters

Bombardment of scutellar tissue of immature embryos: Results from the evaluation of various helium pressures, amounts of gold particles and Pre-and post-bombardment osmotic treatment on transient GUS expression after particle bombardment of scutellar tissue of immature embryos are shown in Table 5.4. These results show that only helium pressure had a significant effect ($P < 0.05$) on the number of blue spots (i.e. *GUS*

Table 5.1. The effect of different concentrations of DNA on transient GUS activity in PEG treated protoplasts of *Triticum tauschii* accession CPI 110649.

DNA amount ($\mu g\ mL^{-1}$)	50	75	100
% of cells with GUS activity	0.0010	0.0050	0.0047

Analysis of variance (2 degrees of freedom) shows no significant difference at the 10% level.

Table 5.2 Percentage of protoplast survived PEG-mediated transformation.

Accession	Protoplasts yield before transformation[1]	Protoplasts yield after transformation[1]	Protoplast survival[2] %
CPI 110649	3.9×10^6	1×10^6	26.6^a
CPI 110813	1.6×10^6	0.3×10^6	19.3^a

[1] Raw mean
[2] Adjusted mean
Means followed by the same superscript are not significantly difference at the 10% level.

Table 5.3 The effect of Macerozyme on the number of protoplasts after transformation of *Triticum tauschii* accession CPI 110649.

Enzymes	Protoplast yield before transformation[1]	Protoplast yield after transformation[1]	Mean[1] protoplast survival[1] %	Mean[1] cells with GUS activity[1] %
CMP	3.54×10^6	1.0×10^6	28.5^a	0.001^c
CP	2.80×10^6	1.4×10^6	51.1^b	0.0033^d

Means followed by the same superscript are not significantly different at the 10% level.
[1] Raw mean
CMP – Cellulase, Macerozyme, Pectolyase
CP – Cellulase, Pectolyase

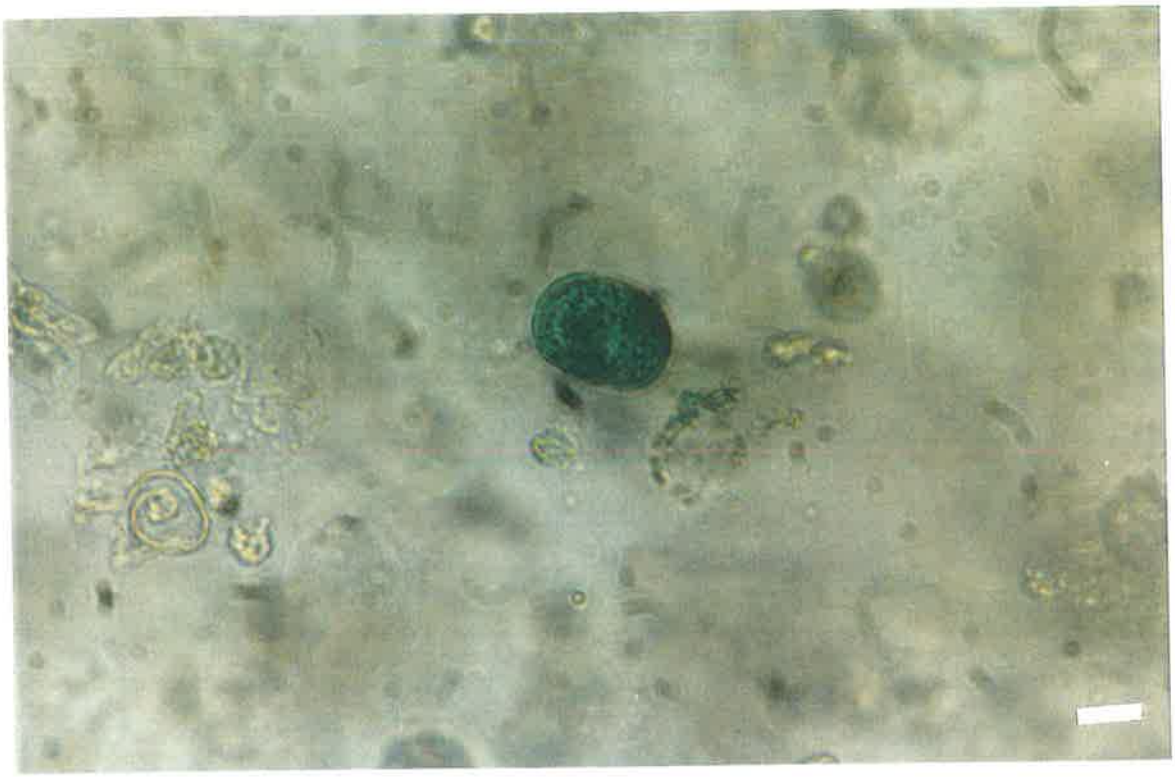


Figure 5.2 Transient GUS activity in a transformed cell of accession CPI 110813, 7 days after protoplast transformation with pAHC25. Scale bar = 10 μm.

expressing cells) per embryo. A higher ($P < 0.05$) number of blue spots was produced with helium pressure of 1300 psi. It was also found that, of the three pressures, only 1100 and 1300 differed ($P < 0.05$) in the number of blue spots produced per embryo (Table 5.4). The level of transient expression amongst embryos was from zero to 100 blue spots per embryo (Figure 5.3).

Microparticle amount (125-500 µg per bombardment) had no effect ($P < 0.05$) on the number of blue spots per embryo (Table 5.4). However, callus was not produced from bombarded immature embryos using pressures between 900 and 1300 psi and particle amounts between 125 and 500 µg per bombardment. In these instances, targeted tissues became brownish after 1-2 weeks (Figure 5.4), as a result to damage caused by bombardment. Subsequent experiments were therefore conducted with the lower gold particle amounts of 60 and 120 µg per bombardment. The number of embryos damaged by these two particle amounts is shown in Table 5.5 and the proportion of total numbers of damaged embryos are plotted by particle amount and accession in Figure 5.5. These results show that particle amount, independent of accession, had a significant influence (χ^2 = 128.181, df = 12, sig. = 0.000) on tissue damage and on the number of scutellar tissues forming callus. Accession was also a significant treatment effect (χ^2 = 13,725, df = 1, sig. = 0.000). The results in Figure 5.5 show that although the proportion of damaged tissues increased with particle amounts for both accessions, the effect of particle amount was stronger on accession CPI 110649 than on CPI 110810. There was a highly significant difference between particle amounts of 60 and 120 µg per bombardment for both accession CPI 110649 (χ^2 = 121.92, df = 1, sig. = 0.000) and CPI 110810 (χ^2 = 23.96, df = 1, sig. = 0.000). The number of scutellar tissues forming callus was significantly (χ^2 = 52.11, df = 1, sig. = 0.000) higher for accession CPI 110649 than for CPI 110810 at particle amount of 60 µg per bombardment. However, there were no significant differences in the number of scutellar tissues forming callus between the two accessions at particle amount of 120 µg per bombardment. The callus produced in all these experiments was neither embryogenic nor transgenic.

Table 5.4 The number of blue spots (showing transient *GUS* expression) per scutellum of an immature embryo of *Triticum tauschii* accession CPI 110649 using different gas pressures, particle amounts and with and without pre- and post-bombardment osmotic treatments.

	+ Osmoticum				- Osmoticum			
Pressure (PSI)	Particle amounts µg/bombardment			Mean	Particle amounts µg /bombardment			Mean
	500	250	125		500	250	125	
900	3.5	2.8	3.0	3.1	2.6	2.8	3.5	3.0
1100	2.7	2.7	2.4	2.6	2.8	3.2	2.7	2.8
1300	3.2	3.7	4.1	3.6	3.2	3.7	3.2	3.2
Mean	3.1	3.1	3.1		2.9	3.2	2.9	
Mean			3.1^{b}				3.0^{a}	

Pressure (PSI)	Grand mean
900	3.0^{ab}
1100	2.7^{a}
1300	3.4^{b}

Particle amounts µg/bombardment	Grand mean
500	3.0^{a}
250	3.1^{a}
125	3.0^{a}

The data was transformed and analysed on a log scale by the LSD procedure.

Significant differences were calculated by analyses of transformed data. Three-way analysis of variance was used. Means followed by the same superscript are not significantly different at the 5% level.

Each value is the average of three replications.

Figure 5.3 Transient *GUS* expression in scutellar tissues of accession CPI 110649 bombarded with plasmid pAHC25. Scale bar = 0.1 mm.

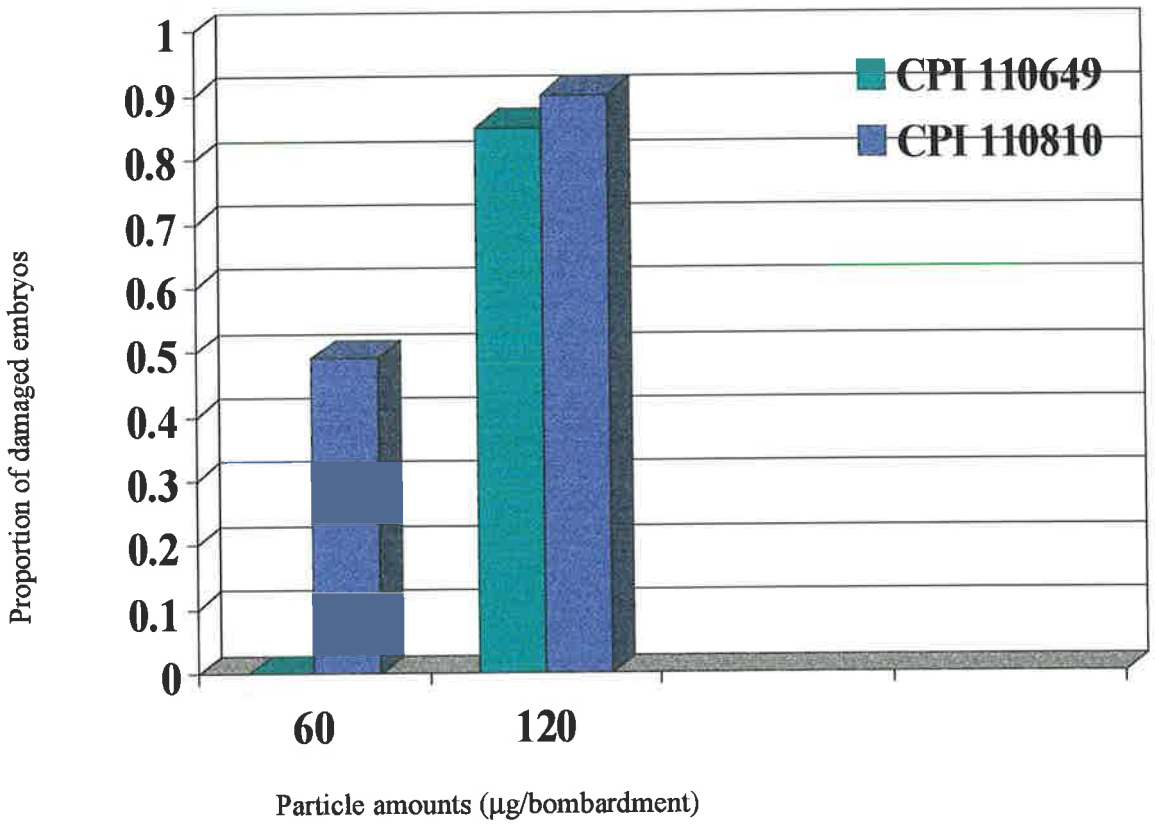
Figure 5.4 A non-bombarded scutellar tissue (right) and a damaged scutellar tissue of accession CPI 110649 bombarded with a pressure of 1300 psi and 125 µg/bombardment particles coated with pAHC25. Scale bar = 1 mm.

Table 5.5 Effect of two particle amounts on two accessions of *Triticum tauschii*.

Accessions	Particle concentration (µg/bombardment)	damaged embryos		Total
		undamaged	damaged	
CPI 110649	60	62	0	62
CPI 110649	120	10	58	68
Total		72	58	130
CPI 110810	60	31	30	61
CPI 110810	120	5	47	52
Total		36	77	113

The proportion of total numbers of damaged embryos by particle amounts and accessions are presented in Figure 5.5.

Figure 5.5 The proportion of damaged embryos (accessions CPI 110810 and CPI 110649) caused by two different particle amounts.



Pre-and post-bombardment osmotic conditioning at different helium pressure and particle amounts did not affect transient *GUS* expression of bombarded immature embryos (Table 5.4).

Bombardment of suspension cultures: Helium pressures, particle amounts and pre- and post-bombardment osmoticum treatments had significant effects ($P < 0.01$) on the overall number of blue spots (i.e. *GUS* expressing cells) in bombarded suspension cultures (Table 5.6). A higher ($P < 0.05$) number of blue spots was obtained using a helium pressure of 1300 and 1800psi compared with pressures of 900, 1100 and 1500 psi (Table 5.6, Figure 5.6). Particle amounts of 125 and 250 μg per bombardment gave a higher ($P < 0.05$) number of blue spots than 500 μg per bombardment (Table 5.6). Pre- and post-bombardment osmotic treatments of suspensions also increased ($P < 0.01$) the number of GUS expressing cells from 1.5 to 3.4 (Table 5.6).

There was also an interaction ($P < 0.01$) between helium pressure and pre- and post-bombardment osmotic conditioning and between particle amounts and pre- and post-bombardment osmotic conditioning for the number of blue spots (Table 5.6). The number of blue spots increased with the increase of helium pressure when pre- and post-bombardment osmotic conditioning was not applied, whereas there was no directional effects on the number of blue spots at different helium pressures when pre- and post-bombardment osmotic conditioning was used. Similarly, the number of blue spots decreased with an increased amount of particles when pre- and post-bombardment osmotic conditioning was not applied, whereas there was no directional effect on the number of blue spots at different amount of particles when pre- and post-bombardment osmotic conditioning was used.

5.3.2.2 Stable transformation

Suspension cell lines of two accessions (CPI 110813 and CPI 110649) were chosen for stable transformation experiments, because of their high regenerative capacity.

Table 5.6 The number of blue spots (transient GUS expression) per 200 mg of cell aggregates from suspension cultures of *Triticum tauschii* accession CPI 110649 bombarded using different gas pressures and particle concentrations and with and without pre- and post-bombardment osmotic treatments.

	+ Osmoticum				- Osmoticum			
Pressure (PSI)	Particle amounts µg/bombardment			Mean	Particle amounts µg /bombardment			Mean
	500	250	125		500	250	125	
900	3.6	3.0	3.9	3.5	0.4	0.6	1.4	0.8
1100	3.1	3.6	3.7	3.4	0.8	1.5	1.8	1.4
1300	3.9	3.5	3.9	3.7	1.1	1.8	2.2	1.7
1500	2.8	3.0	3.7	3.2	1.3	1.3	2.3	1.6
1800	3.8	3.3	2.8	3.3	1.5	2.3	2.5	2.1
Mean	3.4	3.3	3.6		1.0	1.5	2.0	
Mean			3.4^{b}				1.5^{a}	

Pressure (PSI)	Transformed grand mean
900	2.1^{a}
1100	2.4^{ab}
1300	2.7^{b}
1500	2.4^{ab}
1800	2.7^{b}

Particle amounts µg/bombardment	Transformed grand mean
500	2.8^{b}
250	2.4^{a}
125	2.2^{a}

The data was transformed and analysed on a log Scale by the LSD procedure.

Significant differences were calculated by analyses of transformed data. Three-way analysis of variance was used. Means followed by the same superscript are not significantly different at the 5% level.

Each value is the average of three replications.

Figure 5.6 β-Glucuronidase (*GUS*)-expressing blue cells 4 days after bombardment of suspension culture of accession CPI 110649 with microprojectiles coated with plasmid pHC25. Scale bar = 2 mm.

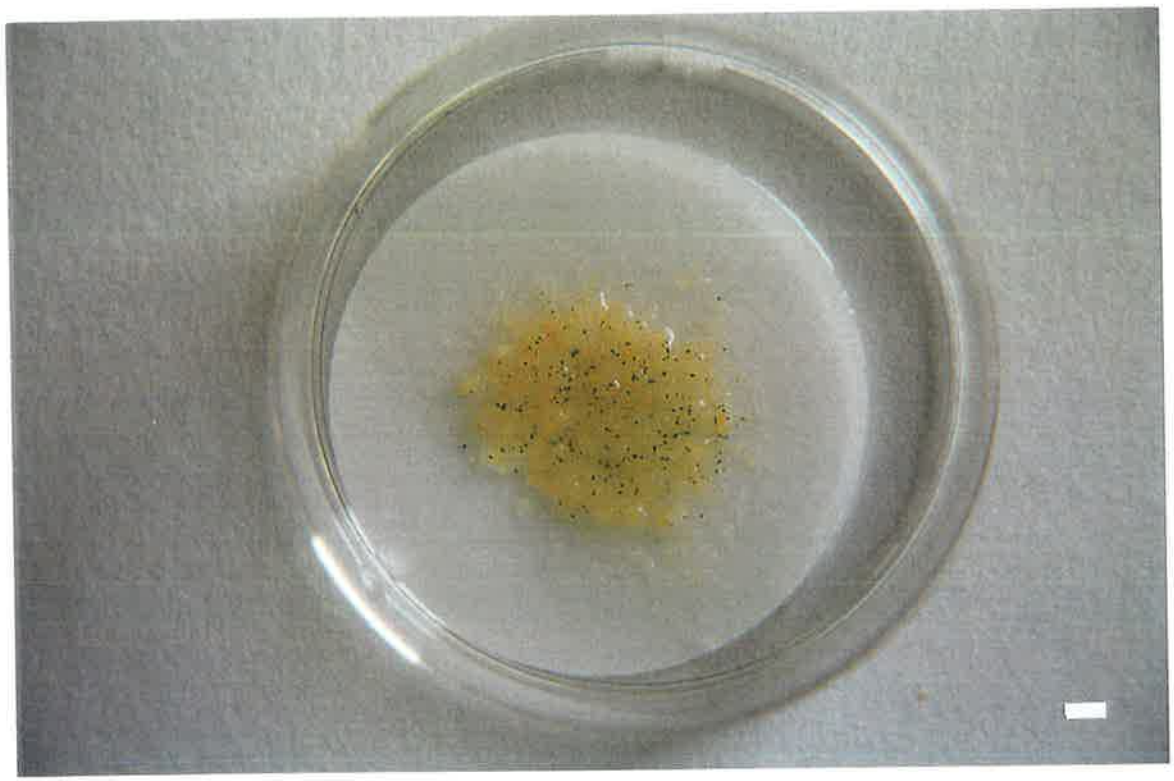


Figure 5.7 β-glucuronidase (GUS)-expressing blue cells evaluated 4 days after bombardment of suspension culture of accession with microprojectile coated plasmid pDM302 and p*Act1*-D. Scale bar = 5 mm.

Histochemical assays two days after bombardment with both *bar* and *GUS* genes using co-transformation with two plasmids pDM302 and p*ACT*1-D showed high transient expression of the *GUS* gene, with around 200 blue cells from accession CPI 110649 and 800 from accession CPI 110813 (Figure 5.7).

Critical kill concentration experiments performed with non-bombarded suspension cultures of *Triticum tauschii* accession CPI 110649 plated on media containing 1 to 4 mg L^{-1} bialaphos indicated that 4 weeks selection with 3 mg L^{-1} bialaphos was enough to inhibit the further growth of non-transformed cell clumps (Figure 5.8).

Colonies resistant to bialaphos were identified 4-6 weeks after bombardment. No colonies survived in the control (non-bombarded) treatment. The resistant colonies were removed from the filters and cultured as individual cell lines on a medium containing 3 mg L^{-1} bialaphos. Seven resistant cell lines survived the herbicide regime after 8 weeks (Figure 5.9). Seven of the lines tested PAT positive, three from accession CPI 110649 and four from accession CPI 110813 (Figure 5.10). One of the seven resistant lines showed GUS activity 12 weeks after bombardment (Figure 5.11).

The resistant lines produced somatic embryoids, but when these putative transgenic callus were transferred to regeneration medium, they differentiated into roots and/or albino shoots (Figure 5.12). However, normal plants regenerated from the control, which were non-bombarded callus from the same suspension culture.

Integration of the pDM302, containing the *bar* gene, into the genome of transformed callus lines was demonstrated by Southern hybridisation (Figure 5.13). Following digestion of the genomic DNA with *Eco*RI and hybridisation with a DIG-labelled probe, the expected 0.87 kb fragment appeared in all callus lines (C1-C7). All lines analysed had additional hybridising bands. No bands were detected in any of the non-transformed callus lines (NC). For copy number estimation, 5 and 20 pg of the plasmid pDM302 correspond to one copy (a) and four copies (b) of plasmid per *Triticum tauschii* genome respectively. Estimation of copy number was based on the intensity and number of bands. Copy number ranged from one (C1) to several copies (C2-C7).

There was sufficient genomic DNA for only one restriction endonuclease digestion.

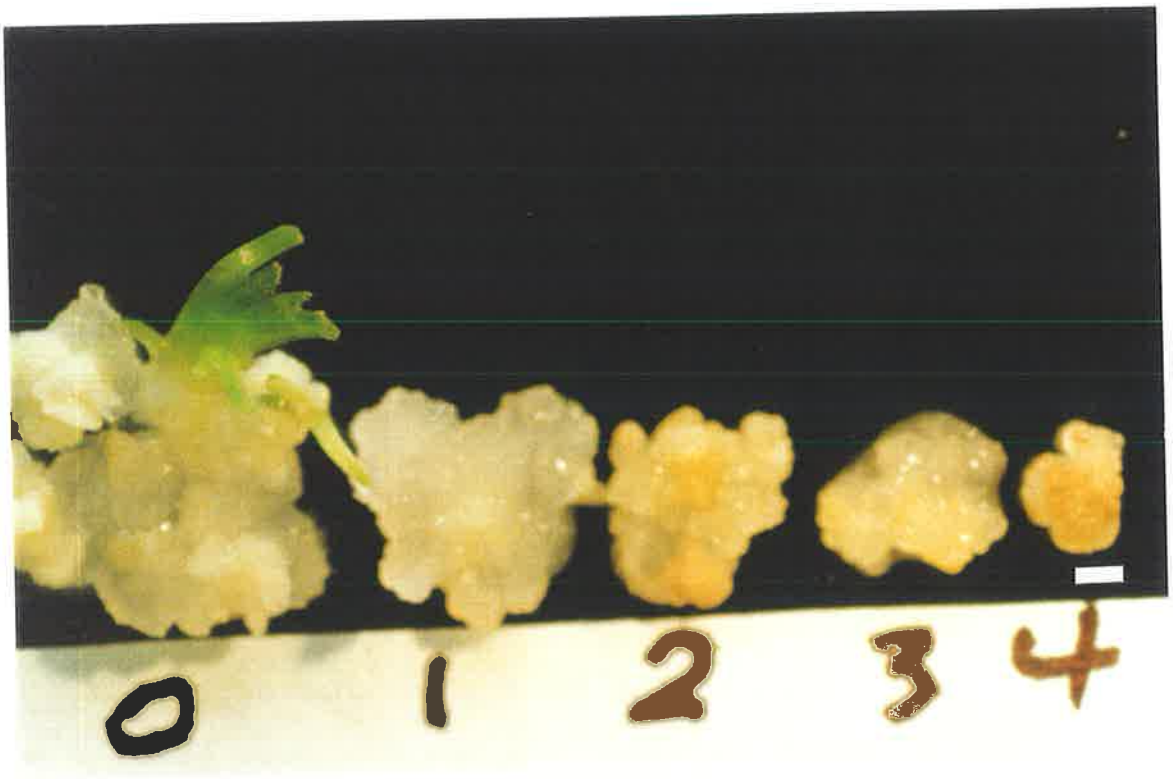


Figure 5.8 The effect of different concentrations of bialaphos (1-4 mg L^{-1}) on a non-bombarded suspension culture of accession CPI 110469. Scale bar = 1 mm.

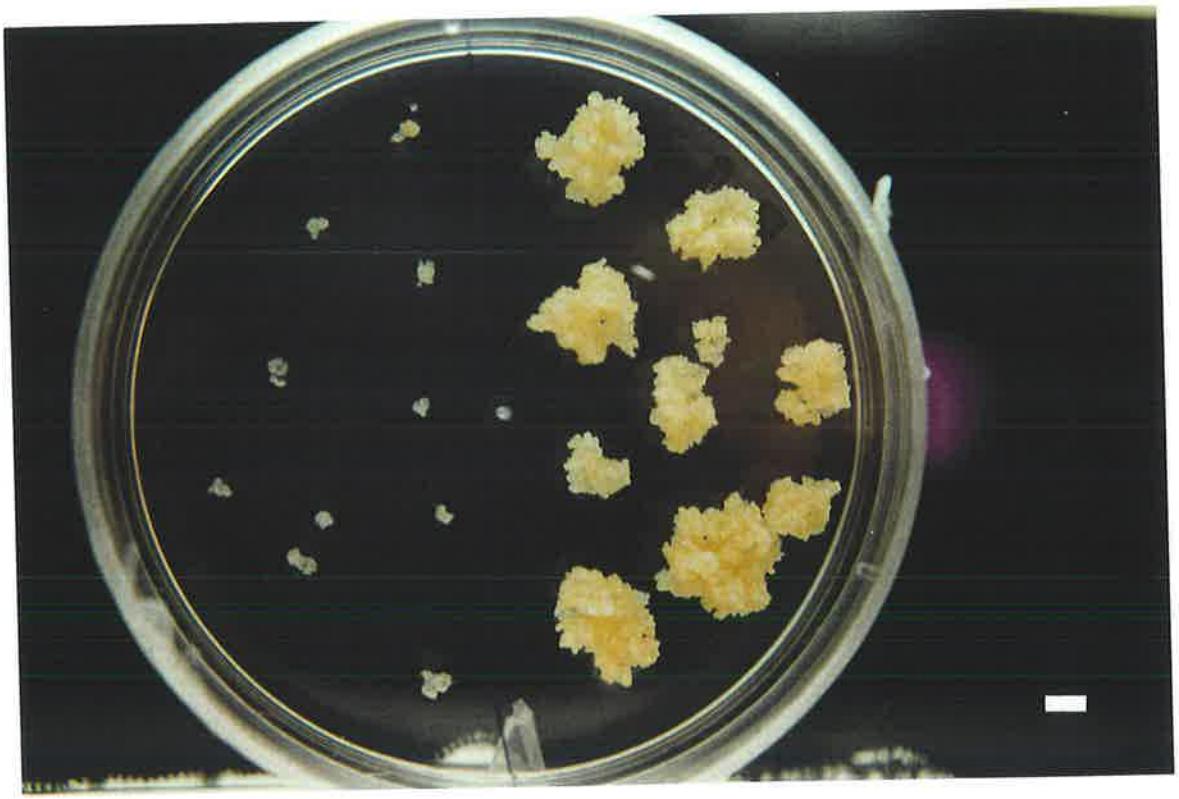


Figure 5.9 A bialaphos resistant line selected on 3 mg L^{-1} bialaphos (right) non-bombarded cell clumps from suspension culture (left) after 4 weeks been growing on a medium containing 3 mg L^{-1} bialaphos. Scale bar = 5 mm.

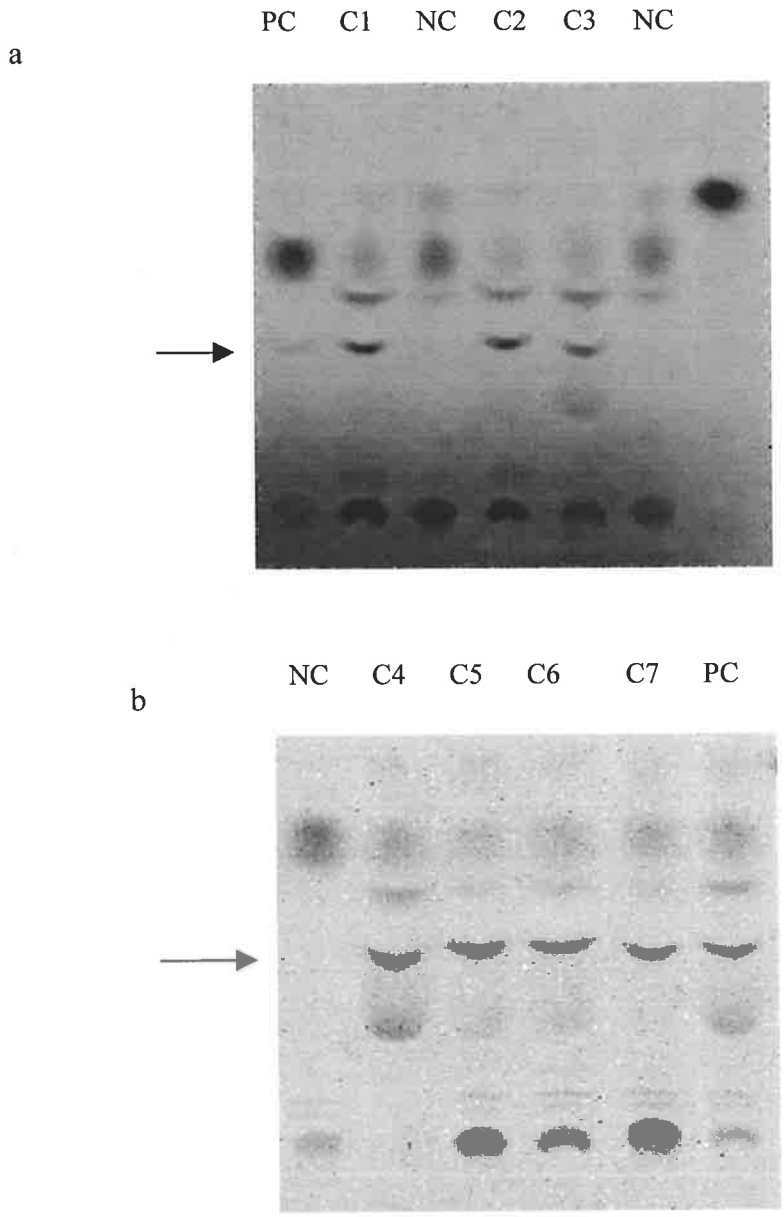


Figure 5.10 PAT activity in bialaphos-resistant callus lines (C1-C7). The band corresponding to acetylated PPT is marked by an arrow; that band is absent in non-bombarded callus (NC). A transgenic barley callus line (R. Singh and G.B. Fincher, unpublished data) in (a) and PAT positive callus line C3 in (b) were included as positive controls (PC). Callus lines C1, C2, C6, and C7 are from *Triticum tauschii* accession CPI110813 and C3, C4 and C5 are from accession CPI110649.

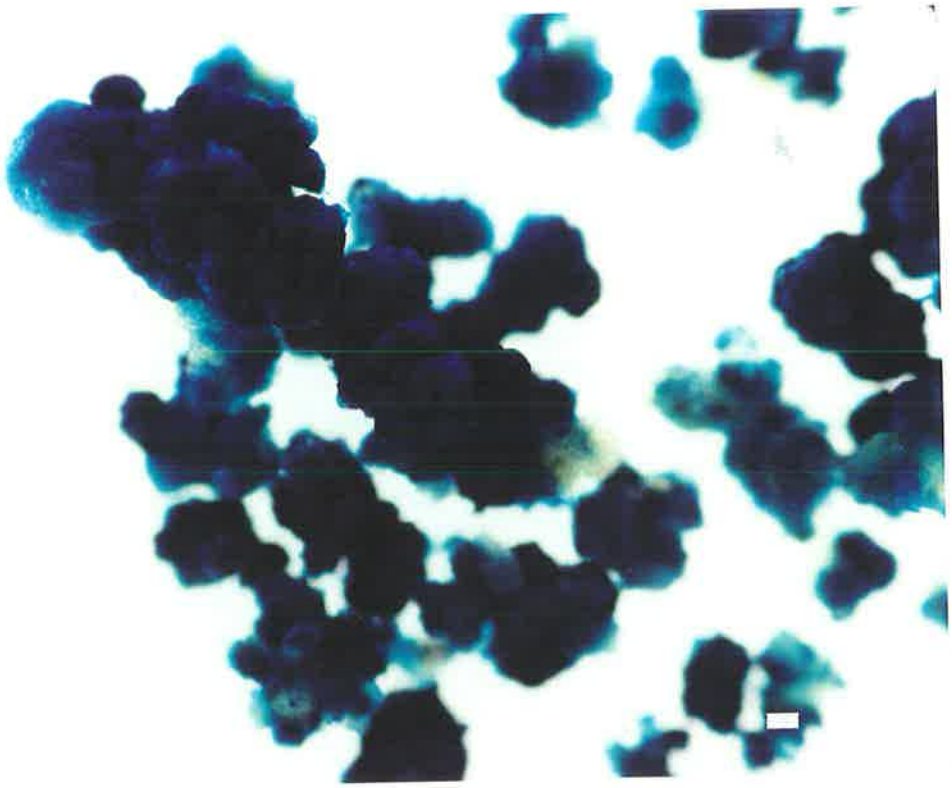


Figure 5.11 Histochemical determination of GUS activity in bialaphos-resistant cell clumps of accession CPI 110649 showing GUS activity 12 weeks after bombardment with plasmid pDM302 and p*ACT* 1-D. Scale bar = 1 mm.

Figure 5.12 Germination of albino shoot from bialaphos resistant cell clumps of accession CPI 110649. Scale bar = 1 mm.

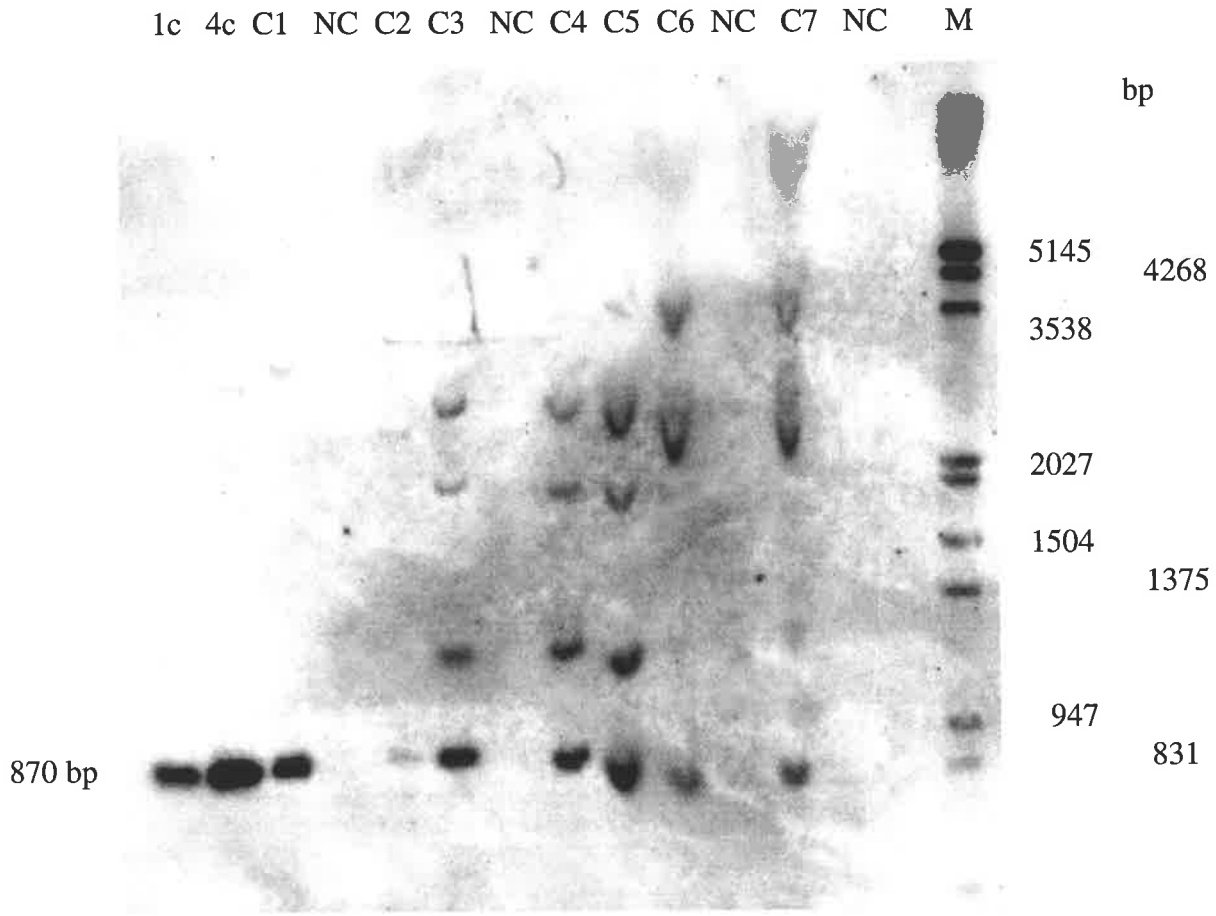


Fig 5.13 Southern blot analysis of DNA isolated from transgenic *Triticum tauschii* callus lines demonstrating integration of the *bar* gene. Genomic DNA from bialaphos-resistant callus lines (C1-C7) and untransformed callus line (NC) (negative control) was digested with *EcoRI*. The blot was hybridised to a DIG labelled *bar* fragment. Lanes designated as1c and 4c contain 5 pg and 20 pg of *EcoRI* digested plasmid pDM302 correspond to one copy (1c) and four copy (4c) of plasmid per *Triticum tauschii* genome respectively. M shows the molecular weight marker (Digoxigenin-labeled marker, Boehringer Mannheim) as size standard. Callus lines C1, C2, C6, and C7 are from *Triticum tauschii* accession CPI110813 and C3, C4 and C5 are from accession CPI110649. The reason why samples on the right ran faster than the others is probably because of impurities in the DNA.

Additional genomic DNA could not be extracted from transgenic callus because callus cultures became contaminated with bacterial. The same filter-bound *Eco*RI-cut DNA was used to determine integration of plasmid p*ACT*1-D. The *bar* probe was stripped from the filter and the filter was hybridised with a DIG-labelled *GUS* probe. All seven transgenic callus lines (C1-C7) contained hybridising bands (Figure 5.14). DNA from non-transformed callus lines (NC) showed no hybridisation to the *GUS* coding region fragment used as probe. All hebicide-resistant cell lines were shown to contain both *bar* and *GUS* genes.

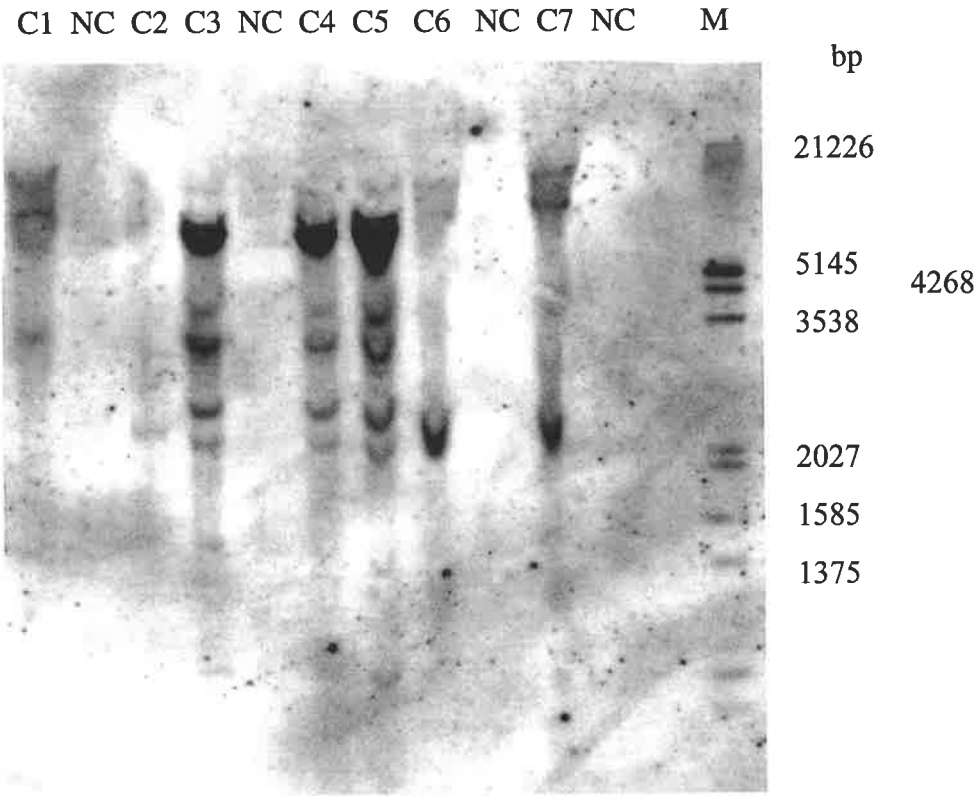


Fig 5.14 Southern blot analysis of DNA isolated from transgenic *Triticum tauschii* callus lines demonstrating integration of the plasmid p*ACT*1-D containing *GUS* gene. Genomic DNA from bialaphos-resistant callus lines (C1-C7) and untransformed callus line (NC) (negative control) was digested with *EcoRI*. The blot was hybridised to a DIG labelled *GUS* fragment. M shows the molecular weight marker (Digoxigenin-labelled marker, Boehringer Mannheim) as size standard. Callus lines C1, C2, C6, and C7 are from *Triticum tauschii* accession CPI110813 and C3, C4 and C5 are from accession CPI 110649.

5.4 Discussion

5.4.1 PEG mediated transformation:

Although PEG-mediated protoplast transformation has been attempted in many cereal species, there are very few reports of the transient expression frequency obtained using this method. Lee *et al.* (1990) compared two protoplast transformation techniques, electroporation and PEG, in wheat and found similar levels of expression with both techniques. In hexaploid wheat, the frequency of transient expression of foreign genes in protoplasts using electroporation was reported to be 1 x 10^{-5} by Lee *et al.* (1990) and 9 x 10^{-3} by He *et al.* (1994). Although the frequency of 1-5 x 10^{-5} obtained with *Triticum tauschii* (Table 5.1) in this study compares favourably with that obtained by Lee *et al.* (1990) with hexaploid wheat, it is lower than that obtained by He *et al.* (1994). The low frequency of transient expression in our study compared with that obtained by He *et al.* (1994) may be due to the fact that most of the *Triticum tauschii* protoplasts aggregated after PEG treatment and subsequently burst after being embedded in agarose medium.

The most commonly used enzyme solutions to isolate protoplasts from cereal suspension cultures contain Cellulase RS and Pectolyase Y23, either supplemented with Macerozyme R 10 (Lazzeri *et al.* 1991; Jähne *et al.* 1991b; Pauk *et al.* 1994) or without Macerozyme (He *et al.* 1992; Qiao *et al.* 1992; Ahmed and Sagi *et al.* 1993). Although the combination of these three enzymes gave high yield of protoplasts from suspension cultures of *Triticum tauschii* (Section 4.3.2), the omission of Macerozyme from the enzyme solution used to isolate protoplasts increased the percentage of protoplasts surviving the transformation treatment from 28% to 51% and also had a positive effect on transient GUS activity (Table 5.3). This finding is consistent with that of Krautwig *et al.* (1994) who observed that the use of Macerozyme resulted in reduced GUS activity in protoplasts. In our study, the percentage (51%) (Table 5.3) of protoplasts surviving the transformation procedure may be compared with the value of 60% obtained in hexaploid wheat by He *et al.* (1994).

Despite using many different culture methods, including the use of feeder cells, cell

division did not occur after PEG treatment. This was not related to the source of protoplasts because protoplasts from the original cell line divided if they were not treated with PEG. In our study, most of the protoplasts aggregated after PEG treatment and subsequently burst after being embedded in agarose medium. Similarly, Lazzeri *et al.* (1991) reported that PEG treatment of barley protoplasts produced very stable protoplast aggregates which were not able to divide and degenerated after they were embedded in agarose medium. It is possible that in our study the density of protoplasts after degeneration of protoplast aggregates was too low for cell division to occur.

Further studies are required to investigate which factors are necessary to develop a stable transformation system for *Triticum tauschii* using protoplasts. The most important barrier to overcome is protoplast aggregation and the subsequent degeneration of protoplasts. Other variables such as plasmid construct, form of plasmid DNA, plating methods and concentration of agarose will then need to optimised.

5.4.2 Microprojectile bombardment

Microprojectile bombardment transformation conditions were evaluated to obtain efficient delivery of DNA into *Triticum tauschii* cells. The efficiency of DNA delivery into intact cells was determined by measuring the number of cells which transiently expressed the *GUS* gene. The level of transient *GUS* expression (up to 100 blue spots per embryo) in scutellar tissue of immature embryos and (up to 800 per plate) suspension cultures of *Triticum tauschii* in our study (Figures 5.3 and 5.7) is comparable to that obtained for scutellar tissue in wheat, 80 and 110 blue spots per embryo (Takumi and Shimada 1996); Altpeter *et al.* 1996), barley 100 blue spot per embryo (Wan and Lemaux 1994) and suspension cultures in rice, 684 and 1152 blue spots per plate (Jain *et al.* 1996; Zhang *et al.* 1996), in maize, 791 blue spots per plate (Taylor *et al.* 1993). Our results indicate that *Triticum tauschii* is as responsive as the major cereal species to delivery of DNA into intact cells.

In our study, particle amount affected callus formation in scutellar tissue of *Triticum*

tauschii. Callus formation ceased when particle amounts greater than 125 µg per bombardment were used (Figure 5.4). At a lower particle amount (60 µg per bombardment), the number of scutella forming callus increased significantly (Table 5.5, Figure 5.5). Becker *et al.* (1994) and Brettschneider *et al.* (1997) also reported that a reduction in particle amount from 116 to 29-30 µg per bombardment was an important step in minimising tissue damage and increasing embryogenic callus formation in wheat and maize. One reason why transgenic cell lines were not produced from bombarded scutellar tissue of *Triticum tauschii* is that high particle amounts used for bombardment. Brettschneider *et al.* (1997) reported that helium pressures had a large effect on stable transformation when using low particle amounts. This may indicate the necessity of testing different helium pressures and particle amount lower than 60 µg per bombardment (perhaps 30 µg per bombardment) to investigate stable transformation of *Triticum tauschii* using scutellar tissues.

Transgenic cell lines were obtained following microprojectile bombardment of embryogenic suspension cultures of *Triticum tauschii*, using a helium pressure of 1300 psi and a particle amount of 125 µg per bombardment (Figure 5.13 and 5.14). The decreased regeneration capacity and the exclusive production of albino shoots from the transgenic cell lines (Figure 5.12), may be due to several factors, including tissue damage caused by the physical bombardment parameters. Physical parameters such as the helium pressure used to accelerate particles, distance to target tissue and particle amount have been shown to reduce or abolish regeneration capacity in cereals such as barley (Koprek *et al.* 1996), wheat (Perl *et al.* 1992; Becker *et al.* 1994; Altpeter *et al.* 1996), maize (Brettschneider *et al.* 1997), and pearl millet (Taylor and Vasil 1991). Becker *et al.* (1994) also reported that faster growing non-regenerable callus was produced in wheat when particle amounts higher than 29 µg per bombardment was used. These results indicate that the 125 µg per bombardment particles used in our study may have affected regeneration capacity of the bombarded suspension cultures. Therefore, stepwise optimisation of transformation parameters for *Triticum tauschii* using particle amounts lower than 125 µg per bombardment will be important for future production of transgenic plants.

The presence of bialaphos (3 mg L^{-1}) in the culture medium is another factor which may influence regeneration capacity and cause production of albino transgenic shoots. Jain *et al.* (1996) reported that shoot formation from putatively transformed callus lines of rice was limited by the presence of ammonium glufosinate in the medium. It is possible that bialaphos and the ammonia released by non-transformed cells during the selection (Tachibana *et al.* 1986) caused a reduction in regeneration capacity and increase in the production of albino shoots.

The osmotic treatment of explants, which was shown to improve transformation efficiency of maize, (Vain *et al.* 1993a), was also tested in the present study. Pre- and post-bombardment osmotic treatments are believed to induce plasmolysis of cells so that fewer cells are severely damaged by the penetrating particles (Armaleo *et al.* 1990; Vain *et al.* 1993a). In our experiments, osmotic treatment did not significantly affect transient *GUS* expression in scutellar tissues after bombardment. The results agree with those for wheat (Altpeter *et al.* 1996) and maize (Brettschneider *et al.* 1997).

In contrast to explant material, enhancement of transient expression in suspension cells by osmotic treatment of target tissues pre- and post-bombardment has been achieved using different osmotically active compounds (sucrose, mannitol, sorbitol and maltose) at various concentrations from 0.25 M to 0.7 M in maize (Vain *et al.* 1993a), forage grasses (Spangenberg *et al.* 1995) and rice (Zhang *et al.* 1996; Jain *et al.* 1996; Nandadeva *et al.* 1999). A corresponding increase in the frequency of stable transformation events was also shown by some of these workers (Vain *et al.* 1993a; Jain *et al.* 1996). The increase in transient expression of up to 8.5 fold (back transformed mean, Table 5.5) obtained in our study with osmotic treatment of suspension cultures is higher than that reported by Vain *et al.* (1993a) in maize and by Zhang *et al.* (1996) in rice, but is similar to that reported by Jain *et al.* (1996) in rice. However, the conditions optimised for transient assays may not be optimal for production of transgenic plants from the same cell type (Nandadeva *et al.* 1999).

In an attempt to obtain stable transformants, suspension cultures were bombarded with particles coated with two plasmids, allowing us to study the co-integration of two

separate gene constructs although selection was only for one. Such a strategy is useful for introduction of genes of interest because progeny containing the gene of interest but lacking a selectable marker gene can be created (Komari *et al.* 1996). This overcomes some of the public concerns of releasing transgenic plants with herbicide resistance or antibiotic resistance genes. The 100% co-transformation frequency obtained in this study is higher than that obtained in maize (77%, Gordon-Kamm *et al.* 1990) and in barley (85%, Wan and Lemaux 1994). However, the number of transformants (seven lines) obtained in our study, is not high enough to accurately predict the frequency of co-transformation of unlinked genes in large-scale experiments. A high frequency of co-transformation is crucial for the introduction of agronomically important genes into *Triticum tauschii*, so that a sufficient number of transgenic plants with the gene of interest, but lacking the selectable marker gene, can be selected.

Despite the fact that Southern analysis indicated the presence of the *GUS* gene in most of the transgenic callus lines, *GUS* activity was detected histochemically in only one callus line of *Triticum tauschii*. The lack of *GUS* gene expression may be because of DNA methylation, which can occur after a sustained period of culture, preventing expression of transgenes (Klein *et al.* 1990; Bochardt *et al.* 1992). Similar observations have been reported by Casas *et al.* (1993) for sorghum.

In summary, both the protoplast transformation and microprojectile bombardment procedures described in this study resulted in the production of a high level of transient gene expression and transgenic callus lines were produced from *Triticum tauschii* using microprojectile bombardment. However, further work is required to develop methods for the production of fertile transgenic *Triticum tauschii* plants.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Summary

The first objective of the work described in this thesis was to develop protocols for cell and tissue culture of *Triticum tauschii*. The second objective was to examine methods for the regeneration of fertile *Triticum tauschii* plants from the tissue-cultured cells. The production of nodular embryogenic callus with sustained competence for regeneration was achieved by culturing 0.5-1.0 mm immature embryos on MS medium supplemented with 24 mg L^{-1} Dicamba® and 725 mg L^{-1} L-proline. This type of embryogenic callus proved to be the most suitable for initiation of suspension cultures. Supplementation of the initiation medium was important for rapid growth of embryogenic suspension cultures. Fine embryogenic suspension cultures were produced in less than six months and the embryogenic capacity of suspension cultures was maintained over three years. Fertile plants with normal morphology were regenerated from suspensions maintained in culture for more than one year.

The third major objective was to transform *Triticum tauschii* using protoplast and microprojectile-mediated transformation systems. The culture system and conditions described in this thesis resulted in high yields of protoplasts from which fertile plants could be regenerated. Transient *GUS* expression was achieved both by treating protoplasts with PEG in the presence of DNA carrying the *GUS* gene, and by microprojectile bombardment of suspension cultures and the scutellar tissue of immature embryos, with similar DNA constructs. Stably transformed cell lines were produced via microprojectile bombardment, but no transgenic plants could be regenerated from the transformed cell lines.

Future Directions

In this research, for first time, *in vitro* systems were developed for the production of fertile plants from embryogenic callus, suspension cultures and protoplast cultures of *Triticum tauschii*. However, furture experiments should be designed to further investigate the effects of media on the production of embryogenic nodular callus, particularly at low Dicamba® and high L-proline concentrations. Any future investigation into plant

regeneration from *Triticum tauschii* protoplasts will need to focus on the fertility of those regenerants. The problem with infertility of plants regenerated from protoplasts isolated from suspension cultures is a common problem amongst cereal species but possible reasons for the infertility are still not clear. It may be useful to try scutellar tissue of immature embryos or primary callus as a source of protoplasts. There are reports of the regeneration of plants from protoplasts isolated directly from scutellar tissue of rice and from primary callus of barley (Ghosh-Biswas *et al.* 1994; Stöldt *et al.* 1996) in which all regenerated plants were fertile.

The demonstration that plasmid DNA can be effectively delivered into *Triticum tauschii* protoplasts and expressed transiently could be used to develop stable transformation systems. However, further work will be required to develop transgenic *Triticum tauschii* plants with this approach. The problem of protoplast aggregation after PEG treatment might be alleviated by modifying PEG concentrations and protoplast plating methods. Factors influencing stable transformation frequency, such as the type of promoter construct, the form of the plasmid, PEG concentration and protoplast source will also need to be investigated. Despite these possibilities, protoplast transformation may not be the best method to transform *Triticum tauschii*, because the procedures involved in the production of suspension cultures and in the isolation of protoplasts are laborious and time-consuming. The problem in infertility of protoplast-derived regenerants also makes this technology inefficient. Furthermore, cytogenetical changes, such as chromosome doubling might occur during the prolonged period required for the isolation and culture of protoplasts (Karp *et al.* 1987; Guiderdoni and Chaïr 1992; Yamagishi *et al.* 1996). The use of transformation methods such as microprojectile bombardment that do not require protoplasts may lead to more efficient production of fertile transgenic plants due to the reduced tissue culture times.

The development of transgenic cell lines with microprojectile bombardment in this research program has brought us to within one step of producing transgenic *Triticum tauschii* plants. Experiments could be performed to expand and complete the optimisation

of bombardment parameters, in particular particle density and helium pressure. There is some evidence that particle densities higher than 30 µg/bombardment cause tissue damage and the formation of non-embryogenic callus (Becker *et al.* 1994; Altpeter *et al.* 1996; Brettschneider *et al.* 1997). At particle densities less than 30 µg/bombardment, optimisation of helium pressure might improve the efficiency of stable transformation.

Additional studies could be focused on using a range of osmotica at various concentrations to test the effect of osmotic treatment on the frequency of stable transformation events. More recently, the role of plasmid DNA structure on integrative transformation frequency has been investigated in wheat (Uzé *et al.* 1999). Transformation frequencies up to 14% were achieved on one occasion when linearized, single-stranded DNA was used. However, the average transformation frequency did not appear to be higher than with other forms of DNA. The use of a single-stranded plasmid in rice resulted in higher stable transformation events than double-stranded DNA (Nandadeva *et al.* 1999). It could be useful therefore to study the effect of different plasmid DNA structures on the frequency of transformation in *Triticum tauschii*.

Finally, in any future investigation into transformation of *Triticum tauschii*, it may be more efficient to focus on target tissues such as scutellar tissue of immature or mature embryos, or primary scutellar callus from mature grains. This would reduce the time required for the regeneration of transgenic plants and eliminate the need for the establishment of suspension cultures.

Another approach would be to investigate *Agrobacterium*-mediated DNA delivery as an alternative transformation method which has not yet been tested with *Triticum tauschii*. This transformation method has been successful for barley, wheat and rice (Section 1.2).

When procedures for the production of transgenic plants are established, the next important step will be the transformation of *Triticum tauschii* with agronomically useful genes such as disease resistance and with genes that improve quality. Several useful genes which are important for the agronomic improvement of bread wheat are available (see Section 1.5).

In conclusion, it should be acknowledged that the original rationale for this study was based on the technical difficulties associated with transformation of wheat. It was proposed that *Triticum tauschii* could be an alternative route for the integration of transgenes into wheat. However, during the course of the project two highly efficient procedures for wheat transformation was published (Uzé *et al.* 1999; Zhang *et al.* 2000). Furthermore, we did not successfully obtain transgenic *Triticum tauschii* plants in the work described in this thesis. Nevertheless, an efficient transformation system for *Triticum tauschii* could still be useful, because this species contains agronomically important genes (Section 1.1). Thus, genetic transformation of *Triticum tauschii* could also be used to introduce transposable elements for functional analysis of the agronomically important genes through gene knock-out experiments (Takumi 1996; Wisman and Hatmann 1998; Nakagawa *et al.* 1999).

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APPENDICES

Appendix 1

Designation and origins of *Triticum tauschii* accessions used in this study.

Accession no.	Country of origin	Variety[A]
CPI 110718	Pakistan	*anathera*
CPI 110809	Iran	*meyeri/typica*
CPI 110810	Iran	*meyeri/typica*
CPI 110813	Iran	*meyeri/typica*
CPI 110909	USSR	*anathera*
CPI 110649	Unknown	*meyeri*
AUS 18911	Iran	*meyeri*
AUS 18912	Iran	*meyeri*
AUS 18913	Iran	*meyeri*
AUS 18914	Iran	*meyeri*
AUS 21714	Iran	*anathera*

[A] Similar to Eig (1929) classification, i.e. *Aegilops squarrosa* ssp *eusquarrosa* var. *meyeri*, var. *anathera*, var. *typica*.

Appendix 2

Compositions of different media used in this study.

Component mg/L	Medium		
	MS	L1	L5
Macro salts			
NH_4NO_3	1650	750	-
KNO_3	1900	1750	-
KH_2PO_4	170	200	300
$MgSO_4.7H_2O$	370	350	350
$CaCl_2.2H_2O$	440	450	350
Micro salts			
H_3BO_3	6.2	5	5
$MnSO_4.H_2O$	16.8	15	15
$ZnSO_4$	8.6	7.5	7.5
KI	0.83	0.75	0.75
$Na_2MoO_4.H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025
Iron			
Na_2EDTA	37	37	37
$FeSO_4.7H_2O$	28	28	28
Vitamins			
Myo-Inositol	100	100	100
Nicotinic acid	50	1	1
Pyridoxin HCl	50	1	1
Thiamine-HCl	10	10	10
Glycine	200	-	-
Ascorbic acid	-	1	1
Biotin	-	0.005	0.005
Ca-pantophenate	-	0.5	0.5
Choline chloride	-	0.5	0.5
Folic acid	-	0.2	0.2
p-aminobenzoic acid	-	1	1
Riboflavin	-	0.1	0.1
Amino acid			
L-Glutamine		750	1500
L-proline		150	300
L-asparagine		100	200

Appendix 3

Solutions

1 x TE

10 mM Tris - HCl (pH 8.0)
1 mM EDTA (pH 8.0)

1 x TBE

0.09 M Tris - HCl (pH 8.0)
0.09 M boric acid
2 mM EDTA