GENETIC TRANSFORMATION OF BARLEY (*Hordeum vulgare* L.) WITH A THERMOSTABLE (1→3,1→4)-β-GLUCANASE GENE

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ABSTRACT

The work described in this thesis evaluated different transformation technologies for the genetic transformation of elite Australian barley cultivars with a mutated barley (1→3,1→4)-β-glucanase gene.

The initial aim of this study was to develop plant regeneration protocols for embryogenic cell suspension and protoplast cultures of barley and to use polyethylene glycol (PEG)-mediated DNA transfer into protoplasts to recover transgenic plants. Although plant regeneration was successfully achieved from the cell suspension cultures, it was accomplished at a much lower frequency for the cell suspension culture-derived protoplasts. This low frequency of plant regeneration from isolated protoplasts prevented the use of protoplasts as target cells for DNA transfer and indicated that alternative technologies were required to produce fertile transformants for the studied cultivars.

Microprojectile bombardment of the suspension-cultured cells demonstrated that DNA was successfully delivered into intact barley cells. The expression of the introduced genes in the callus recovered from selection and the detection of transgene sequences in the genomic DNA isolated from the same callus confirmed that transformed callus lines were produced. Although plants were not regenerated from the transformed callus lines, these results indicated that microprojectile bombardment represented a potentially useful technique for the production of transgenic barley, provided the target cells retained the capacity to regenerate plants following transformation.

The development of a copper sulphate-enhanced plant regeneration system for scutellum-derived embryogenic callus and the use of microprojectile bombardment conditions that combined efficient DNA delivery with minimal damage to the transformed cells subsequently led to the generation of fertile transgenic plants. Biochemical and molecular assays demonstrated the functional expression and nuclear integration of the transgenes in the primary transformants (T_0). The analyses of the first generation of progeny plants (T_1), derived from different primary transformants, confirmed the Mendelian segregation and inheritance of the introduced genes.

In the latter part of this study, microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation were employed to transform barley with a mutated barley (1→3,1→4)-β-glucanase gene. In general, the transformed plants derived from *Agrobacterium tumefaciens*-mediated transformation had simpler transgene insertion patterns compared with the plants recovered from microprojectile bombardment. Reverse transcriptase (RT)-PCR was used to detect mRNA encoding the mutated (1→3,1→4)-β-glucanase enzyme in the germinated T_1 grains of four transformants obtained from *Agrobacterium tumefaciens*-mediated transformation. Molecular and biochemical assays indicated expression of the mutated (1→3,1→4)-β-glucanase gene at the mRNA and protein levels in the homozygous transgenic grain of one transformed plant line.

STATEMENT OF AUTHORSHIP

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

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

Rohan Ranjit Singh

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ABBREVIATIONS

Standard abbreviations are used without definition and non-standard abbreviations are defined below.

2,4-D	2,4-dichlorophenoxyacetic acid
*Act*1	actin gene from rice
Adh-1	alcohol dehydrogenase gene from maize
BAP	benzylaminopurine
bar	phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus*
bp	base-pair(s)
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
dATP	2' deoxyadenosine 5'-triphosphate
dCTP	2' deoxycytidine 5'-triphosphate
dGTP	2' deoxyguanosine 5'-triphosphate
Dicamba	3,6-dichloro-o-anisic acid
dTTP	2' deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*
GS	glutamine synthetase
GUS	β-glucuronidase
hpt	hygromycin phosphotransferase gene from *Escherichia coli*
kb	kilo base(s)
MS	Murashige and Skoog (1962)

*nos*T	termination region of the nopaline synthase gene from *Agrobacterium tumefaciens*
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
Picloram	4-amino-3,5,6-trichloropicolinic acid
PPT	phosphinothricin
(RT)-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SSC	sodium saline citrate
T_0	primary transformant
T_1	first generation of progeny plants of primary transformant
T_2	second generation of progeny plants of primary transformant
T_3	third generation of progeny plants of primary transformant
T-DNA	transfer DNA
Ti	tumour inducing
Ubi-1	ubiquitin gene from maize
uidA	*gusA* or β-glucuronidase gene from *Escherichia coli*
UTR	untranslated region
X-GLUC	5-bromo-4-chloro-3-indolyl-β-glucuronide, substrate for β-glucuronidase

CHAPTER ONE

GENERAL INTRODUCTION

1.1 BACKGROUND

Barley is a cereal that belongs to the family Poaceae and the genus *Hordeum* (Nilan and Ullrich, 1993). Harvested barley and the products derived from it are important raw materials in human and stockfeed diets. In addition, malted barley grain is an essential ingredient in the brewing of beer and the distilling of other alcoholic beverages.

World production of barley for the 2002 season was forecasted to be 131.4 million tonnes, of which Australia would contribute 4.5 million tonnes (International Grains Council, Grain Market Report 316, 2002). The majority of the Australian barley crop is grown in the south-eastern states of the country, and the annual value of the barley industry has averaged $850 million during the last five years (Grains Research and Development Corporation Prospectus, 2000-01). Approximately half of the total Australian barley crop is malting quality; the remainder is feed quality. Traditionally, there has been a $50 per tonne difference in the payments for malting quality barley and the best feed-grade barley (Grains Research and Development Corporation, 1998). Australia controls 32% of the world trade for malting barley and approximately 11% of the world market for export malt (International Grains Council, Grain Market Report 295, 2000). The European Community, Canada and, to a lesser extent, the United States are the other main producers of malting barley for export.

During the 1980s, the consistent supply of superior quality grain from Europe and Canada weakened Australia's position in important world export markets. Although Australia developed a new, rapidly growing market for malting barley with China at this time, its share of the lucrative Japanese market for malt diminished as brewers in that country secured regular supplies of better performing malt from Canada. Since 1986,

the price obtained for Australian malt has consistently been 10-30% lower than those for both European and Canadian malts.

As international beer markets become more discriminating and demands for specific malt characteristics intensify, there is a need to develop premium local cultivars for export and thus to arrest further erosion of Australia's share of the international market. Therefore, the development of technologies that accelerate the release of novel malting barley cultivars through the precise alteration of desirable quality parameters will lend assistance to conventional breeding programs and potentially add value to the end-user products.

For genetic transformation to be routinely applicable to barley breeding, there is a need to establish techniques for the efficient introduction of genetic material into the barley genome. These procedures rely on tissue culture systems to regenerate plants from the cultured cells that carry and express the introduced genes. The work described in this thesis was undertaken to develop *in vitro* culture and genetic transformation systems for elite Australian barley cultivars and to utilise these technologies to insert potentially useful genes into these cultivars.

In the sections below, background information on cereal tissue culture and transformation methodologies is presented.

1.2 PLANT REGENERATION FROM BARLEY CELL CULTURES

Firstly, it is useful to describe some commonly used tissue culture terms. *In vitro* culture is the aseptic growth of isolated plant parts on nutrient medium under controlled environmental conditions. Growth of these tissues in specialised solid culture medium results in the production of a mixed population of undifferentiated cells, referred to as

callus. The key to obtaining whole plants lies in identifying and perpetuating callus sectors composed of embryogenic or regenerable cells. In cereal callus cultures, these sectors are characteristically white to pale yellow in colour and are compact and nodular in appearance (Maddock, 1985). Plant regeneration can be achieved from this callus type by somatic embryogenesis or organogenesis. In the former, distinct embryos form and germinate into plants (Lazzeri *et al.*, 1990), whilst in the latter, shoot emergence is followed by root formation (Bhaskaran and Smith, 1990).

The immature embryo and the isolated immature scutellum have been the most frequently used explants for the initiation of regenerable callus cultures for a diverse range of barley cultivars (Hanzel *et al.*, 1985; Lührs and Lörz, 1987; Bregitzer, 1992; Baillie *et al.*, 1993; Caswell *et al.*, 1995; Koprek *et al.*, 1996; Chang *et al.*, 2003). In addition, most of the regenerated plants were fertile and phenotypically normal.

Recurrent culture of primary callus, derived from immature embryos, has generally led to the production of a friable, embryogenic callus, suitable for the initiation and establishment of cell suspension cultures. These cultures are grown in shaken liquid medium and usually consist of a relatively homogeneous population of small, cytoplasmic-rich cell clusters (Lazzeri *et al.*, 1990). The culture of embryogenic cell suspension aggregates on solid regeneration medium has resulted in the production of fertile plants (Funatsuki *et al.*, 1992; Huang *et al.*, 1993; Singh *et al.*, 1997). These aggregates can also be used as the source tissue for protoplast isolation.

Protoplasts are intact cells whose cell walls have been removed by enzymatic digestion. When cultured, the protoplasts reform their cell walls and divide to produce protoplast-derived callus, from which whole plants can be recovered (Funatsuki *et al.*, 1992; Funatsuki and Kihara, 1994; Kihara and Funatsuki, 1994; Singh *et al.*, 1997).

The ability to regenerate fertile plants from individual cells of embryogenic callus and cell suspension aggregates, and from isolated protoplasts makes it possible to genetically manipulate the genome of a single cell and then obtain a new transgenic barley line via an *in vitro* regeneration pathway. Transformation is defined as the transfer, genomic integration and expression of foreign DNA in a new genetic background. This definition can also describe the situation where a modified gene of the host species is reintroduced into the same species.

In the sections below, several transformation systems are presented, together with a description of promoter sequences and selectable marker and reporter genes that have been successfully used for barley transformation.

1.3 METHODS FOR BARLEY TRANSFORMATION

Three transformation procedures have been developed to produce transgenic barley. These methods are the direct uptake of DNA into protoplasts, the insertion of DNA into cells using microprojectile bombardment, and the transfer of DNA to plant cells by infection with *Agrobacterium tumefaciens*. These are discussed in the subsections below.

1.3.1 Protoplast transformation

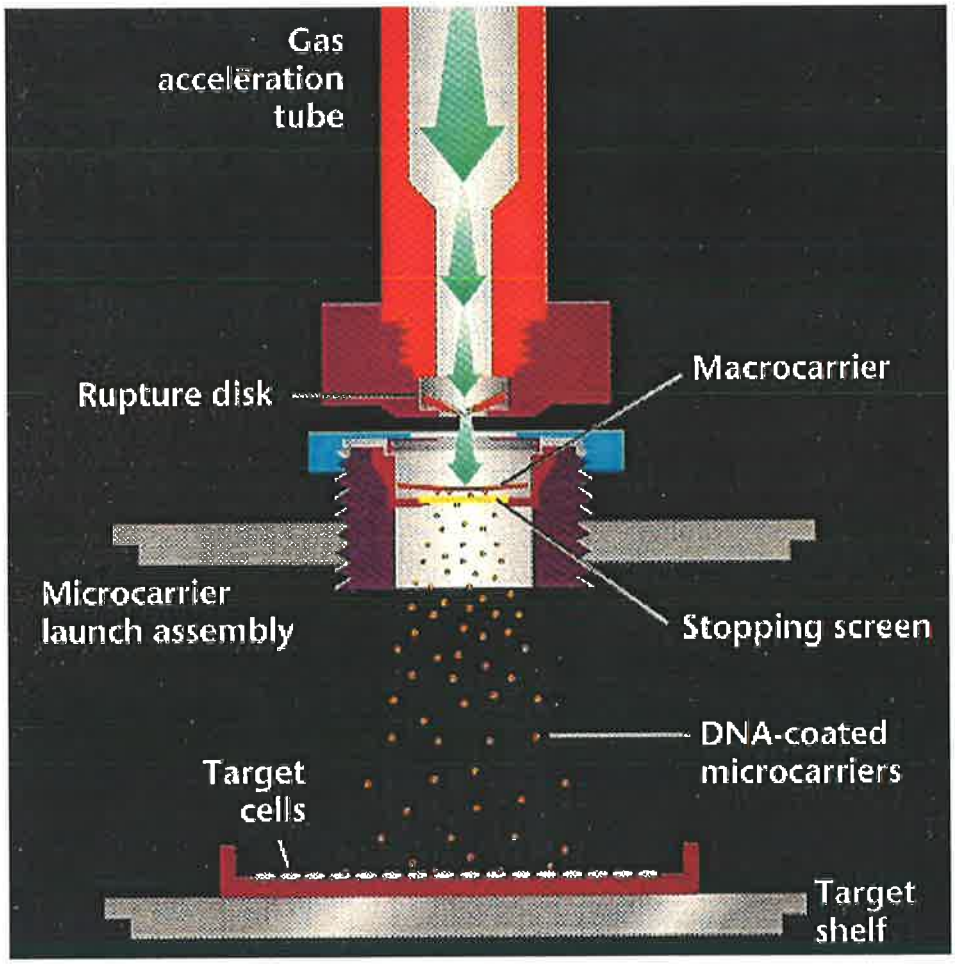
As mentioned previously (Section 1.2), a protoplast is a single cell without a cell wall. Removal of the cell wall eliminates a major barrier to DNA uptake. Reversible permeabilisation of the plasma membrane by polyethylene glycol (PEG) or electroporation enables foreign DNA to enter the protoplast's cytoplasm and nucleus and, at a low frequency, to integrate into the genomic DNA of the recipient cell

(Songstad *et al.*, 1995). Following transformation, the appropriate selection agent is applied during the development of the protoplast-derived callus, and putative transformed callus is induced to regenerate plants (Potrykus, 1995).

Early efforts to transform barley using PEG-induced uptake of DNA into protoplasts resulted in transformed callus lines, but no plants (Lazzeri *et al.*, 1991). Improved plant regeneration protocols for protoplasts isolated from cell suspension cultures (Funatsuki and Kihara, 1994) were later coupled with the procedure for PEG-mediated uptake of DNA to produce fertile transgenic barley (Funatsuki *et al.*, 1995). Furthermore, three groups have reported the regeneration of fertile transgenic plants using barley protoplasts isolated from tissues other than cell suspension cultures (Salmenkallio-Marttila *et al.*, 1995; Kihara *et al.*, 1998 a; Nobre *et al.*, 2000). Salmenkallio-Marttila *et al.* (1995) regenerated transgenic plants via electroporation of protoplasts isolated from microspore-derived callus cultures. Fertile transgenic barley was also successfully regenerated following PEG-mediated uptake of DNA into protoplasts isolated from immature embryo-derived callus (Kihara *et al.*, 1998 a) and the scutellum of the immature embryo (Nobre *et al.*, 2000). Although the protoplast culture system has been employed for barley transformation, this procedure is labour-intensive and technically difficult. Reproducible yields of protoplasts cannot always be achieved, and regeneration of fertile plants from protoplasts remains problematical. The most successful techniques for barley transformation allow transgenes to be inserted into intact, regenerable cells and employ a short tissue culture phase to recover fertile transgenic plants. Therefore, microprojectile bombardment and *Agrobacterium tumefaciens*-mediated gene transfer have become the preferred transformation technologies for barley.

FIGURE 1.1

The PDS-1000/He Biolistic® Particle Delivery System (source: Bio-Rad Products Catalogue, 1998/99)



1.3.2 Microprojectile bombardment

Microprojectile bombardment is the delivery of foreign DNA into target cells using high velocity particles (Gray and Finer, 1993). The PDS-1000/He Biolistic® Particle Delivery System is the most widely used bombardment apparatus for plant transformation (Bio-Rad Laboratories, Hercules, CA, USA). This system uses a sudden release of helium pressure to burst a rupture disk at a pre-determined pressure, and this results in the acceleration of DNA-coated microprojectiles supported on a macrocarrier. The macrocarrier is forced downwards and is retained by the stopping screen, while the microprojectiles pass through the screen and penetrate the cell walls of the target tissues (Figure 1.1). The bombarded tissues are subjected to further culture, in the presence of the appropriate selection agent, to detect stable transformed cells that express the introduced DNA and can regenerate into whole plants.

Using this general tissue culture system, transgenic plants have been produced for a diverse range of cereal species following microprojectile bombardment of the scutellar cells of either the freshly isolated or pre-cultured immature embryo (Christou *et al.*, 1991; Casas *et al.*, 1993; Li *et al.*, 1993; Weeks *et al.*, 1993; Vasil *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994; Castillo *et al.*, 1994; Karunaratne *et al.*, 1996; Ortiz *et al.*, 1996; Takumi and Shimada, 1996; Altpeter *et al.*, 1996; Songstad *et al.*, 1996; Brettschneider *et al.*, 1997; Takumi and Shimada, 1997; Witrzens *et al.*, 1998; Jordan, 2000; Varshney and Altpeter, 2001; Huber *et al.*, 2002). In the case of barley, there have been numerous reports of transgenic plant production using a similar transformation strategy (Ritala *et al.*, 1994; Wan and Lemaux, 1994; Hagio *et al.*, 1995; Koprek *et al.*, 1996; Harwood *et al.*, 2000; Cho *et al.*, 2002; Manoharan and Dahleen,

2002; Harwood *et al.*, 2002). Each report demonstrated the integration and expression of the inserted DNA in the transgenic plants and the transmission of the transgenes to progeny plants.

Transformation frequency (%) is used to measure the efficiency of a studied transformation procedure to produce transgenic plants. It is calculated by dividing the number of independent transgenic lines by the total number of treated explants and multiplying by 100. Transformation frequencies in the range of 0-4.4% have been reported for microprojectile bombardment-mediated barley transformation (Ritala *et al.*, 1994; Wan and Lemaux, 1994; Hagio *et al.*, 1995; Koprek *et al.*, 1996; Jensen *et al.*, 1996; Cho *et al.*, 1998; Ahlandsberg *et al.*, 1999; Manoharan and Dahleen, 2002; Harwood *et al.*, 2002).

1.3.3 *Agrobacterium tumefaciens*-mediated transformation

Agrobacterium tumefaciens is a soil borne pathogen that infects wounded plant cells and causes the development of crown gall tumours on the plant. The *Agrobacterium* cell contains a large (greater than 200 kb) tumour-inducing plasmid (Ti plasmid) that harbours a T-DNA (transfer DNA) region and the virulence *(vir)* gene operon (Zambryski, 1988). When the bacterium infects the plant, the products of the *vir* gene region stimulate the movement of the T-DNA region from the bacterium cell to the genome of the plant cell. The short left and right border sequences (25 bp) that enclose the T-DNA region are responsible for the excision and mobilisation of this region to the nucleus of the plant cell. Once the T-DNA region has integrated into the genome of the infected plant cell, transcription of the genes contained in the T-DNA results in the tumorous phenotype. The transcription of genes involved in auxin and cytokinin

production lead to elevated phytohormone levels in the transformed cells, which cause uncontrollable mitotic activity and tumour formation. The transcription of another set of genes code for the synthesis of opines, a group of compounds used by *Agrobacterium* as a nutrition source (Weising *et al.*, 1988).

Several changes need to be made to the wild-type Ti plasmid in order to use it for plant transformation. Firstly, the tumour-inducing genes must be eliminated from the T-DNA region to prevent crown gall formation that would otherwise interfere with the ability of transformed cells to regenerate into plants. Secondly, the large size of the Ti plasmid prohibits easy *in vitro* manipulation, and in most *Agrobacterium tumefaciens*-based protocols a binary vector system is therefore employed to effect plant transformation. In this case, the *vir* genes are located on a separate Ti plasmid, and the products of these genes act *in trans* to facilitate T-DNA migration from a second Ti plasmid into the genome of the plant cell. The second, smaller Ti plasmid contains a bacterial selectable marker gene to assist with selection of the plasmid in *Escherichia coli (E. coli)*, and a T-DNA region that carries a gene of interest and a plant selectable marker gene to identify transformed cells.

In this transformation procedure, target tissue is coated with a suspension of bacterial cells in a process known as co-cultivation. Following co-cultivation, the explants are transferred to culture medium that contains an antibiotic to kill the *Agrobacterium* and the appropriate selection agent to detect transformed plant cells. During the next tissue culture phase, the transformed cells produce embryogenic callus, which is transferred to regeneration medium containing the selection agent, and plants are eventually recovered (Klee *et al.*, 1987).

Cereals were initially considered to be outside the natural host range of *Agrobacterium tumefaciens* (Potrykus, 1990; Casas *et al.*, 1995), and direct DNA delivery systems, such as protoplast transformation and microprojectile bombardment, were therefore used in attempts to produce transgenic plants. However, transformation of rice (Hiei *et al.*, 1994; Rashid *et al.*, 1996; Aldemita and Hodges, 1996; Komari *et al.*, 1996; Lucca *et al.*, 2001), maize (Ishida *et al.*, 1996; Zhao *et al.*, 1998; Negrotto *et al.*, 2000; Wright *et al.*, 2001; Frame *et al.*, 2002) and wheat (Cheng *et al.*, 1997; Weir *et al.*, 2001; Khanna and Daggard, 2003; Wu *et al.*, 2003) has now been achieved using *Agrobacterium tumefaciens*-mediated gene transfer. The first research group to demonstrate *Agrobacterium tumefaciens*-mediated transformation of barley was Tingay *et al.* (1997). Subsequent reports confirmed that the technique was reproducible, suitable for the generation of large numbers of independent transgenic plant lines, and applicable to different barley cultivars (Horvath *et al.*, 2000; Patel *et al.*, 2000; Wang *et al.*, 2001; Trifonova *et al.*, 2001; Fang *et al.*, 2002). Transformation frequencies in the range of 0.9-6% have been documented for *Agrobacterium tumefaciens*-mediated barley transformation (Tingay *et al.*, 1997; Patel *et al.*, 2000; Wang *et al.*, 2001; Trifonova *et al.*, 2001; Fang *et al.*, 2002). The recent reports of successful barley transformation using *Agrobacterium tumefaciens* will undoubtedly lead to refinements of the procedure that could further improve the transformation frequency.

These transformation strategies employ transformation vectors or plasmids to convey foreign DNA into the genome of the target cell. An important component of the transformation vector is the promoter region, which is involved in the co-ordination of transgene transcription. Since the expression levels of a transgene in particular tissues represent the key to success in transformation technology, considerable attention has

focussed on the development of a variety of DNA vectors in which different promoters direct transgene activity. Some of these are discussed in the following section.

1.4 PROMOTERS

Constitutive promoters are defined as those that direct high-level expression in all tissue types, and their activities are independent of the development stage of the transformed plant (Fütterer, 1995). In practice, however, the activities of so-called "constitutive" promoters actually vary between tissues and at different stages of plant development (Zhong *et al.*, 1996). The constitutive promoter of the cauliflower mosaic virus (CaMV) 35S RNA gene, which has been used extensively for dicotyledon transformation, is a comparatively poor promoter in transformed cereal cells (Last *et al.*, 1991; Chibbar *et al.*, 1993; Schledzewski and Mendel, 1994; Takumi *et al.*, 1994; Ortiz *et al.*, 1997). When the CaMV 35S RNA promoter region was fused to the first intron of the maize alcohol dehydrogenase gene (*Adh-1*; Dennis *et al.*, 1984), transgene expression was significantly enhanced in cereal cells (Callis *et al.*, 1987). This modified promoter region was later successfully used to direct transgene expression in maize (Fromm *et al.*, 1990) and wheat (Vasil *et al.*, 1992). Transgene expression in rice (Cao *et al.*, 1992; Toki *et al.*, 1992; Matsushita *et al.*, 1999), wheat (Weeks *et al.*, 1993; Qureshi *et al.*, 1996), maize (Wan *et al.*, 1995; Brettschneider *et al.*, 1997) and barley (Wan and Lemaux, 1994; Tingay *et al.*, 1997) has also been achieved using the constitutive promoters of the maize ubiquitin (*Ubi-1*) (Christensen *et al.*, 1992) and the rice actin (*Act*1) genes (McElroy *et al.*, 1991).

Other promoter sequences, described as non-constitutive, only activate gene transcription under certain development conditions and in specific cell types (Fütterer,

1995). The promoter region of the barley (1→3,1→4)-β-glucanase isoenzyme EII gene (Wolf, 1991) is an example of a non-constitutive promoter. It directs aleurone-specific gene transcription in the germinated grain (Stuart *et al.*, 1986; Slakeski and Fincher, 1992). Therefore, the choice of an appropriate promoter to co-ordinate transgene expression is critical when a desired plant phenotype is required from genetic transformation.

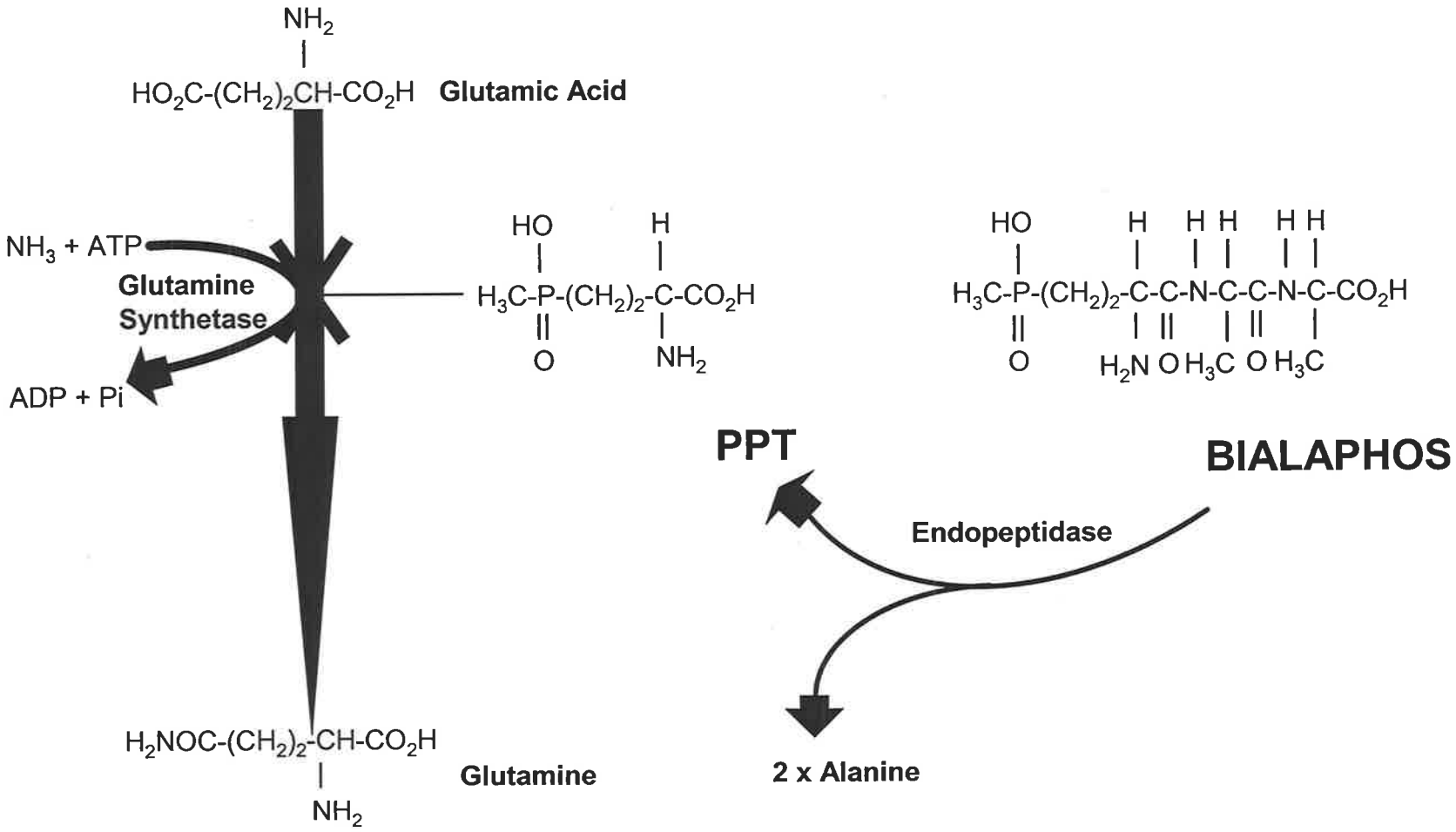
The development of transformation technology is dependent on being able to identify a transformed cell from a vast majority of non-transformed cells and on being able to easily detect expression of the transgene. To facilitate these processes, promoter sequences have been fused to selectable marker genes to identify the transformed cells and to reporter genes to demonstrate that the transgene is being expressed. Commonly used selectable marker and reporter genes are described below.

1.5 SELECTABLE MARKER AND REPORTER GENES

A selectable marker gene is a DNA sequence that encodes a protein, which renders transformed cells and plants resistant to a chemical agent (Christou, 1996). For example, the *hpt* gene encodes the enzyme hygromycin phosphotransferase, and cells transformed with this selectable marker gene are resistant to the antibiotic hygromycin B. The *bar* gene, derived from the soil bacterium *Streptomyces hygroscopicus* (De Block *et al.*, 1987), has been the most commonly used selectable marker gene for cereal transformation (McElroy and Brettell, 1994). The *bar* selectable marker gene may have found widespread use in plant transformation technologies, because selection can be supplied *in vitro* and to plants regenerated from resistant

FIGURE 1.2

The biochemical conversion of bialaphos to phosphinothricin (PPT) (Mullineaux, 1992)

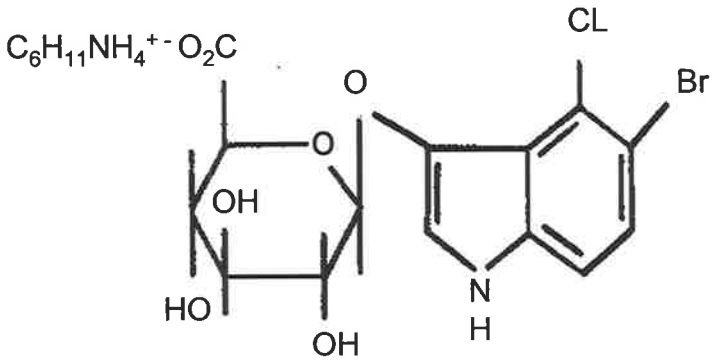


callus that are grown in soil. The *bar* gene encodes resistance to phosphinothricin (PPT), the active ingredient of the selection agent bialaphos and the commercial herbicide formulation, BASTA®. Phosphinothricin is a potent inhibitor of glutamine synthetase (GS) (D'Halluin *et al.*, 1992), and its action results in the accumulation of ammonia in the plant cell (Tachibana *et al.*, 1986), which leads to rapid cell death (Figure 1.2; Mullineaux, 1992). Phosphinothricin acetyltransferase (PAT), the enzyme product of the *bar* gene, catalyses acetylation of the amine side chain of PPT and generates a product that is no longer inhibitory to the activity of GS (De Block *et al.*, 1987). This acetylation process allows the transformed cells that express the *bar* gene to grow on culture medium containing bialaphos, while the development of the non-transformed cells is severely impaired. Furthermore, putative transformants that are transferred to soil can be quickly screened for expression of the *bar* gene by either spraying the whole plant with BASTA® or by localised application of the herbicide to the leaves of the plants (D'Halluin *et al.*, 1992). PAT activity in callus and leaf tissue carrying the *bar* gene can also be detected with a radioactive assay (Spencer *et al.*, 1990).

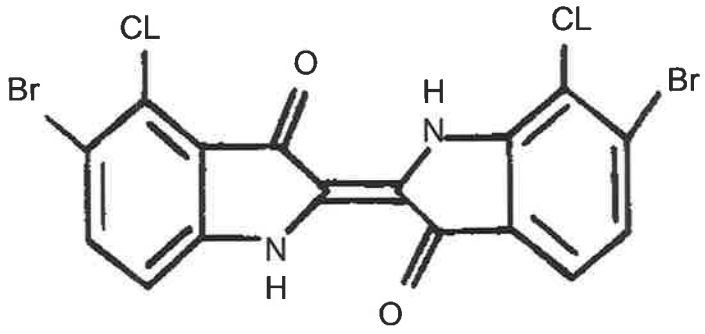
In contrast to selectable marker genes, reporter genes do not confer resistance on transformed cells by the enzymatic modification of a selection agent, but rather code for proteins that can be directly detected or that catalyse specific chemical reactions whose products are easily detectable in transformed cells (Schrott, 1995). The detectable product provides rapid information on the state of transgene expression in the transformed cells (Christou, 1996). Transient expression of the reporter gene can be detected within days of the transformation process to confirm successful DNA delivery into target cells. Stable integration of this DNA into the host's genome permits

FIGURE 1.3

Chemistry of the histochemical β-glucuronidase (GUS) assay and its mode of action (Jefferson, 1987)



5-Bromo-4-Chloro-3-Indolyl-beta-D-Glucuronic Acid,
Cyclohexylammonium salt (X-GLUC)

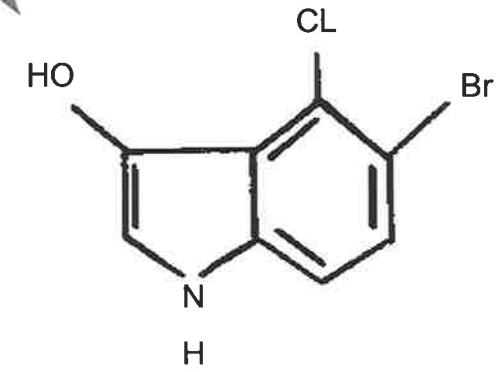


5,5-Dibromo-4,4,-Dichloro Indigo
(blue precipitate)

β-Glucuronidase

Indolyl derivative

Potassium Ferrocyanide and
Potassium Ferricyanide



the expression patterns of the reporter gene to be rapidly monitored in callus, and in plants that survive the selection phase of the transformation process (Hansen and Wright, 1999).

The *uidA* gene from *E. coli*, which encodes the β-glucuronidase (GUS) enzyme (Jefferson, 1987; Jefferson *et al.*, 1987), is the most commonly used reporter gene for plant transformation. Since the GUS enzyme is usually absent from plant cells, or present in very small amounts, expression of the introduced *uidA* gene is easy to detect in transformed cells. The commercially available substrate that has been used for histochemical localisation of β-glucuronidase activity in transformed cells and tissues is 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GLUC). This compound is colourless. Active β-glucuronidase releases the intermediate indolyl derivative, which is also colourless. However, oxidative dimerisation, catalysed by the inclusion of potassium ferrocyanide and potassium ferricyanide in the staining mixture, converts the indolyl derivative into the bright blue, coloured compound, 5,5-dibromo-4,4-dichloro indigo, which can be easily detected by eye (Figure 1.3; Jefferson, 1987).

1.6 BARLEY (1→3,1→4)-β-GLUCANASES

While reporter genes can be used to develop transformation protocols or to study expression patterns of promoters, transformation technology will eventually be applied to crop improvement. Although much of the work undertaken here was involved in the development and assessment of barley transformation systems, an important subsequent objective was to apply the technology through the introduction of a potentially useful transgene. A thermostable barley (1→3,1→4)-β-glucanase gene was used for these "proof of concept" experiments. Background information on the role

of the barley (1→3,1→4)-β-glucanases in the solubilisation of cell wall components during grain germination, together with the application of genetic engineering to increase the thermostability of these enzymes, are now discussed.

Two barley (1→3,1→4)-β-glucanases (EC 3.2.1.73), designated isoenzymes EI and EII, have been purified from aqueous extracts of germinated barley grain (Woodward and Fincher, 1982 a). The function of the (1→3,1→4)-β-glucanases in the germinated grain is to hydrolyse the (1→3,1→4)-β-glucans located in the endosperm cell walls (Henry and Fincher, 1996). This role of the (1→3,1→4)-β-glucanases has important implications for the malting and brewing industries. During malting, the action of the (1→3,1→4)-β-glucanases provides access for other hydrolytic enzymes, namely α-amylase, α-glucosidase and limit dextrinase, to degrade the starch reserves of the endosperm cells into simple sugars that are utilised by yeast for fermentation (Jacobsen *et al.*, 2000). (1→3,1→4)-β-Glucanase activity is again required to remove residual (1→3,1→4)-β-glucans during the mashing process, since aqueous solutions containing high concentrations of this polysaccharide can cause filtration problems in the brewery and undesirable haze formation in the final beer (Fincher, 1994). However, the temperatures employed for malt preparation and for the mashing process quickly inactivate the native barley (1→3,1→4)-β-glucanases, reducing the capacity of these enzymes to efficiently hydrolyse residual (1→3,1→4)-β-glucans. Therefore, it would be advantageous to increase the heat stability of the (1→3,1→4)-β-glucan endohydrolases to ensure that they remain active for longer periods during the mashing steps in the brewery (Bamforth, 1994; Fincher, 1994; MacGregor, 1996).

To enhance the heat stability of these enzymes would require modification of the genes encoding the barley (1→3,1→4)-β-glucanases. The cDNAs and genes encoding the barley (1→3,1→4)-β-glucanases have been cloned and sequenced (Slakeski *et al.*, 1990; Wolf, 1991). Stewart *et al.* (2001) employed protein engineering strategies to design a gene encoding a mutated barley (1→3,1→4)-β-glucanase enzyme, which has a thermostability 3.7°C higher than the wild-type enzyme at elevated temperatures. This mutated DNA has a single base substitution in the endogenous barley (1→3,1→4)-β-glucanase isoenzyme EII gene that results in a histidine to proline change at amino acid residue 300 (H300P) of the thermostable enzyme. A variety of gene vectors, suitable for both microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation procedures, have been constructed to facilitate the transfer of the mutated (1→3,1→4)-β-glucanase gene into barley (Stewart *et al.*, 2001).

The experimental aims of the thesis are listed below, and they include attempts to produce transgenic barley that carries and expresses the gene encoding the mutated barley (1→3,1→4)-β-glucanase H300P enzyme.

1.7 AIMS OF THE WORK DESCRIBED IN THIS THESIS

From a barley breeder's perspective, it would be preferable to incorporate potentially useful transgenes directly into elite breeding lines and cultivars (Professor AR Barr, personal communication). This scheme allows the transgene to be evaluated in a more appropriate genetic background and avoids the need to introgress the transgene into a commercially important genotype from a less desirable, but tissue culture and transformation-responsive cultivar (Lemaux *et al.*, 1999). In recognition of the impact that genetic engineering will have on the

Australian barley industry, the research described in this thesis was undertaken to develop plant regeneration and transformation systems for locally important, elite barley cultivars. The more specific aims of this project were to:

- Develop *in vitro* culture systems for the regeneration of fertile plants from cell suspension and protoplast cultures
- Demonstrate genetic transformation of cell suspension aggregates following microprojectile bombardment
- Use microprojectile bombardment to transform barley with a variety of selectable marker and reporter gene constructs, including a *uidA* gene construct driven by the barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter
- Employ microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation procedures to produce transgenic barley that carries and expresses the gene encoding the mutated barley (1→3,1→4)-β-glucanase H300P enzyme.

Initially, work was directed towards the transformation of barley through direct uptake of DNA into protoplasts. Procedures for the establishment of embryogenic cell suspension cultures and the regeneration of fertile plants from cell suspension culture-derived protoplasts are described in Chapter Two. At this time, success elsewhere with microprojectile bombardment was revolutionising the area of cereal transformation. Since plant regeneration from isolated protoplasts had proved to be difficult, microprojectile bombardment-mediated delivery of DNA into embryogenic suspension-cultured cells became the adopted technique for barley transformation.

The outcomes of these experiments are described in Chapter Three. An improved plant regeneration system for callus cultures, derived from bombarded immature scutella, later led to the production of transgenic barley. The transformation procedure and the analyses used to confirm the genomic integration and expression of the transgenes in the primary transformants (T_0) and their progeny plants (T_1) are presented in Chapter Four. Microprojectile bombardment and the rapidly emerging *Agrobacterium tumefaciens*-mediated transformation technology were used to produce transgenic barley that harboured and expressed the gene encoding the mutated barley (1→3,1→4)-β-glucanase H300P enzyme. The results of this work are described in Chapter Five. Finally, possible future experimental directions, arising from this thesis, are outlined in Chapter Six.

CHAPTER TWO

REGENERATION OF FERTILE PLANTS FROM BARLEY CELL SUSPENSION CULTURES AND CELL SUSPENSION CULTURE-DERIVED PROTOPLASTS

2.1 INTRODUCTION

Cereal cell suspension aggregates and isolated protoplasts are potentially useful target cells for plant transformation. The ability to regenerate fertile plants from these transformed cells is critical if expression of the inserted genetic material is to be achieved in the whole plant.

Embryogenic callus, derived from anthers and immature embryos, has been used successfully to establish barley cell suspension cultures. Jähne *et al.* (1991 a and b) used anther culture-derived callus of cv. Igri to establish embryogenic cell suspension cultures, and plants were regenerated from the cell suspension culture-derived protoplasts. Highly regenerable suspension and protoplast cultures of cv. Dissa (Funatsuki *et al.*, 1992) and long-term embryogenic cell suspension cultures of cv. Igri (Kihara and Funatsuki, 1994) were produced using immature embryo-derived callus. The direct incubation of immature barley embryos in liquid medium has also been used as a means of developing embryogenic cell suspension cultures. Using this methodology, plant regeneration has been reported for cell suspension culture-derived protoplasts of cv. Yong 257 (Yan *et al.*, 1991) and cv. Dissa (Golds *et al.*, 1994).

Four groups have reported the regeneration of fertile plants from barley protoplasts isolated from source tissues other than embryogenic cell suspension cultures (Kihara and Funatsuki, 1995; Salmenkallio-Marttila and Kauppinen, 1995; Stöldt *et al.*, 1996; Nobre *et al.*, 1996). Primary callus derived from isolated microspores of cv. Kympii (Salmenkallio-Marttila and Kauppinen, 1995) and immature embryos of cvs. Igri, Golden Promise and Dissa (Kihara and Funatsuki, 1995; Stöldt *et al.*, 1996) were used to produce protoplasts from which fertile plants

were regenerated. Furthermore, Nobre *et al.* (1996) regenerated fertile plants from scutellum-derived protoplasts of cv. Clipper.

Although the regeneration of cell suspension and protoplast-derived barley plants has been reported in the literature, the genotype specificities of these tissue culture systems have limited the application of these protocols to other barley cultivars (Dahleen, 1995; Jiang *et al.*, 1998). In many cases, the cultivars that performed best in culture were not those used in modern breeding programs. Thus, experimental conditions designed to optimise the *in vitro* culture response of previously untested cultivars need to be developed, in particular for elite cultivars or breeding lines with desirable quality and productivity characteristics.

The work in this Chapter describes the establishment of embryogenic barley cell suspension cultures and the regeneration of fertile plants from isolated protoplasts for the malting quality cultivar, Schooner.

2.2 MATERIALS AND METHODS

2.2.1 Establishment of callus and cell suspension cultures

Donor Schooner plants were grown in a glasshouse. Three grains were sown in 16 cm plastic pots filled with Horsham soil mix (Appendix 1). Developing spikes were harvested from the plants when the immature embryos were approximately 1-2 mm in diameter. The awns were removed and the grains were surface-sterilised with 70% (v/v) ethanol for 3 min and washed extensively with sterile water. The immature embryos were excised from the grains and cultured, scutellum side-up, on callus induction medium, MSB3D (Bregitzer, 1992; Appendix 2). After a three to four week incubation period in the dark at 22-24°C, embryogenic callus sectors were removed and cultured on fresh callus induction medium. When embryogenic callus composed of relatively small and friable sectors was identified, it was transferred to L1D2 liquid medium (Lazzeri *et al.*, 1991; Appendix 2) and maintained with fortnightly subculture. Cell suspension cultures were initiated in plates with six wells (Greiner Labortechnik, Frickenhausen, Germany) using a ratio of 1 g fresh weight tissue: 1.5 ml liquid medium. Once a week during the first month of the cell suspension culture, one third to one half of the liquid medium was exchanged for fresh medium. Every week, for an additional two months, the used medium was removed from the cell suspension culture and replaced with an equivalent amount of fresh medium. After three months, individual cell lines that demonstrated sustained growth and a lack of necrotic tissue were placed in larger culture vessels (approximate volume 100 ml), supplemented with an increased amount of medium (10 ml), and maintained with weekly subculture. Approximately four to six months after cell suspension initiation, large (200-400 μm in diameter), compact, and cytoplasmic-rich cell aggregates were

removed from the "mother" culture using a wide-mouthed pipette and placed in new culture vessels. These "daughter" lines were subcultured twice a week for a further four to five month period to produce fine cell suspension cultures that were suitable for protoplast isolation. For cell suspension cultures older than 10 months, the medium was removed weekly and replaced with enough fresh medium to support 2-3 g fresh weight cells. Cell suspension cultures were placed on an orbital shaker (70 rpm) in the dark at 22-24°C.

2.2.2 Regeneration of plants from cell suspension cultures

Cell suspension samples were plated on L2M medium (Funatsuki *et al.*, 1992; Appendix 2) to assess their capacity for plant regeneration. Embryogenic structures that formed were subcultured on the same medium to induce shoot formation. Callus that produced shoots was transferred to hormone-free MS medium (Bregitzer, 1992; Appendix 2), and the cultures were incubated in the light (16 h day/8 h night photo-period) to promote plant development. Tissue culture-derived plants were initially grown in 10 ml plastic test tubes to ensure good root formation. The plants were later transferred to 100 ml growth vessels. From these growth vessels, tillering plants were transplanted into 8 cm plastic pots containing Horsham soil mix and enclosed within high humidity growth chambers. Once the plants were established in soil, they were transferred into 16 cm plastic pots and grown to maturity in the glasshouse.

2.2.3 Protoplast isolation, culture and plant regeneration

Two to three days after the subculture of the fine cell suspension culture, 0.5 g fresh weight cells were incubated with 5 ml protoplast enzyme solution in a 55 mm x 10

mm plastic Petri dish (Sarstedt Australia, Technology Park, SA, Australia). The enzyme solution contained 1% (w/v) Cellulase RS (Yakult Pharmaceutical Industry Co. Ltd., Tokyo, Japan), 0.5% (w/v) Macerozyme R10 (Serva Feinbiochemica GmbH and Co., Heidelberg, Germany) and 0.5% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan) dissolved in washing solution. The washing solution contained the macro-nutrient, micro-nutrient and amino acids components of L1D2 medium and 100 g/L mannitol (Lazzeri *et al.*, 1991). After 4 h incubation on an orbital shaker (25 rpm) at room temperature, the protoplast enzyme mixture was sequentially filtered through 100, 50 and 30 μm nylon sieves. The protoplasts were harvested by centrifugation (30 x *g*, 10 min) and washed twice with washing solution. The protoplast density was determined with a haemocytometer.

Aliquots of protoplasts (0.5-1 x 10^6) were resuspended in 1 ml 1:1 mixture 2 x L7D0.5 protoplast medium (Dr PA Lazzeri, personal communication; Appendix 2) and 2.5% (w/v) Seaplaque agarose (FMC BioProducts, Rockland, MD, USA). The inclusion of "feeder" cells in the protoplast culture procedure has been shown to dramatically increase the frequencies of protoplast colony formation and plant regeneration from barley protoplasts (Jähne *et al.*, 1991 b; Funatsuki *et al.*, 1992). In recognition of these documented beneficial effects, "feeder" cells were included as part of the standard protocol for the culture of Schooner protoplasts. Protoplast culture was as described by Funatsuki *et al.* (1992), with the following modifications; agarose segments were supplemented with 3 ml 1 x L7D0.5 protoplast medium and approximately 100 mg "feeder" cells derived from a three year old, non-embryogenic Dissa cell suspension culture. Plates were sealed and placed on an orbital shaker (25 rpm) at room temperature for two weeks. After fourteen days, the protoplast medium

and the "feeder" cells were removed, and the plating efficiencies were calculated. Plating efficiency is expressed as the percentage of cultured protoplasts that divided and produced protoplast-derived callus. Three millilitres L1D2 medium was added to the agarose segments, and plates were sealed and returned to the shaker for a further two weeks.

After four weeks, the agarose segments were transferred to L2M medium and broken up with a spatula. Once embryogenic protoplast-derived callus had produced shoots, it was transferred to hormone-free MS medium to induce plant regeneration. The plants regenerated from protoplast culture were handled in a manner similar to the procedure described in Section 2.2.2.

2.2.4 Chromosome analysis of plants regenerated from barley cell cultures

Cytological analyses were performed using the protocol described by Eastwood (1995). Four to six weeks after transfer to the glasshouse, developing plants were transplanted into larger pots. At this stage, roots were collected for chromosome analysis. Harvested roots were soaked in 0.05% (w/v) colchicine for 4 h in the dark before the root tips were removed and stained with 1% (w/v) acetocarmine for a minimum of two days. Stained tips were boiled to evaporation in 45% (v/v) acetic acid and placed on microscope slides where the root caps were removed. The remaining tissue was sliced and macerated before viewing with a Leitz Dialux 22 microscope (Wetzlar, Germany).

FIGURE 2.1

Protoplast culture and plant regeneration in barley

A Immature barley embryos, scale bar = 1 mm.

B Friable embryogenic callus used to initiate cell suspension cultures; scale bar = 1 mm.

C Cell aggregates of established barley suspension culture; scale bar = 30 µm.

D Freshly isolated protoplasts; scale bar = 15 µm.

E Cell wall formation and protoplast division after three days culture; scale bar = 15 µm.

F Actively dividing cell cluster after seven days culture; scale bar = 15 µm.

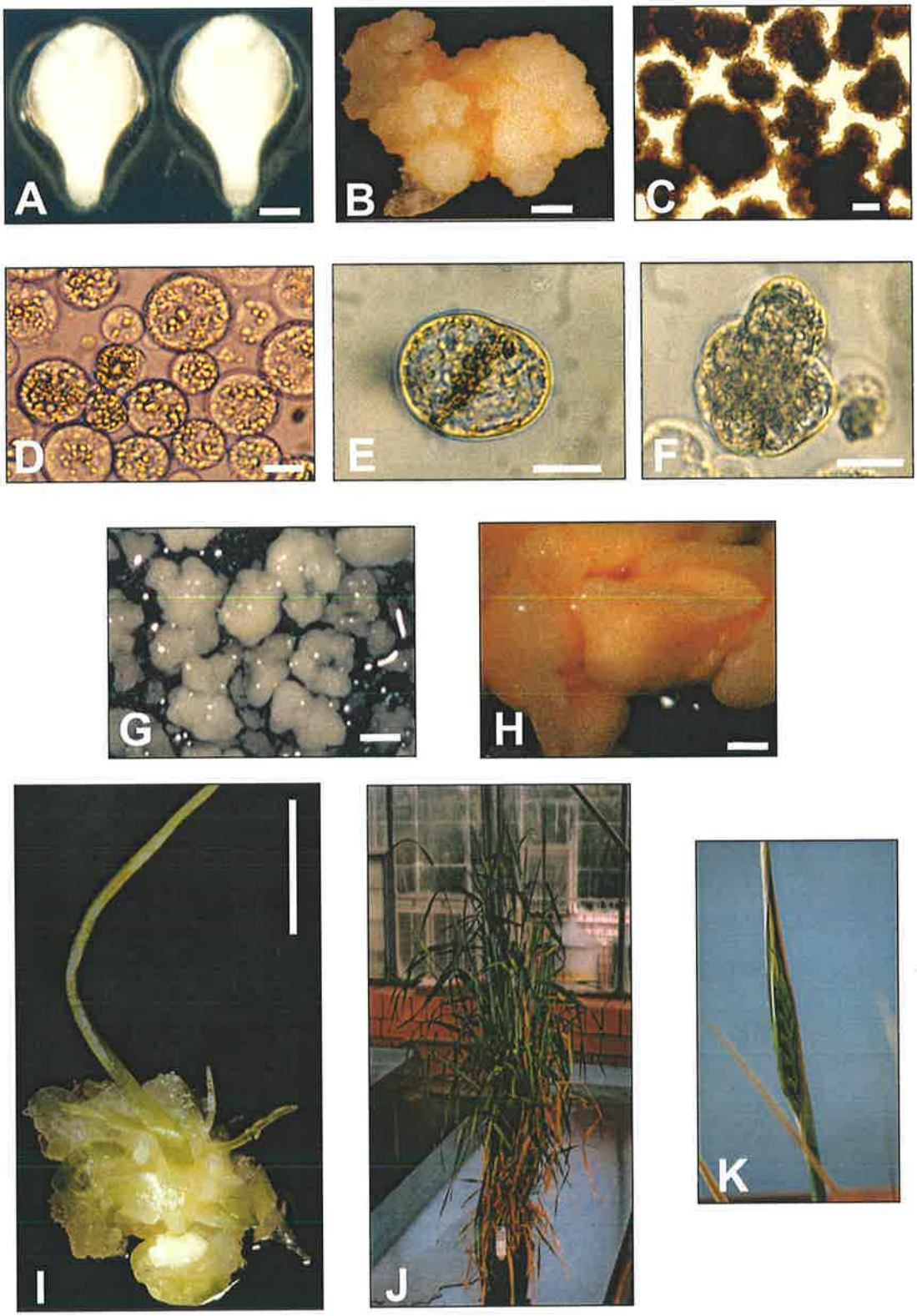
G Protoplast-derived callus colonies after one month culture; scale bar = 1 mm.

H Protoplast-derived embryogenic callus; scale bar = 1 mm.

I Development of a green shoot on protoplast-derived callus; scale bar = 2 cm.

J Fertile protoplast-derived plant in the glasshouse.

K Mature spike of a protoplast-derived plant.


A
B
C
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K

2.3 RESULTS

2.3.1 Establishment of cell suspension cultures

The immature embryos of cultivar Schooner (Figure 2.1 A) produced embryogenic callus at a high frequency after four weeks culture on MSB3D (Table 2.1). Recurrent subculture of the primary callus, derived from an individual embryo, was employed to multiply the callus tissue. During this culture phase, individual callus lines produced fast-growing, friable tissue, composed of both embryogenic and non-embryogenic cell aggregates. This "older", friable callus (Figure 2.1 B) dispersed readily and therefore grew more vigorously in liquid culture compared with the hard, primary callus, which did not proliferate following transfer to liquid medium. However, the proportion of callus lines that principally contained embryogenic tissue decreased with prolonged culture on the callus induction medium (Table 2.1). Sixty-five of the 155 independent callus cultures, produced after 12 weeks on solid medium (Table 2.1), generated sufficient embryogenic tissue for the initiation of the cell suspension cultures.

Upon transfer to L1D2 liquid medium, the external cells of the friable callus began to grow, and the callus pieces became uniformly pale yellow in appearance. As the callus adapted to liquid culture, it released dense, cytoplasmic-rich cell aggregates and elongated, empty single cells into the medium. Weekly subculture was employed to remove the single cells from the liquid culture and leave a suspension composed of cytoplasmic-rich cell aggregates that divided and formed smaller, smooth-surfaced cell clusters. The removal of these cell aggregates to new culture vessels, away from the incubated callus pieces, led to the establishment of cell suspension cultures. The established suspensions consisted mainly of compact cell aggregates with a loose

TABLE 2.1

Production of embryogenic Schooner callus over a 12 week period after culture initiation

Total number of embryos [1]	Number of embryos that produced embryogenic callus after 4 weeks culture	Number of embryos that produced embryogenic callus after 8 weeks culture	Number of embryos that produced embryogenic callus after 12 weeks culture
178	139	59	50
104	81	35	27
148	103	47	35
157	136	55	43
Total 587	**Total 459**	**Total 196**	**Total 155**
Embryogenic callus production at different time intervals as a percentage of the total number of cultured immature embryos			
	78	**33**	**26**

[1] Numbers represent embryos cultured on four separate occasions.

peripheral layer of cytoplasmic-rich cells (Figure 2.1 C). A total of six independent cell suspension cultures that demonstrated sustained growth and long-term regenerative capacity were produced in this study, and these lines were designated Sc 1-6.

2.3.2 Plant regeneration from cell suspension cultures

After four months in suspension culture, tissues tested for plant regeneration capacity often produced albino shoots. It was observed that during the same period, only a small proportion (2-5%) of the plated cell suspension aggregates were capable of plant regeneration. For cell suspension cultures older than eight months, two subculture passages on L2M medium, each of three to four weeks duration, were needed to produce observable embryogenic structures and ultimately shoot formation. More than 110 plants, regenerated from six to eight month old cell suspension cultures, were transferred to soil. Fifty-six of these plants were regenerated from Sc 4. Although all the mature plants were fertile, there were differences in seed set from one plant to another. Each plant produced approximately 30 heads and 3 grains per head. Plant regeneration was still possible from 18 month old cell suspension cultures, but the number of regenerated albino shoots was 3-5 times higher than the proportion of regenerated green plants.

Thirty-three plants, regenerated from Sc 1-5, were analysed for chromosome number. All of these plants possessed the expected 2N=14 chromosome complement.

2.3.3 Protoplast development and characterisation of regenerated plants

When the cell aggregates of four to six month old Schooner suspension cultures were incubated in the enzyme digest mixture, no protoplasts were produced. Prolonged incubation periods and alteration of the base osmoticum from mannitol to KCl did not improve protoplast yield. It was likely that the ability of the enzyme mixture to generate protoplasts was impaired by the compact nature of these cell suspension aggregates. However, after eight to ten months of selective subculture, the increased friability of the cell suspension aggregates was correlated with improved protoplast yields. Using this material, 0.5-5 x 10^6 protoplasts were routinely obtained from 0.5-1 g fresh weight cells (Figure 2.1 D). It was observed that protoplasts were released only from the outer layers of the digested cell suspension aggregates. Fluorescein diacetate staining revealed greater than 95% viability among the isolated protoplasts. Of the six regenerable cell lines produced in this study, three were useful for protoplast culture experiments (Sc 2, 4 and 5). The other cell suspension cultures (Sc 1, 3 and 6) either demonstrated a poor capacity for protoplast production or the cultured protoplasts failed to exhibit sustained cell division.

The purified protoplasts were embedded in solid medium, and after three to five days in culture, they began to form cell walls and divide (Figure 2.1 E). Discrete protoplast-derived callus colonies could be observed in the agarose blocks one week after protoplast isolation (Figure 2.1 F). Using a plating density of approximately 6 x 10^5 protoplasts/ml, plating efficiencies ranged from 0.11-0.46% among the different cell suspension cultures (Table 2.2). After one month in culture, when protoplast-derived callus colonies had grown to approximately 1-3 mm in diameter (Figure 2.1 G), agarose segments were transferred to L2M medium to induce the production of

TABLE 2.2

Plant regeneration from barley protoplasts

Cell suspension line	Number of protoplasts cultured (x 10^5)/ Number of experiments	Plating efficiency (%)	Number of protoplast-derived callus that regenerated albino shoots	Number of protoplast-derived callus that regenerated green shoots [1]	Number of protoplast-derived callus that produced at least one green plant	Number of plants transferred to soil	Number of fertile plants
Sc 2	6.5/1	0.23	76	3	2	1	0
Sc 4	24/3	0.46	281	41	31	22	19
Sc 5	6.0/1	0.11	29	12	9	2	0

[1] Shoots greater than 2 cm in length.

embryogenic structures capable of plant regeneration. Although some of these protoplast-derived callus colonies had developed advanced embryo-like structures during the first month of culture (Figure 2.1 H), the majority consisted of undifferentiated tissue. After a further two months culture on L2M medium to select for embryogenic structures, shoots were regenerated (Figure 2.1 I) and transferred to hormone-free MS medium. Root development was observed two to three weeks after transfer of the protoplast-derived shoots to this medium. In general, plants produced multiple shoots with few roots. Three to five months after protoplast isolation, plants were potted in soil and grown to maturity in the glasshouse (Figures 2.1 J and K).

Both green and albino plants were regenerated from the different protoplast culture experiments (Table 2.2). The proportion of albino shoots was high (7 albino:1 green). From 56 protoplast-derived callus pieces that regenerated green shoots on L2M medium, 42 green plants developed on hormone-free MS medium (Table 2.2). Twenty-five well-developed plants were transferred to soil, and twenty-two plants grew vigorously under glasshouse conditions: all were recovered from protoplast cultures of Sc 4. Among the 22 mature plants, 19 flowered and set seed, while 3 were sterile (Table 2.2). Each protoplast-derived plant produced approximately 27 heads and 3 grains per threshed head. Fourteen protoplast-derived plants were analysed for their chromosome complement. The plants regenerated from Sc 2 and 5 had the normal diploid number, while Sc 4 generated diploid and tetraploid plants in an approximate ratio of 3:1.

One grain harvested from 15 of the 19 fertile protoplast-derived plants was germinated, and the resultant progeny were grown to maturity in the glasshouse. In general, the 15 mature protoplast-derived progeny plants were phenotypically indistinguishable from seed-grown control plants, and each protoplast progeny plant produced approximately 15 heads and 115 grains.

2.4 DISCUSSION

Sixty-five independent cell suspension cultures of Schooner were initiated in this study. Following a six month period of selective subculture, six cell lines were produced whose suspension aggregates routinely developed embryogenic structures from which multiple, fertile plants were regenerated. The frequency of embryogenic cell suspension establishment reported here (approximately 9%) was comparable with those obtained by Lührs and Lörz (1988) (2.6%) and Funatsuki *et al.* (1992) (26.6%). The embryogenic cell suspension cultures provided the source tissue for the isolation of regenerable barley protoplasts.

The age of the cell suspension culture was an important determinant in isolating protoplasts suitable for culture. Four to six month old Schooner suspension cultures, composed of compact cell aggregates, did not yield protoplasts. This failure of "younger" material to yield suitable protoplasts for culture has been reported for other barley cultivars (Satoh *et al.*, 1991; Jähne *et al.*, 1991 b). In the present study, eight to ten month old suspension cultures, composed of more friable, densely cytoplasmic cell aggregates, proved to be the most suitable tissue for protoplast isolation. When this type of material was digested with the enzyme solution, reproducible yields of 0.5-5 x 10^6 protoplasts/g fresh weight cells were obtained. Lührs and Lörz (1988) reported yields of 1 x 10^5-1 x 10^7 protoplasts/g fresh weight cells using similar cell suspension material. It may be argued that "young" cell suspension cultures are not sufficiently adapted to release large numbers of protoplasts suitable for subsequent culture. This represents a serious limitation to this tissue culture system because "older" suspensions, while providing sound protoplasts on the one hand, contain a relatively small population of cells capable of plant regeneration. The gradual loss of

regeneration capacity has been reported previously for long-term barley cell suspension cultures initiated with immature embryo-derived (Lührs and Lörz, 1988), anther culture-derived (Jähne *et al.,* 1991 a) and isolated microspore-derived callus (Lührs and Nielsen, 1992). It was clear that the extended cell suspension culture phase described in this current study resulted in an increased incidence of albino shoot production and a low level of fertile plant regeneration from the cultured protoplasts (Table 2.2).

Here, protoplast-plating density was routinely set within the range of 0.5-1 x 10^6 protoplasts/ml. Protoplast densities less than this figure led to a low incidence of callus formation, while at higher densities competition for available nutrients became a limiting factor for sustained development of protoplast-derived callus. Plating densities for protoplasts have generally been set in the vicinity of 1 x 10^6 protoplasts/ml, although Lührs and Lörz (1988) cultured 2-5 x 10^5 protoplasts/ml.

Unlike the plants regenerated from Schooner cell suspension cultures, which all had the expected 2N=14 complement of chromosomes, tetraploid regenerants were observed among the plants derived from protoplasts. Seed set was observed among all the fertile protoplast-derived plants of Sc 4, regardless of their chromosome status. Chromosomal changes, generated by protoplast culture, have been reported for protoplast-derived callus of wheat (Karp *et al.*, 1987) and plants regenerated from barley protoplasts (Jähne *et al.*, 1991 b). The fusion of two protoplasts, created during the isolation or culture process, and the subsequent growth of this cell product could explain the regeneration of tetraploid protoplast-derived plants observed in the present study.

In this study, the fertility of the protoplast-derived progeny plants approached that observed for seed-grown control plants. These observations contrasted with those made by Kihara *et al.* (1998 b), who reported low rates of fertility among protoplast-derived progeny plants of Igri and Dissa. Undoubtedly, the different cultivars and culture conditions used for cell suspension establishment have significantly influenced the level of somaclonal variation detected in the separate studies.

2.5 SUMMARY AND CONCLUSIONS

Fertile plants were regenerated from both cell suspension and protoplast-derived cultures of the two-row malting barley, Schooner. Embryogenic callus, derived from immature embryos, was used to establish the cell suspension cultures. More than 110 plants, with variable seed set, were regenerated from 6 embryogenic cell suspension cultures. Root tip analysis of plants regenerated from cell suspension cultures revealed the expected 2N=14 chromosome complement. Protoplasts isolated from three cell suspension cultures divided, and both green and albino shoots were produced when the resultant protoplast-derived callus was transferred to regeneration medium. The green shoots were transferred to hormone-free medium, and plants that developed strong root systems were potted in soil and grown to maturity in the glasshouse. Chromosome analysis of protoplast-derived plants showed numerical variation among a proportion of the regenerants. Although seed set for the primary protoplast regenerants was low, the influence of somaclonal variation was less severe on the fertility of their progeny.

Plant regeneration systems are well documented for cell suspension and protoplast cultures of tissue culture-responsive barley cultivars. The work described in this Chapter demonstrates the wider application of these procedures to an agronomically important Australian barley cultivar, Schooner. However, an extended tissue culture phase was required to establish homogeneous cell suspension cultures suitable for protoplast culture. During this culture period, the cells gradually lost their capacity to regenerate into fertile plants, and the small number of plants recovered from the regenerable protoplasts had low fertility. The low frequency of whole plant regeneration reported here for isolated Schooner protoplasts precluded the use of this

protoplast system for the production of fertile transgenic plants.

To obviate the need to regenerate plants from protoplasts, cell aggregates were harvested from liquid cultures during the period prior to the establishment of homogeneous suspensions and subjected to microprojectile bombardment. It was anticipated that microprojectile bombardment-mediated delivery of DNA into these six to eight month old cell aggregates, which possessed a high capacity for plant regeneration (Section 2.3.2), would favour the recovery of transformed barley plants. The outcomes of this experimental strategy are presented in Chapter Three.

CHAPTER THREE

MICROPROJECTILE BOMBARDMENT OF SUSPENSION-CULTURED CELLS TO PRODUCE TRANSFORMED BARLEY CALLUS LINES

3.1 INTRODUCTION

In Chapter Two, methods were described for the initiation and establishment of barley cell suspension cultures. Four to six months after suspension initiation, the immature embryo-derived callus had generated a population of compact and densely cytoplasmic cell aggregates in the liquid medium. These aggregates were subjected to an altered subculture cycle to establish fine cell suspension cultures suitable for protoplast isolation. However, the protoplast-derived callus displayed a limited capacity for plant regeneration (Section 2.3.3). It was anticipated that the transformation and selection processes, coupled with the gradual decline in the regeneration capacity of the long-term suspension cultures, would prevent the useful application of protoplast transformation techniques for the production of transgenic barley. On the other hand, the superior regeneration capacity of the "young" suspension cultures (Section 2.3.2), compared with the cell suspension-derived protoplasts, suggested that DNA delivery into these cell aggregates by microprojectile bombardment could be a more efficient transformation system for the recovery of transgenic plants. Microprojectile bombardment of embryogenic suspension-cultured cells has led to the regeneration of transgenic maize (Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990), rice (Cao *et al.*, 1992; Jain *et al.*, 1996; Xu *et al.*, 1996; Zhang *et al.*, 1996; Chen *et al.*, 1998 a), oat (Somers *et al.*, 1992) and Italian ryegrass (Ye *et al.*, 1997).

In the case of barley, transient expression of various reporter genes has been detected in bombarded suspension-cultured cells (Mendel *et al.*, 1989; Kartha *et al.*, 1989; Chibbar *et al.*, 1993), and microprojectile bombardment of suspension-cultured cells has also produced transformed barley callus (Ritala *et al.*, 1993; Aspegren *et*

al., 1995; Stiff *et al.*, 1995). Although plants were not regenerated from these transformed barley callus lines, it seemed likely that this procedure could be useful for barley transformation, provided the cultured cells retained the capacity to regenerate plants following microprojectile bombardment.

In addition, transformed suspension-cultured cells could represent an easier and quicker method for the analysis of genes discovered in functional genomic programs. For example, if sense or antisense constructs of genes with putative functions in the synthesis of cellulose or other cell wall polysaccharides were introduced into suspension-cultured cells, any effects on wall composition could be detected, without the need to regenerate transgenic plants.

The work described in this Chapter was aimed at the transformation and regeneration of transgenic barley, following microprojectile bombardment of "young" embryogenic cell suspension aggregates.

FIGURE 3.1

Schematic representation of the gene constructs used in the microprojectile bombardment experiments, indicating the position of the restriction enzyme sites used for the molecular analyses

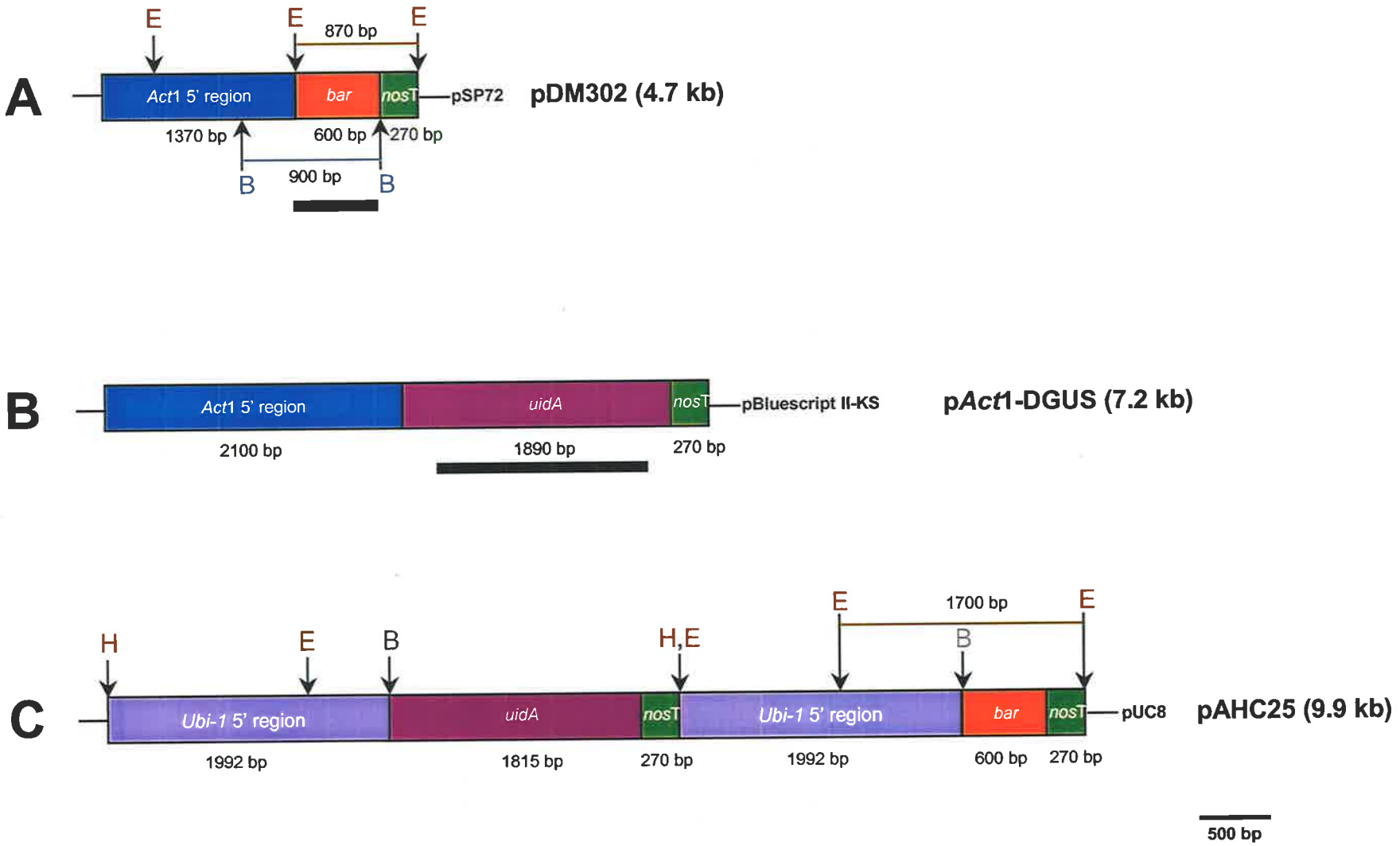
A pDM302.

B p*Act*1-DGUS.

C pAHC25.

Abbreviations: *Act*1: promoter, first exon and intron of the rice actin 1 gene; *bar:* coding region of the phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus*; *nos*T: transcript termination region of the nopaline synthase gene from *Agrobacterium tumefaciens*; *uidA*: coding region of the β-glucuronidase gene from *E. coli; Ubi-1*: promoter, first exon and first intron of the maize ubiquitin 1 gene.

Restriction enzyme sites used for DNA analyses are shown in coloured letters. Solid coloured lines indicate the DNA restriction fragments expected from the digests performed. Abbreviations of the restriction enzymes sites: B: *Bam*HI; E: *Eco*RI; H: *Hin*dIII. The *bar* containing DNA fragment isolated from pDM302 and the *uidA* containing DNA fragment prepared from p*Act*1-DGUS by PCR (indicated by solid black lines) were used as gene probes for Southern analyses.


A
E
E
870 bp
E
Act1 5' region
bar
nosT
pSP72
pDM302 (4.7 kb)
1370 bp
600 bp
270 bp
B
900 bp
B
B
Act1 5' region
uidA
nosT
pBluescript II-KS
pAct1-DGUS (7.2 kb)
2100 bp
1890 bp
270 bp
C
H
E
B
H,E
E
1700 bp
E
B
Ubi-1 5' region
uidA
nosT
Ubi-1 5' region
bar
nosT
pUC8
pAHC25 (9.9 kb)
1992 bp
1815 bp
270 bp
1992 bp
600 bp
270 bp
500 bp

3.2 MATERIALS AND METHODS

3.2.1 Barley cell suspension cultures

Embryogenic callus, derived from immature embryos of Schooner, was used to initiate barley cell suspension cultures as described in Section 2.2.1. Cell aggregates were harvested with a wide-mouthed pipette from the five month old "mother" suspension culture, Sc 4, (Section 2.3.1) and placed in a new culture vessel that contained 7.5 ml L1D2 liquid medium (Lazzeri *et al.*, 1991; Appendix 2). Every week for a further month, the spent medium was removed from the "daughter" culture and replaced with an equivalent amount of fresh medium. After this maintenance period, the suspension culture was subcultured once a week by transferring a 2.5 ml aliquot of the culture to 7.5 ml fresh liquid medium. Two to three days after subculture, cell suspension aggregates were used for microprojectile bombardment.

3.2.2 Gene constructs used for microprojectile bombardment

Plasmids pDM302 (Cao *et al.*, 1992), p*Act*1-DGUS (McElroy *et al.*, 1990) and pAHC25 (Christensen *et al.*, 1992; Christensen and Quail, 1996) were used for microprojectile bombardment (Figure 3.1). Professor R Wu (Cornell University, Ithaca, NY, USA) generously provided plasmids pDM302 and p*Act*1-DGUS. These plasmids contain the *bar* and *uidA* genes, respectively, each under the control of the rice actin (*Act*1) promoter and its first intron. Plasmid pAHC25 contains both the *bar* and *uidA* genes, each driven by the maize ubiquitin (*Ubi-1*) promoter and its first intron. This construct was a gift from Dr PH Quail (Plant Gene Expression Centre, Albany, CA, USA). All the plasmids contain the 3' untranslated region (UTR) and the

polyadenylation signal of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* (Bevan *et al.*, 1983).

Plasmid DNA of the three gene constructs was prepared using the following procedure. An *E. coli* stock, maintained in glycerol at -80°C, was transferred using a sterile inoculating loop into 5 ml Luria-Bertani medium (Sambrook *et al.*, 1989) supplemented with 50 mg/L ampicillin. The culture was incubated with continuous shaking for 16 h at 37°C. Two 1.5 ml aliquots of the culture were transferred to sterile Eppendorf tubes and centrifuged at 18280 x *g* for 30 sec at 4°C. The supernatants were discarded, and the bacterial cell pellets were resuspended in 100 µl GTE solution (25 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM Na_2EDTA). After 10 min at 4°C, 200 µl denaturing solution (0.2 M NaOH, 1% w/v SDS) was added, and the contents of the tube were gently mixed. The tubes were returned to 4°C for 5 min before 150 µl 3M sodium acetate, pH 4.8 was added to precipitate bacterial DNA and other cell debris. The tubes were stored for 30 min at -20°C and centrifuged at 18280 x *g* for 20 min. The supernatants were transferred to new Eppendorf tubes, and 1 volume iso-propanol was added to each tube to precipitate plasmid DNA. The tubes were inverted by hand, stored for 1 h at -20°C and centrifuged at 18280 x *g* for 20 min. The supernatants were discarded, and the plasmid DNA pellets were washed with 70% (v/v) ethanol. The tubes were centrifuged at 18280 x *g* for 10 min. The supernatants were removed, the tubes were inverted, and the plasmid DNA pellets were allowed to air-dry. Plasmid DNA was dissolved in 50 µl R40 buffer (10 mM Tris-HCl buffer, pH 8.0, containing 40 µg/ml pancreatic RNaseA and 1 mM Na_2EDTA) and adjusted to a concentration of 1 µg/µl. The plasmid DNA was stored at -20°C until required for microprojectile bombardment.

3.2.3 Microprojectile bombardment

The gold microcarriers (1 μm diameter, 25 mg) were weighed into a 1.5 ml Eppendorf tube and washed with 1 ml absolute ethanol. The tube was vortexed at high speed for 2 min and centrifuged at 18280 x *g* for 10 sec. The ethanol was replaced, and the washing procedure was repeated. The gold particles were subsequently washed three times with sterile water using the same procedure and finally resuspended in 1 ml sterile water. Aliquots (50 μl) were removed from the continuously vortexed suspension of gold particles and dispensed into microcentrifuge tubes. The aliquots were stored at 4°C until needed for microprojectile bombardment.

For stable transformation experiments, p*Act*1-DGUS was mixed 1:1 with pDM302 and delivered together into cultured cells, while pAHC25 was delivered alone. The DNA-coated microcarriers were prepared, with minor modification, according to the protocol of Klein *et al.* (1988). Gold particles (50 μl; containing 1.25 mg) were continuously vortexed while 5 μg DNA (1μg/μl), 50 μl $CaCl_2$ (2.5 M) and 20 μl spermidine (0.1 M, free base, tissue culture tested) were added, in that order. The complete mixture was vortexed for 3 min, and the gold particles were pelleted at 93 x *g* for 10 sec in a mini-centrifuge. The supernatant was removed, and the DNA-coated microcarriers were resuspended, with gentle vortexing, in 250 μl absolute ethanol. Following centrifugation at 93 x *g* and removal of the supernatant, the microcarriers were finally resuspended in 70 μl absolute ethanol.

The PDS-1000/He Biolistic® Particle Delivery System, marketed by Bio-Rad Laboratories (Hercules, CA, USA), was used for microprojectile bombardment.

For microprojectile bombardment, sterile filter disk papers (4.25 cm, Whatman, Maidstone, Kent, UK) were placed in 55 mm x 14 mm plastic Petri dishes (Sarstedt Australia, Technology Park, SA, Australia) that contained L1D2 medium (Lazzeri *et al.*, 1991; Appendix 2), solidified with 0.8% (w/v) bacteriological agar (GIBCO-BRL®, Life Technologies™, Paisley, Scotland). Approximately 0.5 ml settled cell volume from the subcultured suspension culture was dispensed onto each filter paper. A 10 µl sample of the DNA-coated particles was dispensed onto each prepared macrocarrier. One hundred and seventy-eight µg gold particles and 0.7 µg plasmid DNA were used for each bombardment. The macrocarrier holder was positioned 2.5 cm from the rupture disk holder (gap distance), and the macrocarrier was placed 6 mm from the stopping screen. The suspension-cultured cells were placed on the second shelf below the macrocarrier launch assembly (5 cm from the stopping screen) and bombarded once using 1550 psi rupture disks under a partial vacuum of 28 inch Hg. Each experiment included a control plate of suspension-cultured cells that were not subjected to microprojectile bombardment.

3.2.4 Histochemical β-glucuronidase (GUS) assay

The histochemical GUS assay (Section 1.5) was used to detect expression of the *uidA* reporter gene in transformed cells. Forty-eight hours after bombardment, cells and the supporting filters were transferred to 55 mm x 14 mm plastic Petri dishes and immersed in filter-sterilised GUS staining buffer. This buffer contained 100 mM sodium phosphate, pH 7.0, 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% (v/v) Triton X-100 and 0.1% (w/v) 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-GLUC, Diagnostic Chemicals Ltd.,

Charlottetown, Prince Edward Island, Canada). The sealed Petri dishes were incubated at 37°C for 24 h to reveal transient GUS activity. Following incubation, the buffer was removed and the tissues were covered with 70% (v/v) ethanol. The number of cells that demonstrated transient expression of the *uidA* gene was determined with the aid of a dissecting microscope.

3.2.5 Selection of putative transformed barley callus lines

Following bombardment, the suspension-cultured cells were incubated for two days in the dark at 22-24°C. Cells and filters were transferred to solidified L1D2 medium supplemented with 6.4 μM bialaphos (Shinyo Sangyo Co., Ltd., Tokyo, Japan). The filters carrying the cell aggregates were transferred to fresh selection medium every fortnight. The concentration of bialaphos was increased to 9.6 μM at the second transfer and to 16 μM at the third transfer. Callus that survived this treatment was maintained as a separate line on medium that contained 16 μM bialaphos and was transferred to fresh selection medium every three to four weeks.

To induce plant regeneration, embryogenic sectors of the bialaphos-resistant callus lines were transferred to hormone-free MS medium (Bregitzer, 1992; Appendix 2), supplemented with 3.2 μM bialaphos and incubated in the light (16 h day/8 h night photo-period) at 22-24°C.

3.2.6 Phosphinothricin acetyltransferase (PAT) assay

As described earlier (Section 1.5), cells transformed with the *bar* gene produce the enzyme phosphinothricin acetyltransferase (PAT), which detoxifies phosphinothricin (PPT), and permits herbicide-resistant callus to grow on culture

medium that contains bialaphos. The PAT assay is an experimental technique used to monitor the conversion of PPT to a non-toxic, acetylated form through the transfer of a [^{14}C]-labelled acetyl group from acetyl coenzyme A to the free amino group of PPT. The [^{14}C]-labelled acetylated PPT is detected following the separation of reaction products by thin layer chromatography and autoradiography.

PAT activity assays were performed on protein extracts of bialaphos-resistant callus using a modified version of the procedure developed by Spencer *et al.* (1990). Approximately 50 mg callus was transferred to a 1.5 ml Eppendorf tube and homogenised in 100 µl extraction buffer (50 mM Tris-HCl buffer, pH 7.5, containing 2 mM Na_2EDTA, 0.15 mg/L leupeptin, 0.15 mg/L phenylmethylsulfonyl fluoride, 0.3 mg/L BSA and 0.3 mg/L dithiothreitol) with a plastic micro-pestle. The tube was centrifuged at 16250 x *g* for 10 min at 4°C, and the clarified supernatant was transferred to a new 1.5 ml plastic tube. The total protein content of the extract was determined using the Coomassie® protein assay reagent (Pierce, Rockford, IL, USA; Bradford, 1976). Total protein (25 µg in 20 µl extraction buffer) was combined in a new 1.5 ml tube with 3 µl [^{14}C]-acetyl coenzyme A (60 mCi/mmol, NEN™, Boston, MA, USA) and 2 µl BASTA® (50% w/v concentrate, Hoechst, Victoria, Australia; diluted 1:10 in extraction buffer). The reaction mixture was incubated at 37°C for 30 min. The tube was heated at 100°C for 5 min, the supernatant was collected by centrifugation and evaporated to dryness using a vacuum drier. The reaction products were resuspended in 5 µl extraction buffer and spotted on a silica gel 60 thin layer chromatography plate (Merck, Darmstadt, Germany). Ascending chromatography was performed in a 3:2 mixture of n-propanol and NH_4OH (NH_3 content 28-30%). The chromatography plate was exposed to X-ray film (RX Fuji

Medical X-ray film; RX-U, Tokyo, Japan) for two days, and the film was developed to detect [^{14}C]-acetylated PPT.

3.2.7 Genomic DNA isolation

The method for DNA extraction from barley callus was based on the protocol described by Dellaporta *et al.* (1983). Approximately 0.5 g callus tissue was ground with a pestle to a fine powder under liquid nitrogen. The powder was transferred to a 30 ml centrifuge tube, and 15 ml DNA extraction buffer (100 mM Tris-HCl buffer, pH 8.0, containing 50 mM Na_2EDTA, 500 mM NaCl and 10 mM 2-mercaptoethanol) was added. One ml 20% (w/v) SDS was added, the tube was shaken vigorously and incubated at 65°C for 10 min. Five ml 5 M potassium acetate, pH 4.8 was added, the tube was vortexed, and incubated on ice for 20 min. The supernatant was poured through a Miracloth filter (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) into a clean 30 ml tube that contained 10 ml iso-propanol. The tube was inverted gently and stored at -20°C for 30 min. The tube was centrifuged at 26890 x *g* for 15 min to pellet the DNA. The supernatant was removed, and the tube was inverted for 10 min to air-dry the DNA pellet. The DNA pellet was resuspended in 700 µl TE buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1 mM Na_2EDTA). The solution was transferred to a 1.5 ml Eppendorf tube and centrifuged at 15760 x *g* for 10 min to remove any insoluble debris. The supernatant was transferred to a new 1.5 ml Eppendorf tube, and 75 µl 3 M sodium acetate, pH 4.8 and 500 µl iso-propanol were added. The tube was inverted several times and centrifuged at 15760 x *g* for 5 min to collect the DNA pellet. The pellet was washed with 70% (v/v) ethanol and air-dried for 30 min. The DNA pellet was resuspended in 100 µl TE buffer at 4°C for 18 h.

3.2.8 Southern hybridisation

Digestion of DNA was performed in a total volume of 40 µl by adding 4 µl of the appropriate restriction enzyme buffer (10 x), 2 µl BSA (1 µg/µl), 2 µl spermidine (40 mM), 3 µl of the appropriate restriction enzyme (20 U/µl), 20 µl DNA sample and 9 µl sterile water to a sterile 1.5 ml Eppendorf tube. New England BioLabs (Beverly, MA, USA) supplied the restriction enzymes and buffer solutions. The reaction mixtures were vortexed briefly, centrifuged at 18280 x *g* for 10 sec, and the tubes were incubated at 37°C for 6 h. Eight µl 6 x loading buffer (100 mM Tris-HCl buffer, pH 8.0, containing 0.2 M Na_2EDTA, 30% w/v Ficoll type 4000, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol FF) was added to each tube to terminate enzyme activity.

Gel electrophoresis of digested DNA was performed in a 1% agarose gel (GIBCO-BRL®, Life Technologies™, Grand Island, NY, USA) using 1 x TAE buffer (2 M Tris-acetate buffer, pH 8.0, containing 50 mM Na_2EDTA). A DNA size marker was loaded in the end lane of the gel, while digested DNA samples (30 µl) were loaded sequentially into adjacent gel slots. Electrophoresis was conducted at 45 V for 19 h. Following electrophoresis, the gel was stained with 1 µg/µl ethidium bromide, and the DNA banding pattern was photographed on a UV transilluminator.

Membrane preparation

After staining with ethidium bromide, the gel was treated for 20 min with 200 ml of each of the following solutions; depurinating solution (0.25 M HCl), denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralising solution (0.5 M Tris-HCl buffer, pH 7.2, containing 1.5 M NaCl).

The Southern transfer apparatus (Southern, 1975) was assembled as follows; a sponge and one sheet of Whatman 3MM filter paper, both cut to the size of the gel, were placed in a plastic tray filled with 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). The gel was placed upside down on top of the filter paper. The nylon membrane (Hybond™-N^+; Amersham Pharmacia Biotech Ltd., Little Chalfont, Buckinghamshire, UK) was immersed in water followed by 20 x SSC before being placed over the gel. Any air bubbles trapped between the membrane and the gel were gently removed by rolling a glass rod over the top of the membrane. To complete the blot assembly, another sheet of Whatman 3MM paper, followed by a thick stack of dry paper towels and a glass plate were positioned on top of the membrane. Transfer of DNA from the gel to the membrane was continued overnight.

After DNA transfer, the loading well positions and the side of the membrane that was in contact with the gel were carefully marked. The membrane was rinsed with 2 x SSC, air-dried on paper towel, sandwiched between two sheets of Whatman 3MM paper, and finally baked in a vacuum oven at 80°C for 2 h.

Preparation of radiolabelled DNA probes

The complete *bar* coding region, isolated from plasmid pDM302 as a 600 bp *Bam*HI/*Hin*dIII DNA fragment, was used as a hybridisation probe to detect the selectable marker gene in the genomic DNA of the bialaphos-resistant callus lines. The *uidA* probe was prepared by PCR using primers

5'-GGTGGGAAAGCGCGTTACAAG-3' (forward) and

5'-GTGATGATAATCGGCTGATGC-3' (reverse). These primers were chosen to provide a probe of sufficient size (1.2 kb) for efficient labelling by random priming

(Zainuddin, 2000). The primers had a balanced C+G and A+T content with no obvious internal homologies (Zainuddin, 2000). The reaction mixture for PCR contained 20 ng template DNA (p*Act*1-DGUS), 50 mM KCl, 10 mM Tris-HCl buffer, pH 9.0, 2 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.5 U Taq I DNA polymerase (Promega, Madison, WI, USA) and 10 pmol each of the primers in a final volume of 25 µl. Thermal cycling at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min 30 sec was performed for 35 cycles. The *uidA* PCR product and the *bar* coding region were both resolved by electrophoresis in a 0.8% agarose gel. Both probes were isolated from excised gel fragments using the Bresa-Clean™ Nucleic Acid Purification Kit (Bresatec, Adelaide, SA, Australia), according to the manufacturer's instructions.

Probes were labelled by random priming (Feinberg and Vogelstein, 1983) using the MegaPrime™ DNA labelling system (Amersham). This involved placing 3 µl probe template DNA (*bar* or *uidA*), 5 µl random nonamer primers and 2 µl sterile water in a 1.5 ml Eppendorf tube. The mixture was heated at 100°C for 5 min, chilled on ice for 5 min, and briefly centrifuged to collect the condensation. Four microlitres unlabelled dGTP, dATP and dTTP were added sequentially to the tube, followed by 5 µl reaction buffer, 5 µl [α-^{32}P] dCTP (50 µCi), 2 µl DNA polymerase I Klenow fragment (1 U/µl) and 16 µl sterile water to give a final volume of 50 µl. Pipetting "up and down" mixed the reaction components, and the contents were brought to the bottom of the tube by centrifugation. The tube was incubated at 37°C for 30 min, and 50 µl fractionation dye (1.5% w/v Blue Dextran, 0.5% w/v Orange G in TE buffer, pH 8.0) was added to the tube to terminate the reaction. The entire contents of the tube were loaded onto a column containing Sephadex G100 gel

beads (Pharmacia Biotech, Uppsala, Sweden) to separate the radiolabelled DNA from the unincorporated [α-^{32}P] dCTP. The column was eluted with TE buffer, and the blue fraction that contained the radiolabelled probe was collected in a 1.5 ml Eppendorf tube. This tube was heated at 100°C for 10 min, chilled on ice for 5 min, and the probe was added to the hybridisation solution.

The nylon membrane was pre-hybridised in 6 x SSC, 5 x Denhardht's III reagent (0.2% w/v BSA, 0.2% w/v Ficoll 400, 0.2% w/v polyvinylpyrrolidone, 1% w/v SDS), 20 mM NaH_2PO_4 and 100 µg/ml denatured salmon sperm DNA at 65°C for 20-22 h. Hybridisation was performed at 65°C for 18 h in the same solution used for pre-hybridisation, except that 3 x SSC was used instead of 6 x SSC and the heat denatured radiolabelled probe was included. Following hybridisation, the filter was washed in two changes of 0.5 x SSC, 0.5% (w/v) SDS at 65°C for 30 min. The membrane was air-dried, covered with GLAD WRAP™ (Glad Products, Australia) and exposed to X-ray film (RX Fuji Medical X-ray film; RX-U, Tokyo, Japan) at -80°C.

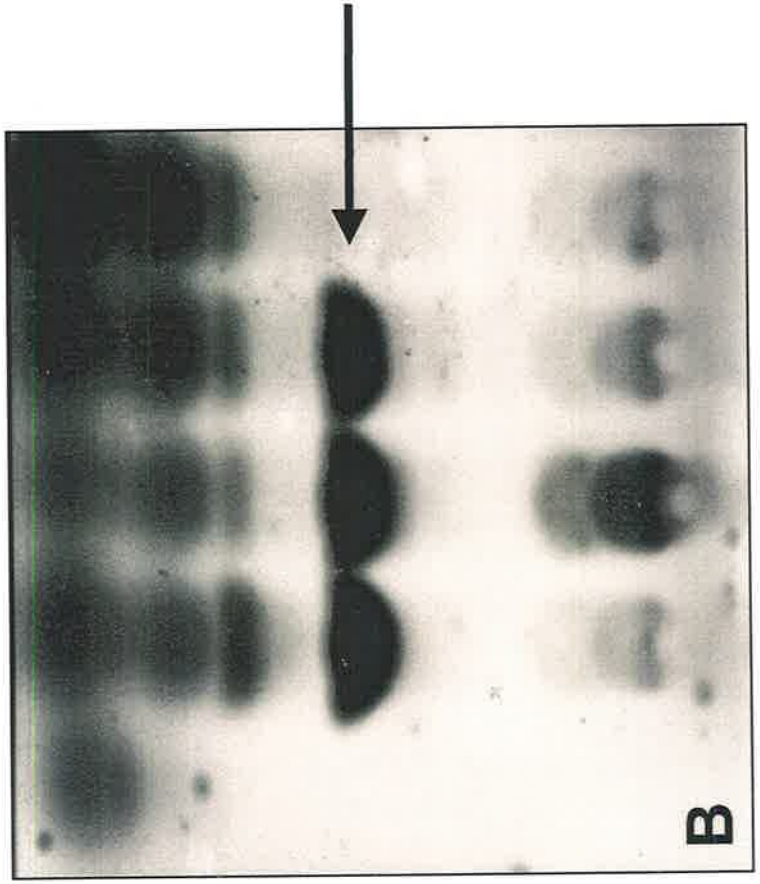
It was possible to perform multiple hybridisation procedures of the same membrane using different DNA gene probes. To strip the probe from the Hybond filter, the membrane was immersed in 200 ml boiling 0.1% (w/v) SDS and 2 mM Na_2EDTA and agitated on a rocking shaker at room temperature for 30 min. The air-dried membrane was sandwiched between two sheets of Whatman 3MM filter paper, wrapped in aluminium foil, and stored at 4 °C until it was reprobed.

FIGURE 3.2

Transgene activity in barley tissues

A Transient expression of the *uidA* gene in suspension-cultured cells, 48 h after microprojectile bombardment with p*Act*1-DGUS.

B PAT activity in bialaphos-resistant callus lines. The first lane was blank, and the reaction mixture was made up to volume with water instead of protein extract. The next three lanes contain protein extracts from three putative transformed callus lines (B1, B2 and B4). The fifth lane contains protein extract from a non-bombarded barley callus (Control). The arrow indicates the band corresponding to acetylated PPT.



CONTROL

B4

B2

B1

BLANK

TABLE 3.1

Transient expression of the *uidA* reporter gene in bombarded barley cells

Plasmid	Number of GUS active cells [1]	The average number of GUS active cells/sample of bombarded cell culture [2]
p*Act*1-DGUS	148 and 77 94 and 201	130
pAHC25	47 and 163 238 and 229	169

[1] The term GUS active cell describes a cell that transcribes the introduced *uidA* gene and translates the mRNA into active β-glucuronidase, which subsequently converts the substrate X-GLUC into 5,5-dibromo-4,4-dicholro indigo, the blue product observed in the cytoplasm of the bombarded cell. Two plates of barley cells were bombarded in two separate experiments with each plasmid.

[2] The average number of GUS active cells/sample of bombarded cell culture was calculated by dividing the total number of GUS active cells by the total number of plates of cell culture bombarded with each plasmid.

3.3 RESULTS

3.3.1 Selection of putative transformed barley callus lines

Two days after microprojectile bombardment, assays for the transient expression of the *uidA* reporter gene were used to monitor the efficiency of DNA precipitation onto the gold microcarriers and to measure the success of DNA delivery into barley suspension-cultured cells. Successful DNA delivery and expression of the *uidA* gene produced a dark blue precipitate in the interior of the bombarded cell (Figure 3.2 A). In most cases, the blue precipitate was localised to single cells, but small blue cell clusters were also observed. Reporter gene expression, driven by the rice actin and maize ubiquitin promoters, was also compared in bombarded suspension-cultured cells. Table 3.1 shows the average number of GUS active cells/sample of bombarded cell culture for two separate experiments in which the two different plasmids were introduced into barley cells. The maize ubiquitin-*uidA* gene construct (pAHC25) showed the higher number of blue spots in the bombarded barley cells, but suspension-cultured cells bombarded with the rice actin-*uidA* gene construct (p*Act*1-DGUS) had nearly the same number of GUS active cells as those bombarded with pAHC25.

The results from the transient GUS activity assays confirmed that the DNA-coated microcarriers were successfully inserted into cultured barley cells by microprojectile bombardment and demonstrated that the *uidA* gene was expressed. The *uidA* reporter gene and the selectable marker gene, *bar*, were simultaneously transferred to the barley cell suspension aggregates in these transformation experiments.

TABLE 3.2

Sensitivity assay for barley cells on solid L1D2 medium (Lazzeri *et al*., 1991) containing different concentrations of the selection agent, glufosinate ammonium

Concentration of glufosinate ammonium (μM)	Average initial fresh weight of cells (g) [1]	Average fresh weight of cells at four weeks (g) [2]	Cell growth expressed as a percentage of the control maintained under non-selection conditions [3]
0	0.206	1.56	100
12.5	0.183	0.926	67 (33)
25	0.196	0.93	62 (38)
50	0.213	0.89	55 (45)
125	0.203	0.565	36 (64)
250	0.186	0.48	34 (66)
500	0.206	0.476	30 (70)

[1, 2] Calculations based on triplicate experiments for each treatment.

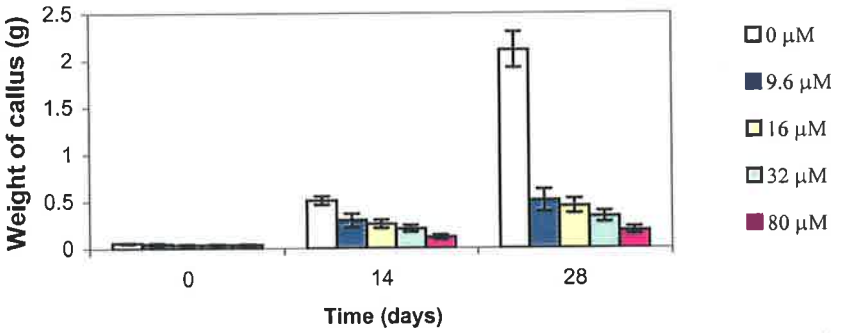
[3] Percentages in parentheses represent the inhibition of cell growth achieved with each tested concentration of glufosinate ammonium.

FIGURE 3.3

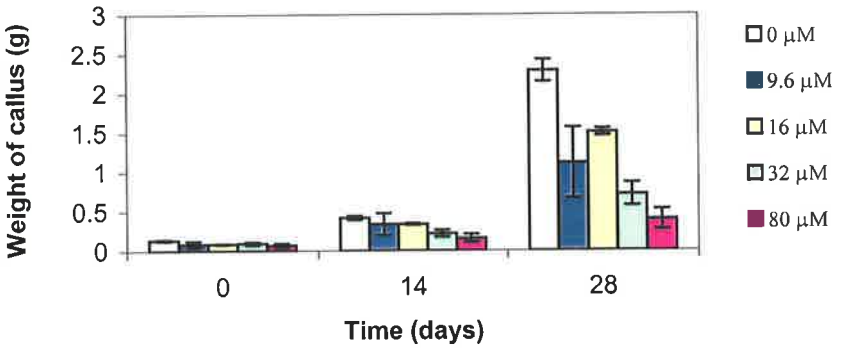
Growth of non-transformed barley callus and putative transformed callus lines, B2 and B3, on solid L1D2 medium (Lazzeri *et al.*, 1991) supplemented with different concentrations of the selection agent, bialaphos

Calculations are based on at least replicate experiments. Measurements were taken at the time of culture initiation (t=0) and at fortnightly intervals (t=14 and t=28) over the course of the incubation period. Values are means ± standard deviations.

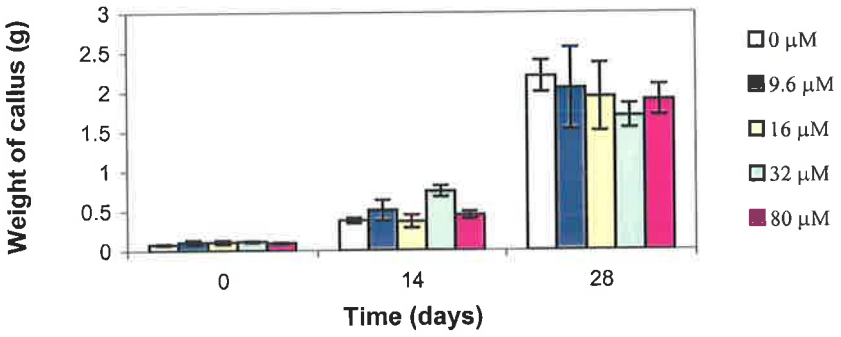
Growth of non-transformed callus on medium that contained different concentrations of bialaphos



Growth of callus line B2 on medium that contained different concentrations of bialaphos



Growth of callus line B3 on medium that contained different concentrations of bialaphos



If the DNA entered the nucleus of the plant cell following microprojectile bombardment, integration of the foreign DNA into the barley genome could occur. In preliminary experiments, sensitivity assays for non-bombarded barley suspension cells were performed with two PPT-based herbicides to determine the minimum concentrations of the selection agents required for the selection of transformants. The two compounds evaluated were glufosinate ammonium (the ammonium salt of PPT) and bialaphos, which consists of PPT linked with two L-alanine residues (Figure 1.2). The growth of non-transformed barley cells was slightly impeded (33%) on medium that contained 12.5 μM glufosinate ammonium, while a concentration of 500 μM inhibited cell growth by 70% compared with cells maintained on non-selection medium (Table 3.2). At low concentrations, bialaphos more effectively impeded the growth of non-transformed cells than glufosinate ammonium. After four weeks culture, callus induction medium that contained 9.6 or 16 μM bialaphos inhibited the growth of non-transformed cells by 75-80% compared with control callus grown under non-selection conditions (Figure 3.3). A six fold increase in the concentration of glufosinate ammonium, compared with bialaphos, was needed to achieve a similar level of growth inhibition for non-transformed barley cells. Consequently, bialaphos was used to select putative barley transformants.

Following bombardment, the first indication of the stable transformation of cells with the *bar* gene was the vigorous growth of bialaphos-resistant callus on selection medium. Although extensive callus inhibition subsequently occurred at each subculture, numerous putative transformants survived the selection procedure. Bialaphos-resistant callus was not observed on plates containing non-bombarded tissues. The two *bar* gene containing constructs (pDM302 and pAHC25) were both

effective in conferring bialaphos resistance to the putative transformed barley callus lines produced during this study. Two of these bialaphos-resistant callus lines, designated B2 and B3, and non-transformed Schooner callus were tested for their ability to grow in the presence of different concentrations of bialaphos (Figure 3.3). After four weeks, the growth of non-transformed callus was strongly inhibited at 9.6 μM bialaphos. The growth of callus line B2 was only retarded at higher concentrations of bialaphos (32 and 80 μM), while the growth of callus line B3 continued with little inhibition at the maximum bialaphos concentration (80 μM; Figure 3.3).

3.3.2 Biochemical and molecular analyses of bialaphos-resistant barley callus lines

3.3.2.1 PAT and GUS activity assays

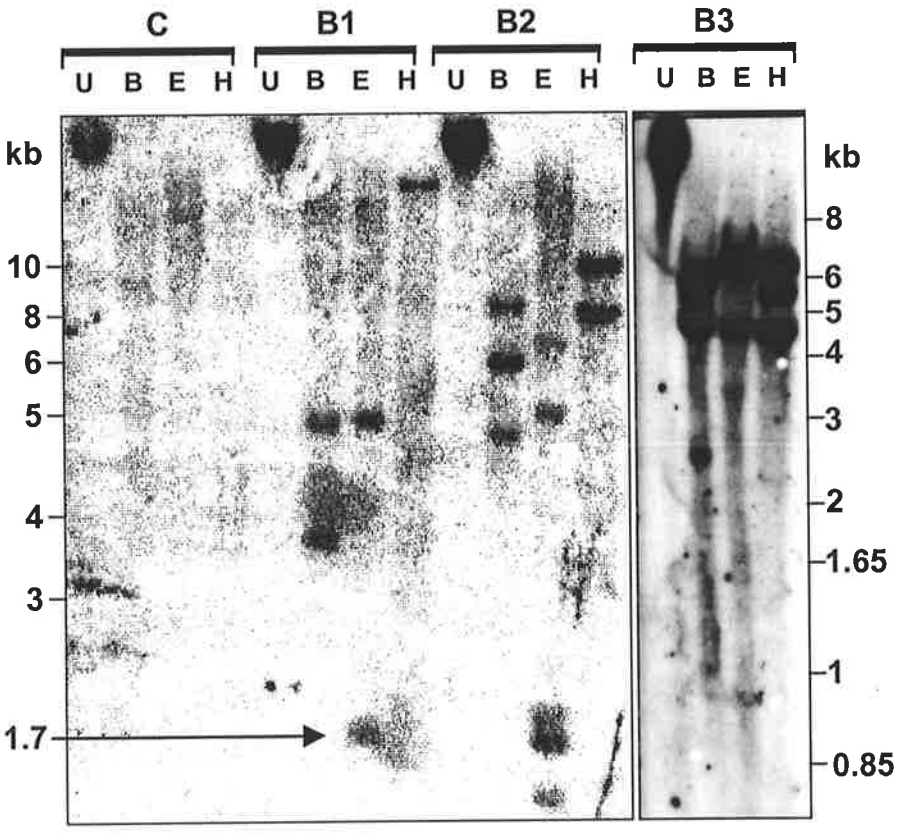
A total of thirteen bialaphos-resistant callus lines (B1-B13) were obtained from the transformation experiments. Of these lines, seven came from cell suspension aggregates bombarded with pDM302 and p*Act*1-DGUS, and six from suspension-cultured cells targeted with pAHC25. Twelve of the 13 bialaphos-resistant callus lines (B1-B11 and B13) contained detectable PAT activity (data not shown). However, PAT activity was observed for only 4 (B1-B4) of the original 13 resistant lines (Figure 3.2 B and data not shown), following long-term maintenance of the putative transformed callus on solid medium supplemented with 16 μM bialaphos. No PAT activity was detected in the protein extract of the non-bombarded barley callus (Figure 3.2 B).

FIGURE 3.4

Southern analysis of independent PAT$^+$ barley callus lines, recovered from suspension-cultured cells bombarded with plasmid pAHC25

Genomic DNA (U: Uncut; B: *Bam*HI-digested; E: *Eco*RI-digested; H: *Hin*dIII-digested) from three putative transformants (B1-B3) and a non-transformed callus (C) was hybridised with a ^{32}P-labelled *bar* gene probe. The arrow indicates the anticipated 1.7 kb *Eco*RI DNA restriction fragment.

The mobility of the DNA marker fragments is shown on the left-hand side of the first membrane and on the right-hand side of the second membrane.


C
B1
B2
B3
U B E H U B E H U B E H U B E H
kb
10
8
6
5
4
3
1.7
kb
8
6
5
4
3
2
1.65
1
0.85

Among the remaining putative transformed callus lines, one ceased to grow, and although the other lines continued to grow on bialaphos-containing medium, no PAT activity was detected in their protein extracts. Further analysis was therefore only continued for callus lines B1-B4. Callus lines B1-B3 were recovered from suspension-cultured cells bombarded with pAHC25, and callus line B4 originated from cultured cells targeted with pDM302 and p*Act*1-DGUS.

The PAT$^+$ callus lines, B1-B4, were also assayed for expression of the *uidA* reporter gene using the histochemical GUS staining technique. None of these callus lines showed any GUS activity, despite the fact that three putative transformed lines (B1-B3) were recovered from suspension-cultured cells bombarded with pAHC25, which carries both the reporter and selectable marker genes.

3.3.2.2 Southern analyses of PAT$^+$ callus lines

Southern hybridisation was used to confirm the presence of the *bar* gene in the genomic DNA of putative transformed callus lines B1-B4. After hybridisation with the *bar* probe, strong hybridisation signals, corresponding to high molecular weight DNA, were detected in the gel tracks that contained undigested genomic DNA of the four callus lines (Figure 3.4 and data not shown). As predicted from the restriction map of pAHC25 (Figure 3.1), the *bar* probe hybridised to a 1.7 kb DNA fragment in the *Eco*RI-digested genomic DNA of callus lines B1 and B2 (Figure 3.4). In addition to this anticipated *bar* hybridising DNA restriction fragment, extra hybridising fragments were also observed in the *Eco*RI gel tracks of callus lines B1 and B2 (Figure 3.4). The *bar* probe detected multiple *Eco*RI DNA restriction fragments larger than the expected 1.7 kb DNA fragment in the genomic DNA of callus line B3

(Figure 3.4).

As expected from the restriction map of pAHC25 (Figure 3.1), the *Bam*HI digest of the genomic DNA isolated from callus lines B1-B3 was predicted to generate restriction fragments larger than 0.87 kb that hybridised to the *bar* probe, while it was anticipated that the *Hin*dIII digest would produce *bar* hybridising fragments larger than 2.8 kb. These predicted DNA restriction fragments were based on the assumption that the expression cassette of the selectable marker gene was intact, and contained the promoter, the protein coding region, and the terminator region. The *Bam*HI and *Hin*dIII DNA restriction fragments detected in the genomic DNA of callus lines B1-B3 with the *bar* probe were larger than the minimum size hybridising fragments (Figure 3.4) expected from the restriction map of pAHC25 (Figure 3.1).

Genomic DNA isolated from callus line B4 was also digested with *Eco*RI and *Bam*HI and hybridised with the *bar* probe. These restriction enzymes were expected to release 0.87 kb and 0.9 kb DNA fragments respectively, from an intact insertion of pDM302 (Figure 3.1). A single, hybridising DNA restriction fragment, of the correct size, was identified in both the *Eco*RI and *Bam*HI gel tracks of callus line B4 (data not shown).

When the same membranes were stripped and probed with the *uid*A probe, no hybridising fragments were detected for any of the *bar* containing callus lines. Each callus line had a different hybridisation pattern, which was consistent with the expectation that these bialaphos-resistant lines were derived from independent transformation events.

When embryogenic callus sectors of these four transformants were transferred to regeneration medium, callus line B1 produced albino shoots (data not shown). The other lines exhibited a complete loss of plant regeneration capacity and continued to grow as undifferentiated callus.

3.3.3 Summary of the microprojectile bombardment experiments

Fifteen samples of barley suspension-cultured cells were bombarded to produce 4 *bar* containing callus lines. The assays for transient expression of the *uidA* reporter gene detected about 200 GUS active cells/sample of bombarded cell culture. The "conversion" rate from transient expression to stable transformation was determined by dividing the number of independent transgenic callus lines (4) by the total number of GUS active cells (15 x 200 = 3000). This resulted in an approximate transformation frequency of 1 transgenic callus line per 750 GUS^+ cells (or 0.13%), provided all transient GUS active cells possessed the capacity to divide and produce a resistant callus under selection conditions.

3.4 DISCUSSION

The plasmids pAHC25, pDM302 and p*Act*1-DGUS have been used successfully to drive high-level transgene expression in a variety of cereal suspension-cultured cells transformed by microprojectile bombardment (McElroy *et al.*, 1991; Cao *et al.*, 1992; Chibbar *et al.*, 1993; Taylor *et al.*, 1993; Chen *et al.*, 1998 a). In the current study, barley suspension-cultured cells bombarded with the maize ubiquitin-*uidA* gene construct (pAHC25) demonstrated a similar level of transient GUS activity to cultured cells targeted with the rice actin-*uidA* gene construct (p*Act*1-DGUS) (Table 3.1). These preliminary experiments identified bombardment conditions that directed transient expression of the *uidA* gene in regenerable barley cell suspension cultures and suggested that the promoters would also be suitable to direct high-level expression of the selectable marker gene, *bar*, in transformed barley cells.

It has been reported that glufosinate ammonium and bialaphos are equally effective as selection agents for the *bar* gene in maize (Dennehey *et al.*, 1994), wheat (Altpeter *et al.*, 1996) and barley transformation (Jähne *et al.*, 1994; Wan and Lemaux, 1994). However, the present study demonstrated that the growth of non-transformed barley cells was more efficiently inhibited with bialaphos than glufosinate ammonium, on a molar basis (Table 3.2 and Figure 3.3). This difference in the capacity of bialaphos and glufosinate ammonium to inhibit the growth of non-transformed cells could be explained by the amount of active PPT in the two selection agents. Although the two selection agents contain the same active ingredient (PPT), their chemical structures are different. Glufosinate ammonium is the salt of PPT (Dennehey *et al.*, 1994), while bialaphos consists of PPT linked with

two L-alanine residues (D'Halluin *et al.*, 1992). Bialaphos only becomes active after intracellular peptidases have cleaved the two alanine residues from the substrate (Figure 1.2; Mullineaux, 1992). Dennehey *et al.* (1994) reported that alanine is also an inhibitor of glutamine synthetase (GS). Alanine released in the plant cell from the enzymatic breakdown of bialaphos could increase the level of GS inhibition above that achieved with PPT alone, and this could explain the superiority of bialaphos in retarding the growth of non-transformed cells. Furthermore, all of the PPT in bialaphos exists as the biologically active L-isomer, while glufosinate ammonium is a commercial preparation that contains a mixture of the active L-isomer and the inactive D-isomer (Dennehey *et al.*, 1994). It was therefore anticipated that the use of bialaphos in the selection procedure would greatly facilitate the identification of transformed cells by more efficiently impeding the growth of non-transformed cells.

However, the efficient inactivation of bialaphos by transformed cells is also likely to protect neighbouring, non-transformed cells during the selection process, and this could allow the non-transformed cells to grow in culture. Resistant callus, composed of transformed and non-transformed cells, could have been removed from the bombarded culture and maintained as an individual line at the end of the sixth week of selection. During subsequent callus maintenance on selection medium, the large population of non-transformed cells could have proliferated further at the expense of the smaller population of transformed cells. This could explain the absence of PAT activity in the other nine bialaphos-resistant callus lines recovered from selection (Section 3.3.2.1). It has been reported that the "cross-protection" of non-transformed cells by transformed cells has allowed the regeneration of transformed and non-transformed plants during rice transformation (Christou *et al.*,

1991) and the regeneration of non-transformed plants at high frequencies (95-100%) during tobacco transformation (Park *et al.*, 1998).

Southern hybridisation was performed to demonstrate that the observed PAT activity of callus lines B1-B4 was the result of the integration of the *bar* gene into the genomic DNA. An *Eco*RI DNA restriction fragment was detected in the genomic DNA of callus lines B1 and B2 that corresponded in size to the portion of pAHC25 that contains the 3' end of the first intron of the maize ubiquitin gene, the *bar* gene, and the *nos* termination region (Figure 3.4). In addition, the presence of extra, *bar* hybridising DNA restriction fragments in the *Eco*RI gel tracks of callus lines B1-B3, which were of approximately equal intensity to the expected DNA fragment, indicated that the *bar* gene could have undergone molecular rearrangement prior to or during genomic integration. Although the diagnostic *Eco*RI restriction sites in pAHC25 (Figure 3.1) could have been altered following transformation, the coding region of at least one insertion of the *bar* gene must have been intact, because callus lines B1-B3 exhibited PAT activity. DNA restriction fragments that generated weak hybridisation signals were also detected following Southern analysis (e.g. in callus lines B2 and B3; Figure 3.4). These restriction fragments may be the product of incomplete digestion of genomic DNA or represent insertions of *bar* gene fragments that were generated by physical disintegration of the intact plasmid during microprojectile bombardment.

The integration of fragments of the original gene construct into the plant genome has been commonly observed among the plants transformed by microprojectile bombardment (Gordon-Kamm *et al.*, 1990; Becker *et al.*, 1994; Wan and Lemaux, 1994; Jähne *et al.*, 1994; Hagio *et al.*, 1995). Since this transformation

method relies on physical force to introduce DNA into plant cells, it is highly probable that these hybridising fragments of lower intensity could be the result of transgene shearing, prior to genomic integration (Pawlowski and Somers, 1996).

The *uidA* gene was neither integrated into the genomic DNA nor expressed in the stained tissues of callus lines B1-B3. Regardless of the events that occurred during transformation, incomplete integration of the entire pAHC25 construct meant that only the *bar* containing elements of this plasmid DNA were successfully transferred into the genomes of callus lines B1-B3. The *Bam*HI and *Hin*dIII digests provided estimates of the number of insertion sites for the *bar* gene in the genomic DNA of callus lines B1-B3. The restriction fragments detected by Southern hybridisation were bordered at one end by the restriction enzyme site located in the ubiquitin-*bar* region of pAHC25 (Figure 3.1) and at the other end by the site located in the plant genomic DNA, near the insertion site of the transgene. The number of *bar* gene insertion sites ranged from one to three among these callus lines (Figure 3.4).

Callus line B4 was recovered from a co-transformation experiment, meaning that two separate gene constructs were simultaneously delivered to suspension-cultured cells. The results from Southern analyses and transgene activity assays demonstrated that only the *bar* containing gene construct (pDM302) was successfully transferred to the genome and expressed in the bialaphos-resistant callus of this transformed line. The molecular analyses also ruled out the possibility that the *uidA* gene had been integrated into the genomic DNA of callus line B4, but was not expressed.

Attempts to regenerate plants from the four independent transformed callus

lines were unsuccessful. When the bialaphos-resistant callus was transferred to culture medium to induce plant regeneration, it had been subjected to a similar period of time in culture as the cell suspension cultures that were used successfully for plant regeneration from isolated protoplasts (Section 2.3.3). Therefore, it seemed unlikely that the prolonged tissue culture phase was the single major reason for the lack of plant regeneration observed here. It was possible that the bombardment conditions were sub-optimal for obtaining transformed barley callus suitable for plant regeneration. While transient expression of the *uidA* gene could be enhanced by the use of more severe bombardment conditions (i.e. higher rupture pressure and more gold per bombardment), GUS active cells could have been severely damaged and unable to regenerate plants.

Based on the results of Southern hybridisation, the transformation frequency observed here (0.13%) was lower than those reported for non-regenerable cell suspension cultures of maize (Spencer *et al.*, 1990; 5-10%) and barley (Ritala *et al.*, 1993; a "conversion" frequency of 1.3%). On the other hand, Gordon-Kamm *et al.* (1990) reported a similar transformation frequency following microprojectile bombardment of embryogenic suspension-cultured cells of maize (one transformant was recovered from 1000 cells that transiently expressed the *uidA* gene). In future studies, the use of more "gentle" bombardment conditions in combination with an altered selection protocol, which either uses a liquid culture-based system to improve cell-to-selection medium contact (Vasil *et al.*, 1991) or a higher concentration of selection agent to eliminate the growth of non-transformed cells, will be required to increase the transformation frequency and improve the likelihood that the target cells retain the capacity to regenerate plants following transformation.

3.5 SUMMARY AND CONCLUSIONS

The work described in this Chapter demonstrates the integration and functional expression of the selectable marker gene, *bar*, in transformed barley callus produced by microprojectile bombardment of suspension-cultured cells. The transformants were characterised by the growth of resistant callus on bialaphos-containing medium, the production of the acetylated-PPT derivative in the PAT assay and the detection of transgene sequences following Southern hybridisation. However, the transformed callus lines exhibited limited regenerative potential, and only albino shoots were produced from one transformed line.

Since the establishment of embryogenic barley cell suspension cultures has proved difficult and long-term cultures possessed a limited capacity for plant regeneration (Chapter Two), it was reasoned that the callus produced by the immature scutellum, shortly after isolation, could be a more receptive target tissue for barley transformation. The use of this type of callus for microprojectile bombardment would eliminate the need to establish embryogenic cell suspension and protoplast cultures prior to transformation and would employ a short *in vitro* culture phase and the high regeneration capacity of the scutellum-derived callus to regenerate fertile plants.

In Chapter Four, the DNA delivery and selection systems developed here have been applied to isolated immature scutella to generate self-fertile, transgenic barley.

CHAPTER FOUR

THE PRODUCTION OF TRANSGENIC BARLEY BY MICROPROJECTILE BOMBARDMENT OF CULTURED IMMATURE SCUTELLA

4.1 INTRODUCTION

Gene transfer by microprojectile bombardment of the scutellar region of the immature embryo has been routinely employed for cereal transformation (Li *et al.*, 1993; Vasil *et al.*, 1993; Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.*, 1996; Takumi and Shimada, 1996; Ortiz *et al.*, 1996; Brettschneider *et al.*, 1997; Witrzens *et al.*, 1998; Jordan, 2000; O'Kennedy *et al.*, 2001; Huber *et al.*, 2002).

Using this procedure, barley has been transformed with selectable marker and reporter genes, and potentially useful genes responsible for pest resistance and quality improvement (Wan and Lemaux, 1994; Ritala *et al.*, 1994; Hagio *et al.*, 1995; Koprek *et al.*, 1996; Jensen *et al.*, 1996; Nuutila *et al.*, 1999; Harwood *et al.*, 2000). The successful transformation of barley has generally relied on the use of immature embryos of the agronomically less desirable cultivar, Golden Promise. This cultivar was chosen for transformation on the basis of its superior *in vitro* culture response, compared with other cultivars examined (Lemaux *et al.*, 1999). However, the insertion of genes into this tissue culture-responsive barley cultivar requires the subsequent and time-consuming transfer of the transgenes into agronomically important germplasm by conventional crossing. If transgenes are closely linked to undesirable traits, the backcrossing program could be protracted. It would therefore be beneficial to apply transformation methods directly to elite breeding lines. Although a number of other barley cultivars have been transformed by microprojectile bombardment, including the Finnish cultivar, Kymppi (Ritala *et al.*, 1994), and the North American cultivars, Galena, Harrington and Conlon (Cho *et al.*,

1998; Manoharan and Dahleen, 2002), no success has been reported for commercially important Australian cultivars.

In this study, tissue culture and microprojectile bombardment-mediated transformation protocols are described for the immature scutella of two elite Australian barley cultivars, namely Schooner and Sloop. These protocols led to the regeneration of transformed plants. DNA analyses and enzyme activity assays were used to demonstrate the integration and functional expression of the *bar* and *uidA* genes in the primary transformants (T_0). The transmission and expression of both transgenes were also examined in the progeny plants (T_1) derived from two independent primary transformants.

4.2 MATERIALS AND METHODS

4.2.1 Isolation and culture of immature scutella for microprojectile bombardment

The growth conditions for the donor Schooner and Sloop plants, and the procedure used for grain surface-sterilisation were described in Section 2.2.1. Using a dissecting microscope, the immature embryos were aseptically excised from the grains, and the scutella were isolated by carefully removing the entire embryonic axis. The isolated scutella were cultured scutellum side-up on callus induction medium, MSB3D (Bregitzer 1992; Appendix 2), supplemented with 10 μM $CuSO_4$ (MSB3D10Cu). The medium was solidified with 0.8% (w/v) Difco Bitek™ agar (Becton Dickinson, Sparks, MD, USA), and the pH was adjusted to 5.8 prior to steam sterilisation. The cultures were incubated in the dark at 22-24°C.

4.2.2 Gene constructs used for microprojectile bombardment

The details of pDM302 and p*Act*1-DGUS are described in Section 3.2.2. Plasmid pIGNEII was constructed by Dr KJ Nunan (University of Adelaide, unpublished). To produce this gene construct, the promoter region of the barley (1→3,1→4)-β-glucanase isoenzyme EII gene, starting from the transcription start point and including 1006 bp of the 5' upstream region of the gene's promoter, was inserted into the promoter-less plasmid pIGN. The pIGN vector contains the first intron of the maize alcohol dehydrogenase gene (*Adh-1*) linked to the *uidA* gene (Olive *et al.,* 1990) and was kindly donated by Dr JV Jacobsen (CSIRO Plant Industry, Canberra, ACT, Australia). This plasmid contains the 3' untranslated region (UTR) and the polyadenylation signal of the nopaline synthase (*nos*) gene from

FIGURE 4.1

Schematic representation of the gene constructs used for the microprojectile bombardment experiments, indicating the position of the restriction enzyme sites used for the molecular analyses

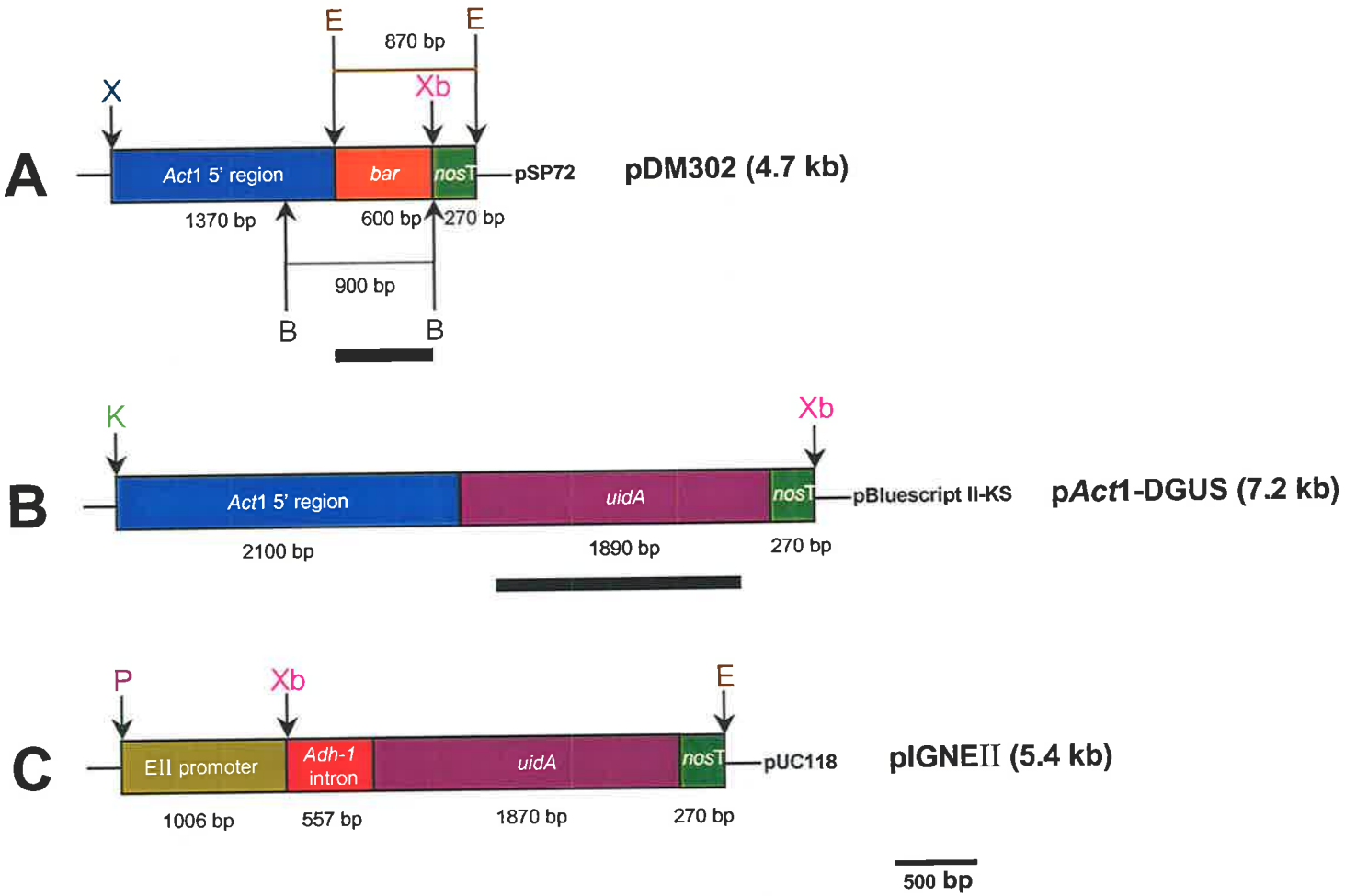
A pDM302.

B p*Act*1-DGUS.

C pIGNEII.

Abbreviations: *Act*1: promoter, first exon and intron of the rice actin 1 gene; *bar:* coding region of the phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus*; *nos*T: transcript termination region of the nopaline synthase gene from *Agrobacterium tumefaciens*; *uidA*: coding region of the β-glucuronidase gene from *E. coli*; EII: promoter region of the barley (1→3,1→4)-β-glucanase isoenzyme EII gene; *Adh-1*: the first intron of the maize alcohol dehydrogenase gene.

Restriction enzyme sites used for DNA analyses are shown in coloured letters. Solid coloured lines indicate the DNA restriction fragments expected from the digests performed. Abbreviations of the restriction enzyme sites: B: *Bam*HI; E: *Eco*RI; K: *Kpn*I; P: *Pst*I; X: *Xho*I; Xb: *Xba*I. The *bar* containing DNA fragment isolated from pDM302 and the *uidA* containing DNA fragment prepared from p*Act*1-DGUS by PCR (indicated by solid black lines) were used as gene probes in the Southern analyses.


A
E
E
870 bp
X
Xb
Act1 5' region
bar
nosT
pSP72
pDM302 (4.7 kb)
1370 bp
600 bp
270 bp
900 bp
B
B
B
K
Xb
Act1 5' region
uidA
nosT
pBluescript II-KS
pAct1-DGUS (7.2 kb)
2100 bp
1890 bp
270 bp
C
P
Xb
E
EII promoter
Adh-1 intron
uidA
nosT
pUC118
pIGNEII (5.4 kb)
1006 bp
557 bp
1870 bp
270 bp
500 bp

Agrobacterium tumefaciens (Bevan *et al.*, 1983). Schematic maps of the three gene constructs are shown in Figure 4.1. Plasmid DNA of the three gene constructs was prepared using the procedure described in Section 3.2.2.

4.2.3 Microprojectile bombardment

The DNA-coated gold particles were prepared according to the protocol described in Section 3.2.3, with minor modifications. To a 1.5 ml Eppendorf tube containing 50 µl vortexed gold particle suspension (1.25 mg), 3.5 µl pDM302 and p*Act*1-DGUS or pIGNEII (1 µg/µl) were added. Gold particles were coated with the plasmid DNA by adding 100 µl $CaCl_2$ (2.5 M) and 40 µl spermidine (0.1 M; free base, tissue culture tested), in that order. The tube was vortexed continuously for 3 min and centrifuged at 93 x *g* for 10 sec. The supernatant was removed and the DNA-coated microcarriers were resuspended in 250 µl absolute ethanol with gentle vortexing. Following centrifugation and removal of the supernatant, the microcarriers were finally resuspended in 250 µl absolute ethanol.

The scutella were bombarded five to fourteen days after culture initiation. Twelve to fifteen scutella were arranged scutellum side-up in the centre of a 55 mm x 14 mm plastic Petri dish (Sarstedt Australia, Technology Park, SA, Australia) containing MSB3D10Cu supplemented with equimolar amounts of sorbitol and mannitol at a final concentration of 0.4 M. The osmoticum treatment started 4 h before bombardment and continued for 20 h after bombardment. The PDS-1000/He Biolistic® Particle Delivery System (Section 3.2.3) was used for microprojectile bombardment. For each bombardment, 10 µl DNA-coated microcarrier solution (50 µg gold particles and 0.28 µg mixed DNA) was dispensed onto a macrocarrier. The

macrocarrier holder was positioned 2.5 cm (gap distance) from the rupture disk holder, and the macrocarrier was placed 6 mm from the stopping screen. The scutella were placed on the second shelf below the macrocarrier launch assembly (5 cm from the stopping screen) and bombarded once with either 900 or 1100 psi rupture disks under a partial vacuum of 28 inch Hg. Each experiment included control plates of either non-bombarded scutella or scutella bombarded with only gold particles.

Immediately after the post-bombardment incubation on the osmoticum treatment, the scutella were transferred to 90 mm x 14 mm plastic Petri dishes containing fresh MSB3D10Cu medium, and callus formation was induced for three to four weeks in the dark at 22-24°C.

4.2.4 Selection of bialaphos-resistant callus and regeneration of putative transformants

The herbicide, bialaphos, (Shinyo Sangyo Co. Ltd., Tokyo, Japan) was used to select putative transformed callus and regenerated plants.

The bialaphos concentration used for selection was based on an experiment where 2-4 mm pieces of non-transformed embryogenic callus were cultured on callus induction medium containing different concentrations of bialaphos (0, 3.2, 6.4, 16, 32, and 80 μM). The experiment was continued for two subcultures, made at monthly intervals, to monitor callus development. The growth of non-transformed cells was efficiently inhibited with 16 μM bialaphos, and this concentration was therefore used for the selection of putative transformed callus.

Selection commenced three to four weeks after bombardment. The callus derived from an individual scutellum was broken into 2-4 mm pieces and transferred to MSB3D medium that contained 1 μM $CuSO_4$ and 16 μM bialaphos (MSB3D1CuBL16). The cultures were incubated for three weeks in the dark at 22-24°C. At the end of the first subculture on selection medium, light-yellow callus that showed vigorous growth was separated from brown, necrotic tissue and cultured on fresh selection medium for a further four weeks. During the subsequent selection passages, callus that continued to grow was handled in a similar manner. Using the procedure developed by Wan and Lemaux (1994), multiplication of the original piece of putative transformed callus led to the generation of a uniformly growing, bialaphos-resistant callus line. The resistant callus was maintained by monthly subculture on MSB3D1CuBL16.

For plant regeneration, embryogenic sectors of the bialaphos-resistant callus line were transferred to hormone-free MS medium that contained 1 μM $CuSO_4$ and 3.2 μM bialaphos (MSB01CuBL3.2) and cultured in the light (16 h day/8 h night photo-period) at 22-24°C. All the plants regenerated from the same bialaphos-resistant callus were considered clonal plants of the same transformation event, until shown otherwise by Southern analysis. After six to eight weeks, the putative transformed plants were transferred to soil and maintained according to the procedure outlined in Section 2.2.2.

4.2.5 Histochemical β-glucuronidase (GUS) assay

GUS activity was detected using the technique described in Section 3.2.4. Methanol was included in the staining solution at a final concentration of 15% (v/v) to

suppress endogenous β-glucuronidase activity (Kosugi *et al.*, 1990). The expression of the *uidA* gene was assayed 48 h after bombardment by incubating the scutella in the staining solution at 37°C for 24 h. GUS activity in the bialaphos-resistant callus and the tissues of the putative transformants was studied in the same manner described for the bombarded scutella. To detect GUS activity in the leaves of the putative transformants and their progeny plants after staining, the chlorophyll was extracted from the leaf pieces by incubating them in 70% (v/v) ethanol at 37°C for 24 h (Zainuddin, 2000).

4.2.6 Phosphinothricin acetyltransferase (PAT) assay

PAT activity assays for bialaphos-resistant callus and putative transformed plants were performed using the procedure described in Section 3.2.6.

4.2.7 Small scale isolation of plant genomic DNA

Total genomic DNA was extracted from leaf tissue using the method described by Langridge *et al.* (1997). Approximately 0.2 g leaf tissue was ground with a pestle to a fine powder under liquid nitrogen. The powder was transferred to a 2 ml Eppendorf tube that contained 600 µl DNA extraction buffer (100 mM Tris-HCl buffer, pH 8.5, containing 1% w/v sarkosyl, 100 mM NaCl and 10 mM Na_2EDTA). The sample was homogenised using a hand-held plastic micropestle. Phenol:chloroform (1:1; 600 µl) was added to the sample, and the tube was shaken vigorously and placed on ice for 5 min for partial phase separation. The tube was centrifuged at 18280 x *g* for 10 min to separate the phases. The upper, aqueous phase was removed with a micropipette and transferred to a new plastic tube. Sodium acetate (60 µl, 3 M, pH 4.8) and iso-

propanol (600 µl) were added to the new tube. The tube was inverted gently and placed at -20°C for 30 min to precipitate the genomic DNA. The tube was centrifuged at 18280 x *g* for 10 min, the supernatant was poured off, and the DNA pellet was washed with 70% (v/v) ethanol. The tube was centrifuged at 18280 x *g* for 5 min, the supernatant was removed, and the DNA pellet was air-dried for 30 min. The DNA pellet was resuspended overnight in 30 µl R40 buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1 mM Na_2EDTA and 40 µg/ml pancreatic RNaseA).

4.2.8 Southern hybridisation

Digestion of DNA was performed according to the procedure of Langridge *et al.* (1997). For each sample, 6 µl DNA, 1 µl 10 x restriction enzyme buffer, 1 µl acetylated BSA (1 mg/ml), 1 µl 40 mM spermidine and 1 µl restriction enzyme (10 or 20 U/µl, depending on the enzyme) were added to a 1.5 ml Eppendorf tube. All restriction enzymes and buffers were purchased from New England BioLabs (Beverly, MA, USA). The reaction mixtures were vortexed briefly, centrifuged at 18280 x *g* for 10 sec, and the tubes were incubated at 37°C for 5 h. To terminate enzyme activity, 2 µl 6 x loading buffer, described in Section 3.2.8, was added to each tube.

Gel electrophoresis was performed using the procedure described in Section 3.2.8. The Southern blotting protocol (Southern, 1975) was performed as described in Section 3.2.8, except that the transfer solution was 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0).

After DNA transfer, the membrane was rinsed with 5 x SSC and air-dried on paper towel. The membrane, with the DNA side-up, was placed on a stack of

Whatman 3MM filter paper (Maidstone, Kent, UK) soaked with 0.4 M NaOH for 20 min. The membrane was transferred to 200 ml neutralising solution (0.5 M Tris-HCl buffer, pH 7.2, containing 1.5 M NaCl and 1 mM Na_2EDTA) for 5 min followed by a rinse with 2 x SSC for 2 min. The membrane was air-dried on paper towel and prepared for hybridisation.

The preparation of the radiolabelled probes, the hybridisation procedure, the washing conditions used for the membrane, and autoradiography were performed as described in Section 3.2.8.

4.2.9 Isolation and incubation of aleurone layers of T_1 grains for histochemical GUS analysis

The native barley (1→3,1→4)-β-glucanase isoenzyme EII gene is normally transcribed in the aleurone layer of the germinated grain (Stuart *et al.*, 1986; Slakeski and Fincher, 1992). To determine whether the promoter region of the pIGNEII gene construct regulated expression of the *uidA* reporter gene in a similar manner, aleurone layers were isolated from T_1 grains and subjected to the histochemical GUS assay.

The barley aleurone layers were isolated according to the procedure of Chrispeels and Varner (1967), with minor modifications. The embryo and distal end were removed from non-transformed barley grains and from the T_1 grains of the transformed plant line. The grain sections were sterilised in 2% (v/v) Domestos® bleach (Lever Rexona, NSW, Australia; active ingredients 5.25% w/v sodium hypochlorite, 1.25% w/v sodium hydroxide and 0.5 g/L "alkaline salts") for 40 min. The grain sections were washed three times with sterile 10 mM HCl, once with sterile

water, once with sterile 10 mM HCl and finally four times with sterile water (Stuart *et al.*, 1986). The sections were sandwiched between moist sterile filter paper contained in 90 mm x 14 mm plastic Petri dishes (Sarstedt Australia) and incubated for three days in the dark at room temperature. After this incubation period, the aleurone layers were prepared by cutting along the creases of the grains and gently scraping away the endosperm with a scalpel and forceps. The isolation of the aleurone layers was performed under sterile conditions. Twenty layers were placed in a 25 ml conical flask and incubated in 2.5 ml sterile 10 mM $CaCl_2$, pH 5.5, which contained 100 mg/L neomycin sulphate, 100 mg/L chloramphenicol and 20 mg/L nystatin (Stuart *et al.*, 1986). The layers were incubated on an orbital shaker (40 rpm) for up to four days in the dark at 25°C. Using the X-GLUC solution described in Section 3.2.4, GUS activity was monitored in freshly isolated layers and in layers removed at 24 h intervals after the commencement of incubation.

TABLE 4.1

Callus induction and plant regeneration frequencies for non-bombarded immature scutella of cv. Schooner cultured on various concentrations of copper sulphate

Copper concentration (μM)	Number of cultured scutella	Number of scutella that produced embryogenic callus	Mean number of viable plants produced per cultured scutellum
0.1 [1]	60	39	0.60
10	54	40	3.09
50	55	37	0.78

[1] Standard MS concentration.

4.3 RESULTS

4.3.1 Improved plant regeneration frequencies for barley callus using elevated levels of copper sulphate ($CuSO_4$)

The effect of increased copper sulphate concentration on embryogenic callus production was investigated for immature scutella of cv. Schooner. The production of embryogenic callus was not dependent on the concentration of $CuSO_4$ in the callus induction medium, which was in the range of 0.1 μM and 50 μM (Table 4.1). However, the highest number of regenerated plants was achieved from callus cultures initiated on medium containing 10 μM $CuSO_4$. At this concentration, there was a 4-5 fold increase in the number of plants regenerated per scutellum compared with the scutellum-derived callus produced on culture medium that contained either 0.1 μM or 50 μM $CuSO_4$ (Table 4.1). A similar observation was made for Sloop, the other cultivar used for the transformation experiments (data not shown).

Although a comparative study of bombarded scutella was not performed, this modification to the culture conditions was incorporated into the transformation and selection experiments with the expectation that it would assist with the recovery of plants from putative transformed barley callus.

4.3.2 Bombardment and selection of putative transformed callus lines

To demonstrate that the modified copper content of the culture medium would be useful for the development of a transformation protocol for cvs. Schooner and Sloop, immature scutella were bombarded with two different plasmids, and putative transformed callus was monitored for improvement in plant regeneration. One

FIGURE 4.2

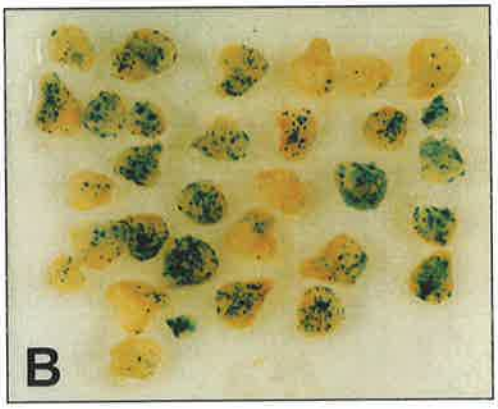
Histochemical GUS analyses in bombarded immature scutella and putative transformed callus

A and B Two days after bombardment, scutella transformed with pIGNEII (A) and p*Act*1-DGUS (B) were assayed for transient expression of the *uidA* gene.

C GUS activity, driven by the rice actin promoter, in putative transformed callus line 3 (top of the plate).

Non-transformed barley callus treated with the X-GLUC staining solution is shown at the bottom of the plate.


A


B

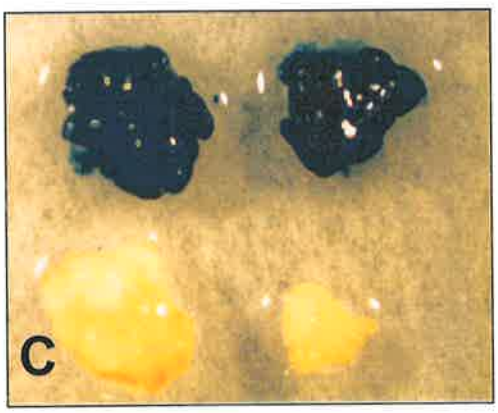

C

FIGURE 4.3

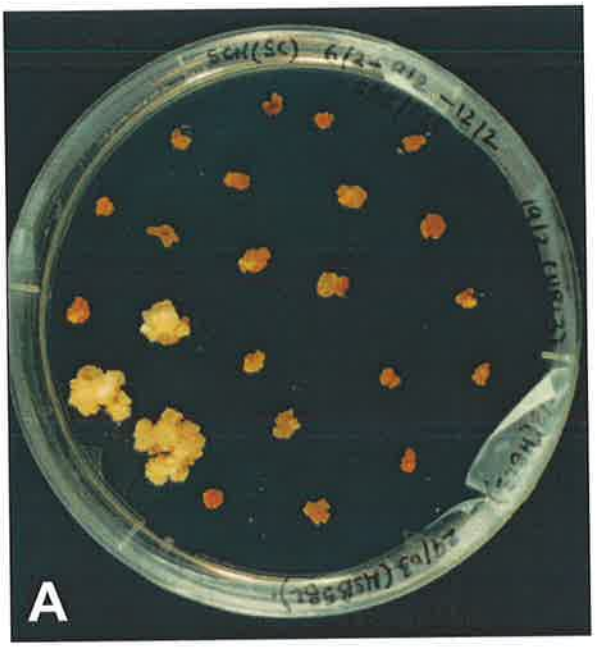
Selection of putative transformed barley callus and the regeneration of bialaphos-resistant plants

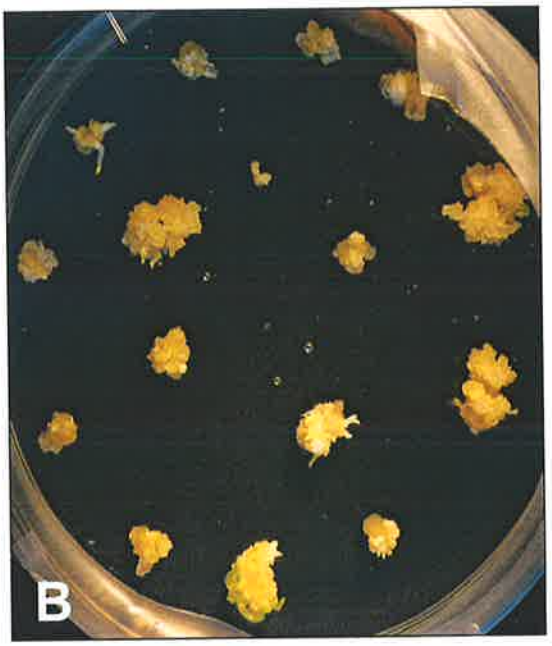
A Emergence of bialaphos-resistant callus.

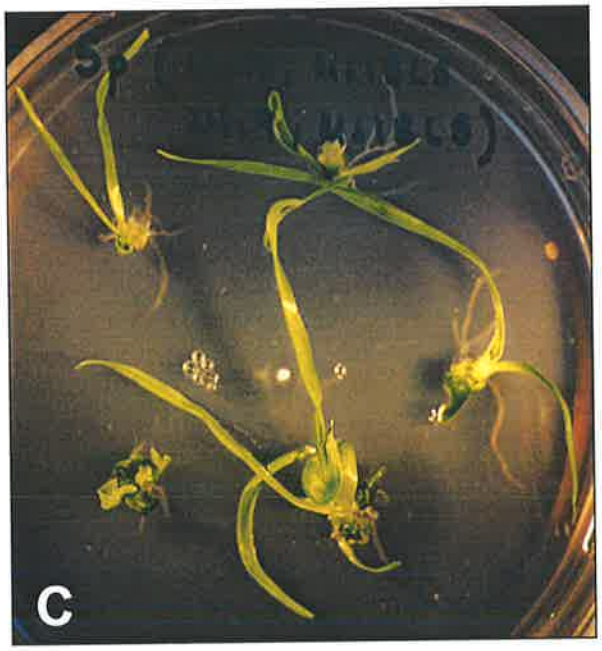
B Growth of a bialaphos-resistant callus line on culture medium supplemented with 16 μM bialaphos.

C Regeneration of putative barley transformants on hormone-free culture medium that contained 3.2 μM bialaphos.

D Fertile putative primary transformant established in soil.


A


B


C


D

plasmid carried the selectable marker gene, *bar*, and the other contained the reporter gene, *uidA*.

Two days after bombardment, a random selection of bombarded scutella was chosen from each experiment and subjected to histochemical GUS analysis. Although there was variation in the number of blue spots per scutellum, both the barley (1→3,1→4)-β-glucanase and rice actin promoters directed transient expression of *uidA* gene in the bombarded scutella (Figure 4.2 A and B).

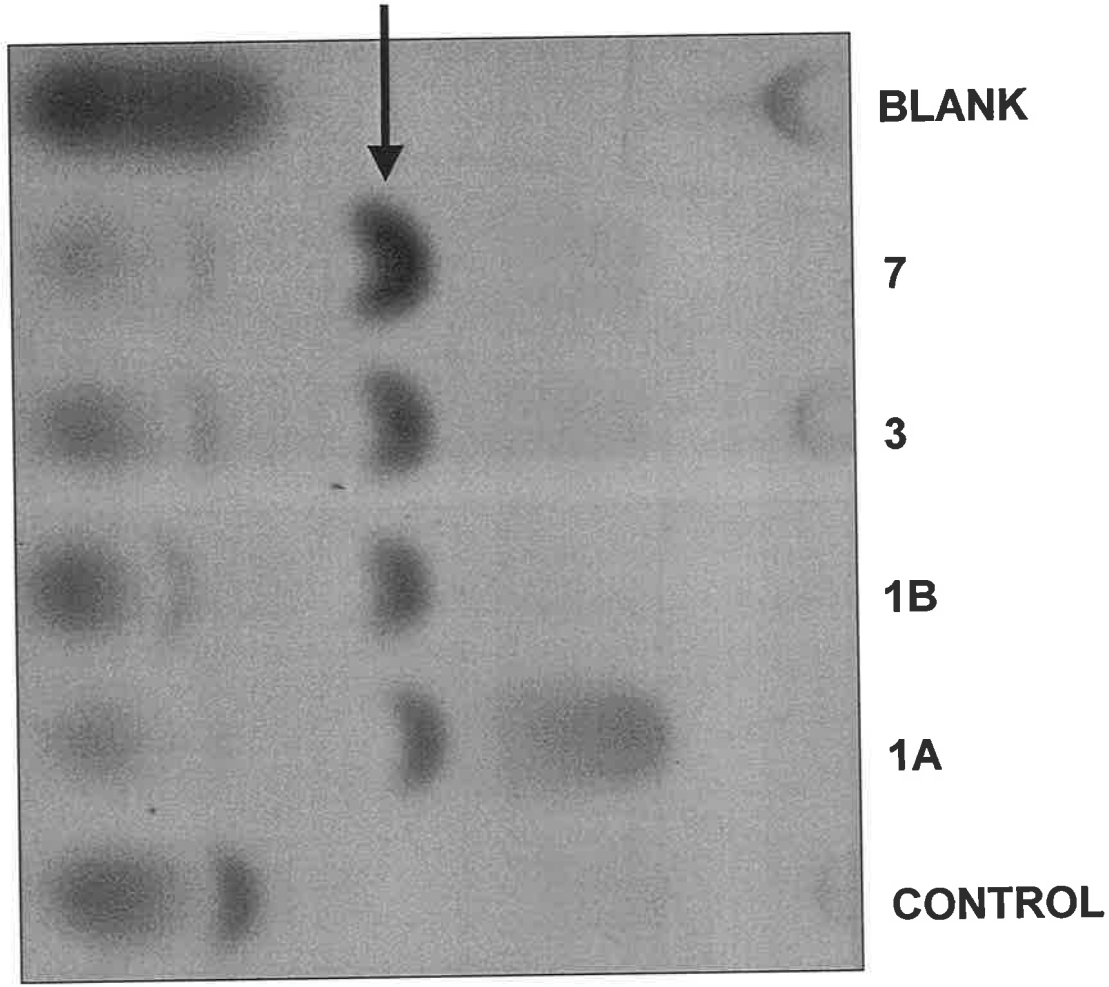
The barley scutella that were not used for the histochemical GUS analysis were cultured on callus induction medium for three to four weeks. During the post-bombardment culture period without selection, the bombarded scutella showed levels of callus formation similar to non-bombarded tissues. The callus produced from the bombarded scutella was broken into small pieces and transferred to selection medium.

The putative transformed callus was initially identified by its growth on bialaphos-containing medium. After four weeks of selection, the majority of the callus pieces had died, while only a few pieces of callus remained healthy and yellow in appearance. The necrotic pieces were discarded, while the surviving callus sectors were removed, broken into small pieces and transferred to fresh selection medium. During the second and third selection cycles, some callus pieces grew vigorously, while the growth of the remaining pieces was severely inhibited (Figure 4.3 A). The bialaphos-resistant callus was divided into small pieces and transferred to fresh selection medium for a number of additional selection cycles to obtain four putative transformed lines devoid of non-transformed tissue and composed of healthy, fast-growing callus (Figure 4.3 B). The bialaphos-resistant callus lines were

FIGURE 4.4

PAT activity in protein extracts of independent bialaphos-resistant callus lines

The first lane contains extract from non-bombarded barley callus (Control). The next four lanes contain extracts from four putative transformed callus lines (1A, 1B, 3 and 7). The sixth lane was blank and the reaction was made up to volume with water instead of cellular extract. The arrow indicates the band corresponding to acetylated PPT.


BLANK
7
3
1B
1A
CONTROL

assigned a number that corresponded to the bombardment experiment, while a letter was used to identify different lines derived from the same experiment. The putative transformed callus lines are hereafter referred to as 1A, 1B, 3 and 7.

When the non-bombarded scutella and the scutella bombarded with only gold particles were subjected to the same selection procedure, no bialaphos-resistant callus was recovered.

4.3.3 Expression of the *bar* and *uidA* genes in bialaphos-resistant callus lines

Transient GUS assays demonstrated that the foreign DNA was successfully delivered into the cells of the barley scutella following microprojectile bombardment (Figure 4.2 A and B). The subsequent growth of callus on bialaphos-containing medium provided strong evidence for the stable integration and functional expression of the selectable marker gene, *bar*, in the barley genome of the putative transformants (Figure 4.3 A and B). Protein extracts of callus lines 1A, 1B, 3 and 7 were shown to contain detectable levels of PAT activity using the chromatographic assay method (Figure 4.4). No PAT activity was detected in the protein extract of the non-transformed barley callus.

A small amount of tissue from the four bialaphos-resistant callus lines was analysed for histochemical expression of the *uidA* reporter gene. Callus line 3 demonstrated very strong expression of the reporter gene with all tested callus pieces displaying uniform, dark-blue staining (Figure 4.2 C). This callus line was identified as being PAT^+ and GUS^+, because it expressed both the selectable marker gene and the reporter gene. However, the bialaphos-resistant callus lines 1A, 1B and 7, which were recovered from scutella bombarded with the pIGNEII gene

construct, remained yellow-white in colour in the presence of the X-GLUC substrate. These callus lines that expressed *bar* but not *uidA* were scored as being PAT^+ and GUS^-. The callus pieces derived from the non-bombarded scutella did not exhibit histochemical GUS activity when they were incubated in the X-GLUC staining solution (Figure 4.2 C).

4.3.4 Regeneration of putative transformed plants

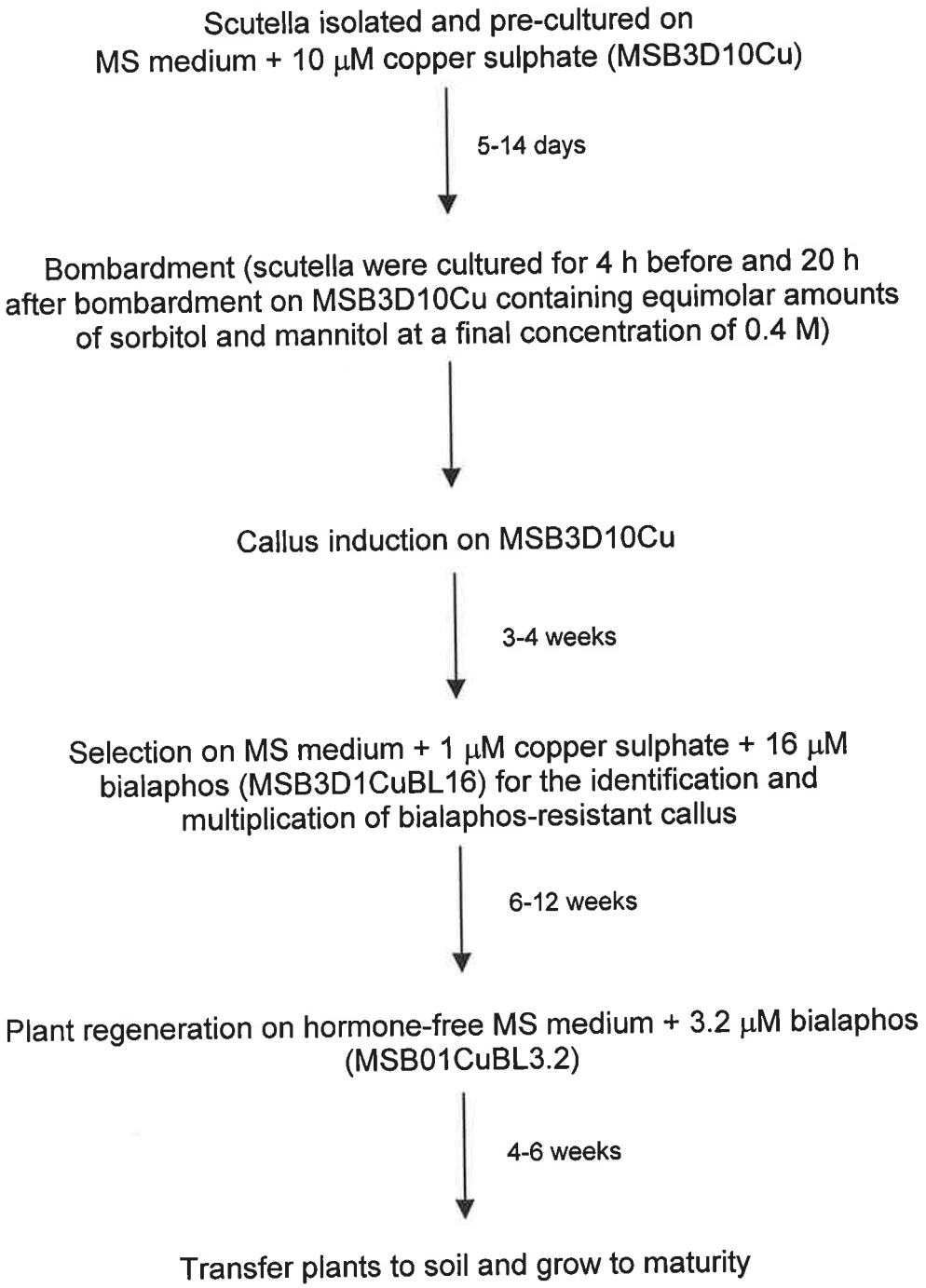
The repeated subculture of bialaphos-resistant callus on selection medium, MSB3D1CuBL16, inevitably led to the formation of embryogenic callus sectors (Figure 4.3 B) that produced plants on regeneration medium, MSB01CuBL3.2 (Figure 4.3 C).

Although plants were regenerated from all of the putative transformed callus lines, the ability of the different callus lines to produce plants was highly variable. For example, two lines produced a single plant, while another extremely responsive line regenerated greater than 150 tissue culture-derived plants. One hundred and ninety-two plants were regenerated from the four bialaphos-resistant callus lines.

The putative transformed plants were transferred to soil and grown to maturity (Figure 4.3 D). Although seven plants recovered from callus line 3 were morphologically abnormal and unable to set seed, most primary transformants were phenotypically similar to seed-grown, non-transformed plants and were self-fertile, despite differences in seed set. The variation in seed set appeared more pronounced in the plants recovered from different lines than among the plants regenerated from the same bialaphos-resistant callus line. However, there were two cases where the same bialaphos-resistant callus line regenerated both fertile and

FIGURE 4.5

Flow diagram of the processes used for the production of bialaphos-resistant barley plants



sterile plants. No albino shoots or plants were produced, despite the fact that the plant regeneration capacity of the bialaphos-resistant callus lines declined with prolonged culture.

The average time required for each step involved in the regeneration of the putative barley transformants is summarised in Figure 4.5. In general, the time frame from microprojectile bombardment of the pre-cultured scutella to anthesis of the regenerated plants was six to eight months. The exact times varied for each putative transformed line, depending on how quickly the bialaphos-resistant callus emerged on selection medium and the regenerated plants developed in culture.

4.3.5 Analysis of putative transformed plants

The plants recovered from selection and successfully transferred to soil were tested for the functional expression of both the *bar* and *uidA* genes.

Leaf samples were taken from a single plant regenerated from three different PAT^+ callus lines (1A-1, 3-3 and 7-1) and assayed for PAT activity; the acetylated form of PPT was detected for plants 3-3 and 7-1, but not for plant 1A-1 (data not shown).

Histochemical GUS activity was initially scored in the young leaf samples of plants established in soil. One hundred and twenty-eight plants, recovered from the four bialaphos-resistant callus lines, were tested for expression of the *uidA* reporter gene; 104 regenerated plants scored GUS^+ and the remaining 24 plants scored GUS^-, following the incubation of the leaf tissues in the X-GLUC staining solution. The intensity and location of reporter gene expression was highly variable in the stained leaves of the plants regenerated from the different callus lines. The six

FIGURE 4.6

Histochemical GUS activities in different tissues of putative barley transformants

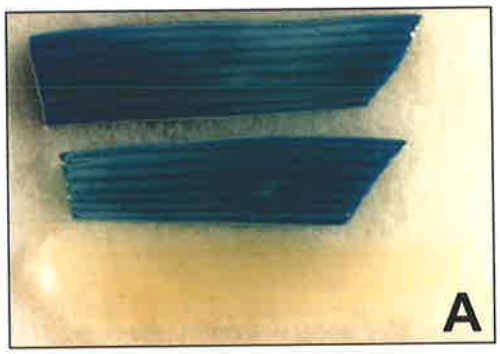
A Histochemical GUS activity, driven by the rice actin promoter, in the leaf tissue of primary transformant 3-3. Control leaf tissue at the bottom.

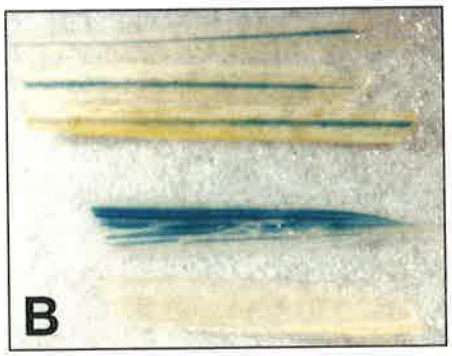
B GUS activity, directed by the (1→3,1→4)-β-glucanase promoter, in the leaf segments of primary transformant 1A-1. Control leaf tissue at the bottom.

C A tissue culture plant regenerated from callus line 7 showing variable expression of the *uidA* gene.

D GUS activity, driven by the rice actin promoter, in the roots isolated from primary transformant 3-3. Control root at the bottom.

Histochemical GUS activity in intact reproductive organs (anthers and ovary, **E**), sectioned immature T_1 grains (**F**) and isolated aleurone layers of T_1 grains of primary transformant 1A-1 (right-hand well, **G**). Aleurone layers isolated from non-transformed grains are shown in the left-hand well.


A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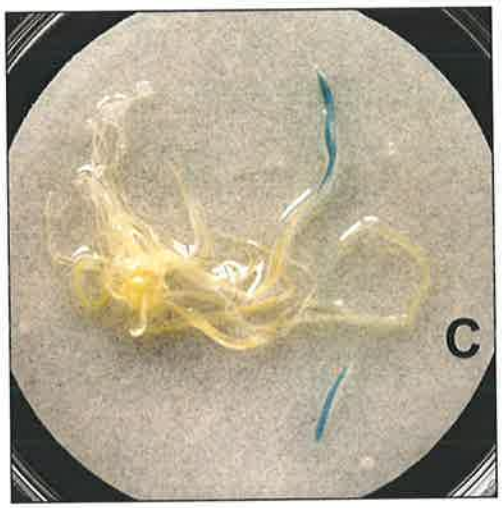
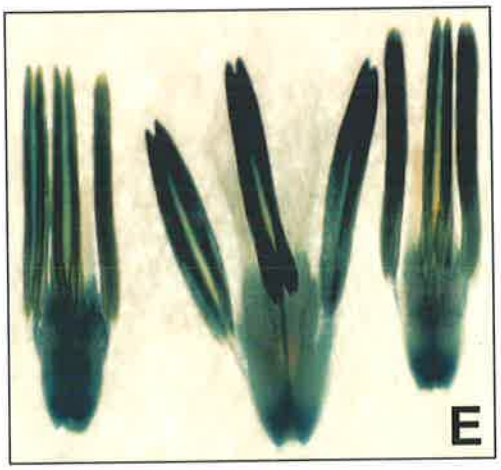

C


D


F


E


G

plants regenerated from GUS^+ callus line 3 showed very strong, dark-blue staining in the young leaves (Figure 4.6 A). The other GUS^+ plants were regenerated from GUS^- callus lines 1A and 7. The GUS activity for plant 1A-1 was predominantly observed in the vascular tissue of the stained leaves (Figure 4.6 B). The 24 GUS^- plants were recovered from callus lines 1B and 7. The 17 GUS^- plants regenerated from callus line 7 were later shown to be GUS^+ in the floral tissues, suggesting that there were GUS^- leaves present in the regenerated plants. When whole tissue culture plants derived from callus line 7 were incubated in the X-GLUC staining solution, irregular GUS activity was detected in the stained leaves (Figure 4.6 C). No GUS activity was detected in any of the stained tissues of the seven plants recovered from callus line 1B.

The p*Act*1-DGUS and pIGNEII gene constructs were also expressed in other differentiated tissues of the putative transformants. For p*Act*1-DGUS, these tissues included the young roots (Figure 4.6 D) and the floral parts (data not shown) of plant 3-3. The barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter directed GUS activity in the stained floral tissues (Figure 4.6 E), the sectioned immature T_1 grains (Figure 4.6 F), and the isolated aleurone layers of mature T_1 grains of regenerated plant 1A-1 (Figure 4.6 G). Similar levels of GUS activity were detected in the isolated aleurone layers of T_1 grains that carried the pIGNEII gene construct, regardless of the duration of pre-culture prior to staining with the X-GLUC substrate.

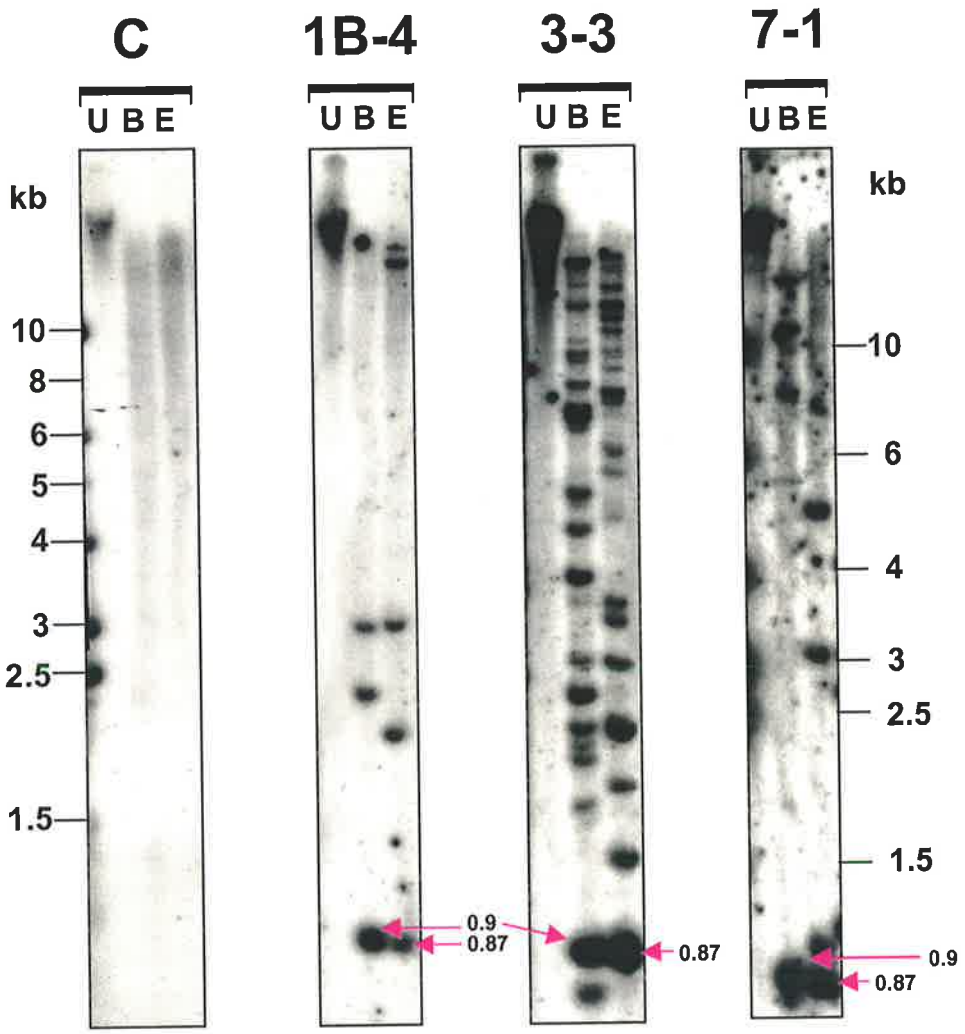
The transgene expression studies of the four bialaphos-resistant callus lines revealed that callus line 3 expressed the *bar* and *uidA* genes (Section 4.3.3). However, transgene expression studies of the putative transformants revealed that the plants regenerated from two bialaphos-resistant callus lines (3 and 7) expressed

FIGURE 4.7

Southern analysis of putative transformed plants regenerated from three independent PAT$^+$ callus lines

Genomic DNA (U: Uncut; B: *Bam*HI-digested; E: *Eco*RI-digested) of three putative transformants (1B-4, 3-3, 7-1) and a non-transformed barley plant (C) was hybridised with a ^{32}P-labelled *bar* gene probe. The arrows indicate the expected *bar* hybridising fragments in the *Bam*HI (0.9 kb) and the *Eco*RI (0.87 kb) tracks.

The mobility of the DNA marker fragments is included on the left-hand side of the first panel and on the right-hand side of the last panel.


C
1B-4
3-3
7-1
U B E
U B E
U B E
U B E
kb
10
8
6
5
4
3
2.5
1.5
0.9
0.87
0.87
kb
10
6
4
3
2.5
1.5
0.9
0.87

both the selectable marker gene and the reporter gene. Southern hybridisation was used to confirm the stable integration of the transgenes in the genomic DNA of plants that expressed the *bar* and *uidA* genes.

To detect rearrangements in the coding region of the *bar* gene, plant genomic DNA was digested with restriction enzymes that release DNA fragments containing the intact coding sequence of the selectable marker gene. For plasmid pDM302, the coding region-releasing restriction enzymes are *Bam*HI and *Eco*RI. It was anticipated that the *bar* probe would hybridise to a 0.9 kb *Bam*HI restriction fragment. This hybridising fragment contains the coding region of the *bar* gene (0.6 kb) and the 3' end of the first intron of the rice actin gene (0.3 kb) (Figure 4.1). In the same way, the *bar* probe was expected to hybridise to a 0.87 kb *Eco*RI restriction fragment. This fragment contains the coding region of the *bar* gene (0.6 kb) and the *nos* termination region (0.27 kb) (Figure 4.1). Southern analysis detected these diagnostic DNA restriction fragments and suggested the presence of multiple intact *bar* coding regions in the genomic DNA of three plants, 1B-4, 3-3 and 7-1 (Figure 4.7). Other hybridising *Bam*HI and *Eco*RI DNA restriction fragments were also detected in the plant genomic DNA of these lines (Figure 4.7).

To estimate the number of selectable marker gene insertions for the three plants, genomic DNA was digested with restriction enzymes that cut pDM302 once and probed with the coding region of the *bar* gene. For pDM302, the restriction enzymes that cut the plasmid once are *Xba*I and *Xho*I (Figure 4.1). The size of the hybridising band(s) is determined either by the distance between the restriction site of the plasmid and the nearest site in the plant DNA flanking the inserted plasmid or by the distance between the unique restriction sites of adjacent insertions of the

FIGURE 4.8

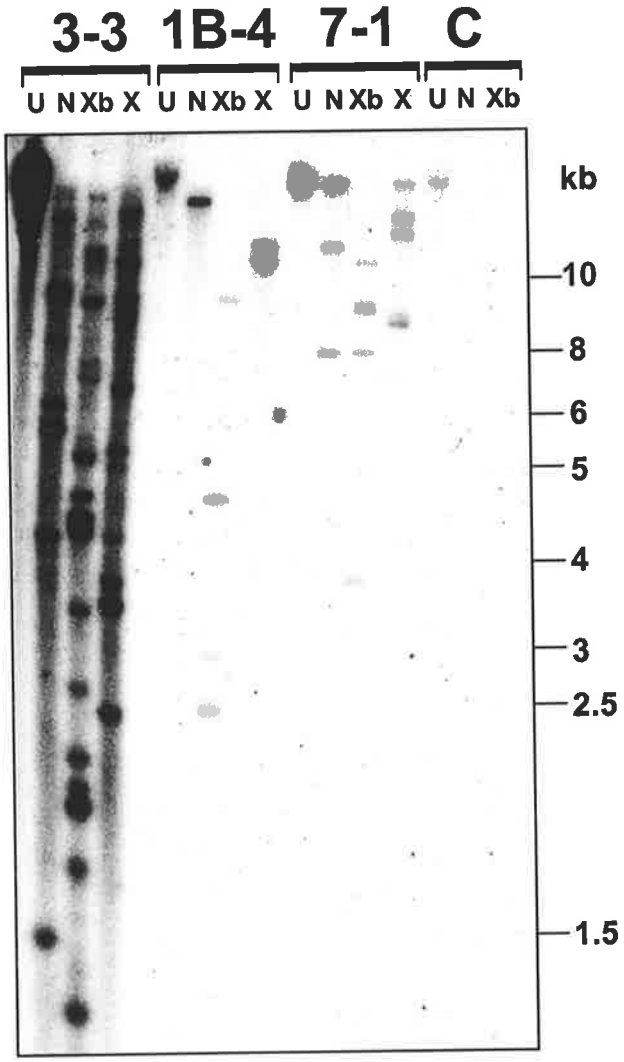
Autoradiographs of Southern blots hybridised with the *bar* gene fragment from pDM302 and the *uidA* gene fragment from p*Act*1-DGUS

A Genomic DNA (U: Uncut; N: *Nco*I-digested; Xb: *Xba*I-digested; X: *Xho*I-digested) of three putative transformants (3-3, 1B-4, 7-1) and a non-transformed barley plant (C) was hybridised with a ^{32}P-labelled *bar* gene probe.

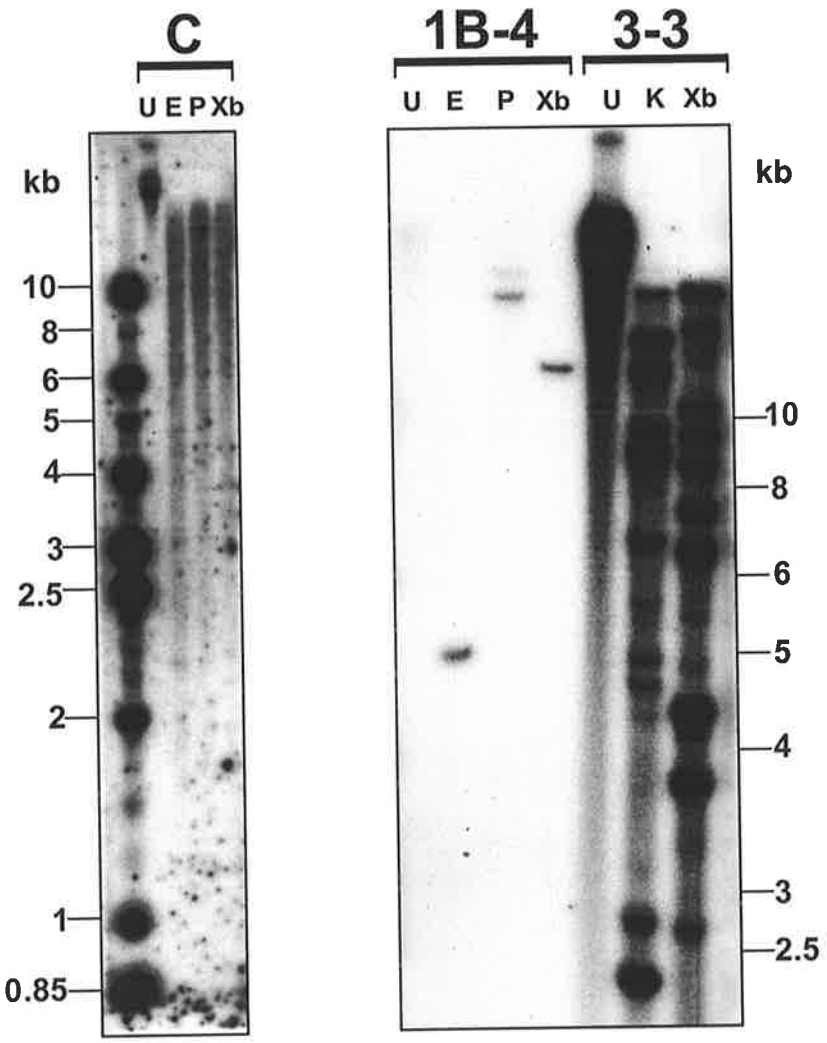
B Genomic DNA (U: Uncut; E: *EcoR*I-digested; K: *Kpn*I-digested; P: *Pst*I-digested; Xb: *Xba*I-digested) of two putative transformants (1B-4 and 3-3) and a non-transformed barley plant (C) was hybridised with a ^{32}P-labelled *uidA* gene probe.

The mobility of the DNA marker fragments is included on the right-hand side of panel **A** and on the left- and right-hand sides of the panels in **B**.

A



B



pDM302 gene construct. Southern analysis detected multiple *bar* hybridising fragments in the *Xba*I and *Xho*I-digested genomic DNA of the three plants (Figure 4.8 A). The presence of five hybridising bands in the *Xba*I and *Xho*I-digested DNA of transformant 7-1 suggested that five *bar* coding regions were inserted in the plant genome (Figure 4.8 A). The estimated number of *bar* gene insertions for plant 3-3 was greater than 10 and between 2-4 for plant 1B-4 (Figure 4.8 A).

The *Bam*HI and *Eco*RI-digested DNA of these plants revealed extensive rearrangement of the *bar* coding region, and this indicated that the *bar* hybridising fragments detected in the *Xba*I and *Xho*I-digested DNA corresponded to full-length and rearranged transgene insertions. In the case of plant 7-1, four *bar* hybridising fragments were detected in *Bam*HI and *Eco*RI-digested DNA. The 0.9 kb *Bam*HI fragment and the 0.87 kb *Eco*RI restriction fragment corresponded to a full-length *bar* coding region; the other three fragments represented rearrangements of the *bar* coding region (Figure 4.7). When the genomic DNA of this line was digested with *Xba*I and *Xho*I and hybridised with the *bar* probe, each digest revealed the presence of five *bar* coding regions (Figure 4.8 A). Therefore, the results from the different DNA digests strongly suggested the insertion of three rearranged *bar* coding regions and two intact *bar* coding regions in the genomic DNA of this transformant.

Genomic DNA from plants 1B-4, 3-3 and 7-1 was also digested with *Nco*I and probed with the *bar* coding region. Plasmid pDM302 does not contain any *Nco*I restriction sites. The hybridising fragments would therefore be terminated at either end by *Nco*I sites located in the plant genomic DNA flanking the inserted *bar* coding region. A single hybridising band larger than 10 kb was detected in the genomic DNA of plant 1B-4, and three bands greater than or equal to 8 kb were observed in

the *Nco*I-digested DNA of transformant 7-1 (Figure 4.8 A). Southern analysis revealed at least 7 *bar* hybridising fragments, ranging in size from approximately 1.5 kb to greater than 10 kb, in the *Nco*I-digested genomic DNA of plant 3-3 (Figure 4.8 A).

As mentioned earlier (Section 4.3.4), 192 plants were transferred to soil. A total of 143 plants were subjected to Southern analysis, and all the plants contained the *bar* coding region. In addition, multiple plants regenerated from callus line 3 had identical *bar* hybridisation patterns. On the other hand, two distinct hybridisation profiles for the *bar* gene were detected among plants recovered from callus lines 1B and 7 (data not shown). Thus, molecular analysis identified a total of six independent transformation events among the primary transformants recovered from the four bialaphos-resistant callus lines.

Southern hybridisation was also used to estimate the number of *uidA* coding regions inserted in the genomic DNA of plants 3-3 and 1B-4. The reporter gene construct for transformant 3-3 was p*Act*1-DGUS, and the restriction enzymes that cut the plasmid once are *Kpn*I and *Xba*I (Figure 4.1). The estimated number of insertions for the *uidA* coding region was greater than 10 for plant 3-3 (Figure 4.8 B). Plants 1B-4 and 7-1 were co-transformed with the pIGNEII gene construct, and genomic DNA was digested with *Eco*RI, *Pst*I and *Xba*I to predict the number of *uidA* coding regions (Figure 4.1). The detection of a single hybridising band in all three digests provided strong evidence for the insertion of one *uidA* coding region in the genomic DNA of plant 1B-4 (Figure 4.8 B). The detection of five hybridising bands in the digested DNA of plant 7-1 suggested the insertion of five *uidA* coding regions (data not shown).

TABLE 4.2

Estimated number of transgene insertion sites in barley transformants produced by microprojectile bombardment with two separate gene constructs

Transformant	Number of *bar* insertion events	Number of *uidA* insertion events
1B-4	2-4	1
3-3	greater than 10	greater than 10
7-1	5	5

TABLE 4.3

Summary of transformation experiments

Bombardment number	Cultivar	Number of bombarded scutella	Pre-culture time (days) on callus induction medium	Construct combination	Number of transformation events [1]	Number of events producing plants	Total number of plants in soil	Number of plants tested for GUS activity [2]	Number of GUS$^+$ plants
1	Schooner	150	5	pDM302 + pIGNEII	3	3	9	8	1
2	Schooner	49	6	pDM302 + pIGNEII					
3	Schooner	120	5	pDM302 + p*Act*1-DGUS	1	1	6	6	6
4	Schooner	50	7	pDM302 + p*Act*1-DGUS					
5	Sloop	238	6	pDM302 + pIGNEII					
6	Sloop	120	7	pDM302 + pIGNEII					
7	Sloop	80	14	pDM302 + pIGNEII	2	2	177	114	114
	Total	**807**			**6**	**6**	**192**	**128**	**121**

[1] Determined by Southern hybridisation to the *bar* coding region.

[2] Young leaf segments and floral tissues were tested for histochemical GUS activity.

Although Southern analysis detected the reporter gene in the genomic DNA of these plants, the chosen restriction enzymes did not establish whether the DNA sequences responsible for expression of the *uidA* gene were intact or had undergone rearrangement. Histochemical GUS assays confirmed that transformants 3-3 and 7-1 carried at least one intact *uidA* expression cassette, because GUS activity was detected in the stained tissues of these plants. On the other hand, transformant 1B-4 contained a hybridising *uidA* coding region, but exhibited no detectable GUS activity. The estimated numbers of insertions for the two transgenes were similar for each transformed plant (Table 4.2).

4.3.6 Summary of transformation experiments

The outcomes of the bombardment experiments are presented in Table 4.3. To determine the transformation frequency (%) for the generation of transformed barley, the number of independent transgenic lines (6) was divided by the total number of bombarded scutella (807) and multiplied by 100. This resulted in a transformation frequency of 1 transgenic line per 135 bombarded scutella or 0.74 %.

4.3.7 Transgene expression and segregation in the T_1 generation

Definitive evidence for the transgenic status of a primary transformant was confirmed by the transmission of the transgenes to the T_1 progeny plants. Lines 1A-1 and 7-1 were chosen to analyse the expression and inheritance of the *uidA* and *bar* genes in the T_1 progeny plants.

Fifteen randomly chosen T_1 grains of primary transformants 7-1 and 1A-1 were germinated, and the plants were grown to maturity in the glasshouse. All the T_1

progeny plants reached maturity, had near full seed set and demonstrated the same morphology as non-transformed, control plants. Histochemical GUS analyses demonstrated expression of the *uidA* reporter gene in the stained leaf tissues and the excised floral parts of the parent plants. The GUS^+ T_1 progeny plants of transformed line 7-1 had reporter gene expression patterns similar to their T_0 parent. Eleven of the 15 T_1 progeny plants of transformant 7-1 produced histochemically detectable GUS activity in the stained leaves. The same 11 plants also scored GUS^+ in the stained floral tissues. However, there was one instance of a T_1 progeny plant that produced no detectable GUS activity in the stained leaves, but expressed the *uidA* gene in the reproductive organs. As a result of staining different tissues, only three T_1 progeny plants of line 7-1 were scored as GUS^-. None of the T_1 progeny plants of line 1A-1 demonstrated GUS activity in either the leaves or the floral tissues.

No PAT activity was detected in the tested T_1 progeny plants of line 1A-1, and the T_1 progeny plants of line 7-1 were not subjected to the chromatographic PAT assay.

For each transformed line, the segregation patterns of the transgenes in the T_1 progeny plants were investigated by Southern analyses. Genomic DNA was digested with *Nco*I and probed with the coding regions of the *uidA* and *bar* genes. Two *uidA* hybridising *Nco*I restriction fragments and three *bar* hybridising *Nco*I restriction fragments were detected in the genomic DNA of plant 7-1 (Figure 4.9 A and B). Southern analysis established that the genomic DNA of the 12 GUS^+ T_1 progeny plants of 7-1 contained *uidA* coding regions, whereas the three GUS^- plants produced no hybridisation signal. For primary transformant 1A-1, Southern

FIGURE 4.9

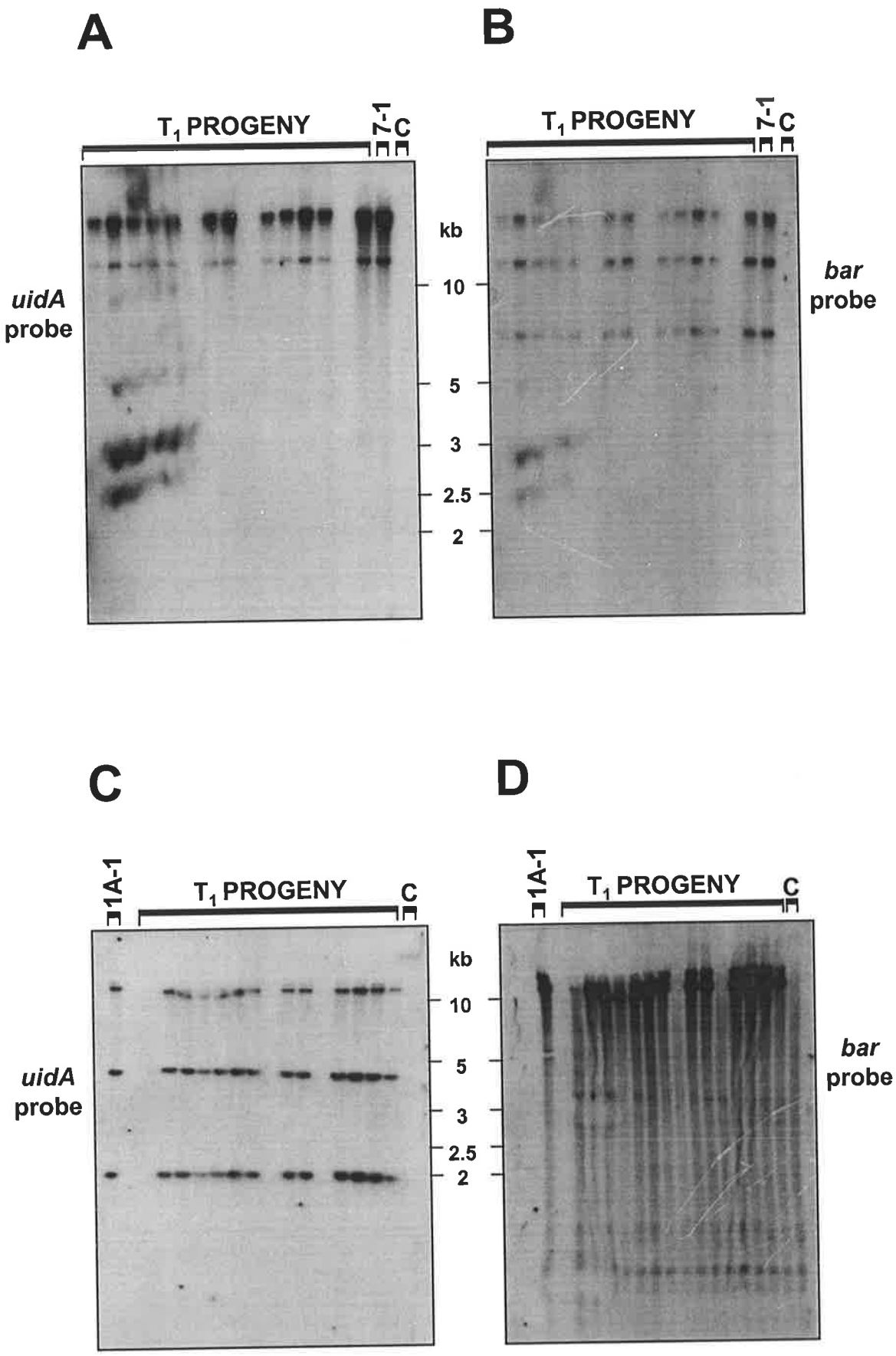
Southern analyses of the T_1 progeny plants derived from two primary transformants, 7-1 and 1A-1

Genomic DNA of the primary transformants (7-1 and 1A-1), their T_1 progeny and a non-transformed barley plant (C) was digested with *Nco*I and sequentially hybridised with a ^{32}P-labelled *uidA* gene probe and a ^{32}P-labelled *bar* gene probe.

Panels **A** and **B**: Parent plant 7-1 and its T_1 progeny plants.

Panels **C** and **D**: Parent plant 1A-1 and its T_1 progeny plants.

The mobility of the DNA marker fragments is indicated between the two panels of each transgenic line.


A
B
T1 PROGENY
7-1
C
kb
10
5
3
2.5
2
uidA probe
bar probe
C
D
1A-1
T1 PROGENY
C
kb
10
5
3
2.5
2
uidA probe
bar probe

hybridisation revealed three *uidA* hybridising *Nco*I restriction fragments and two *bar* hybridising *Nco*I restriction fragments (Figure 4.9 C and D). Hybridising *bar* and *uidA* restriction fragments were detected in the genomic DNA of 12 T_1 progeny plants, although the transgenes were not expressed in the tissues of these plants.

Southern hybridisation also revealed that the *uidA* coding regions co-segregated with the *bar* coding regions in the T_1 progeny plants of both analysed lines; all T_1 progeny plants that contained the *uidA* coding sequences also contained the *bar* coding regions. The co-segregation of the different hybridising restriction fragments accounted for the observation that the genomic DNA of the T_1 progeny plants either contained all the transgene insertion events or none at all (Figure 4.9 A-D).

The evidence from the molecular assays of both families of progeny plants strongly suggested that all the transgene insertions were located at a single locus or were closely linked on the same chromosome. Self-pollination of the heterozygous primary transformants was therefore expected to yield 25% homozygous T_1 transgenic progeny, 50% heterozygous T_1 transgenic progeny, and 25% homozygous T_1 non-transgenic progeny. Overall, the ratio of transgenic T_1 progeny plants to non-transgenic T_1 progeny plants would be 3:1. The observed numbers of transgenic and non-transgenic T_1 progeny plants for lines 1A-1 and 7-1 were in close agreement with the ratio expected for transgene co-segregation (Lines 1A-1 and 7-1, χ^2 0.2, $0.9<P>0.5$; Table 4.4).

TABLE 4.4

Genetic analysis of the transgene sequences in T_1 progeny plants of selected primary transformants

Transformant	Transgenic [1]	Non-transgenic [1]	Total	Expected ratio	χ^2	Probability
1A-1	12	3	15	3 Transgenic: 1 Non-transgenic	0.2	0.9<P>0.5
7-1	12	3	15	3 Transgenic: 1 Non-transgenic	0.2	0.9<P>0.5

[1] In the case of transformant 1A-1, the observed numbers were determined following Southern hybridisation. Molecular analyses and GUS activity assays were used to distinguish the transgenic and non-transgenic T_1 progeny plants of transformant 7-1.

4.4 DISCUSSION

The bombardment conditions used in this study aimed to efficiently deliver DNA to the largest number of scutellar cells, while ensuring that the bombarded scutella remained viable for embryogenic callus production and plant regeneration. To achieve this outcome, 50 µg DNA-coated microcarriers were used per bombardment, and the pre-cultured scutella were treated with high-osmoticum medium before, during, and after DNA delivery. These parameters have been shown to be important for the production of embryogenic callus and for the recovery of plants following microprojectile bombardment of cereal scutellar tissues (Vasil *et al.*, 1993: Becker *et al.*, 1994; Takumi and Shimada, 1996; Brettschneider *et al.*, 1997; Cho *et al.*, 1998). The composition of the culture medium was also investigated in an effort to further enhance the probability of plant regeneration from barley callus cultures.

Several studies have demonstrated that the frequency of plant regeneration from barley callus is improved by increasing the concentration of copper sulphate in the culture medium (Dahleen, 1995; Castillo *et al.*, 1998; Bregitzer *et al.*, 1998; Cho *et al.*, 1998; Nuutila *et al.*, 2000). The precise role of copper sulphate in plant regeneration from barley callus cultures remains unclear. Since copper is known to be a component and/or elicitor of important enzymes involved in many biosynthetic pathways (e.g. the cytochrome oxidase system), it has been suggested that these enzymes could play a role in plant regeneration from cereal callus cultures (Purnhauser and Gyulai, 1993; Castillo *et al.*, 1998). The results described in the current study also indicated that modification to the copper content of the callus induction medium was important for plant regeneration from the immature scutellum-

derived callus of cvs. Schooner and Sloop (Table 4.1). Culture medium supplemented with 100 times the standard MS medium copper concentration (0.1 μM) increased the number of plants regenerated from the callus cultures of both cultivars (Table 4.1 and data not shown). At 10 μM, this copper concentration was higher than the optimum used for the Finnish cultivar, Kymppi (5 μM; Nuutila *et al.*, 2000), but was lower than the best performing concentration for the North American cultivar, Hector (50 μM; Dahleen, 1995). The data reported in the current study also supported the opinion expressed in earlier investigations that the copper concentration employed for enhanced plant regeneration was dependent on the tested cultivar (Dahleen, 1995; Bregitzer *et al.*, 1998; Nuutila *et al.*, 2000). The copper-enhanced plant regeneration system was successfully combined with microprojectile bombardment to recover putative transformed barley of the two elite Australian breeding lines.

Southern hybridisation was used to demonstrate the incorporation of the *bar* and *uidA* genes into the genomic DNA of the putative transformants regenerated from the bialaphos-resistant callus lines. The *bar* and *uidA* gene fragments hybridised to the high molecular weight undigested DNA of a non-transformed barley plant (Figures 4.7 and 4.8 A and B). This hybridisation could be due to homology of the *bar* and *uidA* gene probes with a gene or repetitive DNA sequence in the barley genome. The *bar* and *uidA* gene probes detected a variety of hybridisation patterns in the genomic DNA of the putative transformants (Figures 4.7 and 4.8 A and B). Southern hybridisation, with restriction enzymes predicted to release a full-length transgene fragment from pDM302, confirmed the presence of an intact *bar* gene in the genomic DNA of all the putative transformants (Figure 4.7). In addition to the

expected *bar* hybridising fragment, Southern analysis identified other transgene hybridising fragments, most frequently larger than the full-length transgene, in the *Bam*HI and *Eco*RI-digested DNA samples (Figure 4.7). The *Bam*HI and *Eco*RI DNA restriction fragments that produced weak hybridisation signals could either represent incompletely digested DNA or rearranged insertions of the *bar* coding region. The integration of transgene fragments into the plant genome has been commonly observed in the transgenic plants produced by microprojectile bombardment, because this method employs physical force to deliver DNA to plant cells, and transgene shearing could occur before insertion into the genome (Pawlowski and Somers, 1996). The extra hybridising bands therefore represented additional insertions of the *bar* coding region that had either undergone rearrangement during transformation or lost diagnostic restriction enzyme sites during integration into the barley genome.

Southern analysis with the *bar* probe was used to detect differences in the integration patterns of the selectable marker gene in plants regenerated from the same bialaphos-resistant callus line. The primary transformants recovered from callus lines 1B and 7 had two different *bar* hybridisation patterns (data not shown). These lines probably developed from two independently transformed cells located very close to each other on the same scutellum (Wan *et al.*, 1995; Bower *et al.*, 1996; Zhang *et al.*, 2000). It was possible that the callus produced by the two transformed cells was maintained as one line during selection, and plants were subsequently regenerated from the different transformation events. On the other hand, if the callus lines originated from single-cell transformation events, the primary transformants that exhibited different hybridisation patterns could have been

regenerated from cells that lost insertions of the *bar* coding region after integration (Spencer *et al.*, 1992).

Although Southern analysis with the *uidA* gene probe was designed to estimate the number of transgene insertions, the DNA sequences responsible for expression of the *uidA* gene must have been intact to direct GUS activity in the stained tissues of plants 3-3 and 7-1. Multiple insertions of the *bar* and *uidA* genes were detected in the genomic DNA of the analysed transformants (Table 4.2). The similar numbers of *bar* and *uidA* coding region insertions in an individual transformant could be due to the two separate plasmids forming concatemers by either homologous or illegitimate recombination prior to genomic integration (Spencer *et al.*, 1990; Pawlowski and Somers, 1996).

Based on the results of the Southern analyses, a 100% co-transformation frequency was achieved for the unlinked *bar* and *uidA* genes in the analysed transformants. Goto *et al.* (1993) also reported a 100% co-integration frequency in rice for introduced genes carried on separate plasmids. The co-transformation frequency for unlinked genes obtained in the present study was higher than those reported for transformed barley (85%; Wan and Lemaux, 1994) and maize (77%; Gordon-Kamm *et al.*, 1990) callus lines.

The expression of the inserted genes was monitored by the application of the marker gene assays to the transformed plants. Although the selectable marker gene was detected in the genomic DNA of the primary transformants, there were inconsistencies with the use of the PAT assay to observe the expression of the *bar* gene. PAT activity was at its highest level in the bialaphos-resistant callus lines (Figure 4.4), but was either undetectable, in the case of transformant 1A-1, or

present at low levels in plants 3-3 and 7-1 (data not shown). Despite this loss or gradual decline in the expression of the *bar* gene, PAT enzyme levels were sufficient to confer resistance to bialaphos, because these plants were regenerated on selection medium. Weeks *et al.* (1993) and Karunaratne *et al.* (1996) reported problems with the reliability of the PAT assay after they detected *bar* coding regions in PAT^- wheat transformants. On the other hand, the inactivation of *bar* gene expression could be attributed to gene silencing (see below).

The development of a barley transformation system allowed the 5' regulatory elements of the p*Act*1-DGUS and pIGNEII gene constructs to be assessed in a range of transformed tissues. Consistent with earlier reports (Becker *et al.*, 1994; Takumi and Shimada, 1996; Qureshi *et al.*, 1996; Brettschneider *et al.*, 1997), p*Act*1-DGUS directed GUS activity in a variety of barley tissues, including leaves, roots (Figure 4.6 A and D) and reproductive organs (data not shown). The pIGNEII gene construct expressed the *uidA* reporter gene in the leaves, anthers, ovary and sectioned immature T_1 grains (Figure 4.6 B, E and F) of primary transformants 1A-1 and 7-1, and the aleurone layers isolated from mature T_1 grains of plant 1A-1 (Figure 4.6 G). These observations were unexpected, because expression of the native barley (1→3,1→4)-β-glucanase isoenzyme EII gene is normally restricted to the aleurone layer of the germinated grain (Stuart *et al.*, 1986; Slakeski and Fincher, 1992). The behaviour of the pIGNEII gene construct in transformants 1A-1 and 7-1 can be rationalised by three alternate explanations:

- A 5' flanking region of the barley (1→3,1→4)-β-glucanase isoenzyme EII gene of 1006 bp was used to prepare the pIGNEII gene construct. Although

transcriptional factors responsible for regulated expression of the native EII gene were present in the transformed plants, the absence of important DNA sequence(s) from the promoter region of pIGNEII suggested that crucial DNA-protein interactions needed for proper regulation of reporter gene expression were not possible;

- Last *et al.* (1991) reported that the promoter-less pIGN plasmid produced low level GUS activity in transformed suspension culture-derived protoplasts of maize, wheat, ryegrass and rice. The presence of transcriptional enhancer elements in either the maize *Adh-1* intron, which has been shown to stimulate a 100 fold increase in foreign DNA expression in transformed maize cells (Callis *et al.*, 1987), or the pUC118 cloning vector sequence could have directed GUS activity in a wide variety of cell types;
- The chromosomal environment surrounding the transgene insertion site could have contributed to the deregulated expression of the pIGNEII gene construct in the two transformants. In the same way, the lack of GUS activity in the stained tissues of plant 1B-4 could relate to the integration site of the *uidA* gene in this transformant, although other explanations, such as the insertion of a rearranged, non-functional *uidA* expression cassette cannot be discounted. The influence of "position effects" has been frequently cited as a factor that causes the variation in transgene expression commonly observed among plants regenerated from different transformation events (De Block, 1993; Casas *et al.*, 1995).

During the course of this study, 807 scutella were bombarded with various gene constructs in 7 experiments. The results from the Southern analyses confirmed that successful transformation was achieved for only three experiments (Table 4.3). Four independent Schooner transformants were recovered from a total of 369 bombarded scutella, while 438 Sloop scutella were bombarded to regenerate 2 independent transformants (Table 4.3). The transformation frequency obtained for Schooner was 1.1%, while it was 0.45% for Sloop. These frequencies were within the range (0-4.4%) reported elsewhere for transgenic barley produced by microprojectile bombardment (Section 1.3.2).

Southern hybridisation was used to examine the transgene integration patterns in two primary transformants, 1A-1 and 7-1, and their T_1 progeny plants. In the current study, both lines transmitted their parental hybridisation patterns to all their transgenic T_1 progeny (Figure 4.9 A-D). The progeny analyses also demonstrated co-segregation of the *bar* and *uidA* genes in both lines. These observations suggested that the transgenes of both lines were probably located on the same chromosome and genetically linked. The numbers of transgenic and non-transgenic T_1 progeny plants for both self-pollinated primary transformants closely agreed with the Mendelian ratio expected when a single, dominant and heterozygous locus is responsible for the inheritance of the transgenic phenotype (3:1; Table 4.4). Previous studies have also demonstrated that multiple insertions of unlinked transgenes tended to be clustered at the same locus and transmitted together to progeny plants (Walters *et al.*, 1992; Goto *et al.*, 1993; Qu *et al.*, 1996; Chen *et al.*, 1998 a).

The expression of the *uidA* gene was also investigated in the T_1 progeny plants of the primary transformants. In the case of line 7-1, the parent plant and the *uidA* containing T_1 progeny plants had similar levels of GUS activity in their stained leaf sections, indicating that transgene expression was stable through a single generation. Moreover, the expression of the *uidA* gene in this transgenic line was more likely to be detected in the reproductive tissues than the leaves. The reason for this observation is unclear, but could relate to the position of the transgene insertion site in the barley genome. It is possible that this "position effect" could cause unpredictable expression patterns of the *uidA* gene in this transgenic barley line. Other reports have also indicated variable GUS activity in different tissues of the same transgenic plant (Zhong *et al.*, 1996; Rooke *et al.*, 2000). On the other hand, there was complete loss of GUS activity in the transgenic T_1 progeny plants of line 1A-1. Since this population of transgenic progeny had the same *uidA* hybridisation profile as the parent plant, it seemed unlikely that deletion or rearrangement of the DNA sequences responsible for expression of the *uidA* gene was the reason for the absence of GUS activity. It appeared that the expression of the *uidA* gene was silenced in the T_1 generation.

The silencing of transgene expression has been detected in a diverse range of transgenic plants produced by microprojectile bombardment (Register *et al.*, 1994; Karunaratne *et al.*, 1996; Kumpatla *et al.*, 1997; Chen *et al.*, 1998 a; Chen *et al.*, 1998 b; Kluth *et al.*, 1998; Kohli *et al.*, 1999; Gahakwa *et al.*, 2000). Several mechanisms have been proposed to explain transgene silencing, including anti sense RNA production, *trans* inactivation (also termed co-suppression), methylation of the transgene's promoter region, and "position effects" (Meyer, 1995; Stam *et al.*,

1997; Kumpatla and Hall, 1998; Zhong, 2001). In the present study, the basis for transgene silencing in the T_1 transgenic progeny plants is unknown. Furthermore, the inactivation of transgene expression is not always confined to the T_1 progeny plants, with transgene instability reported for the T_2 and T_3 generations of transgenic cereal crops (Register *et al.*, 1994; Srivastava *et al.*, 1996; Kumpatla and Hall, 1998). At present, the processes involved in transgene silencing remain poorly understood, and extensive assessment of successive generations of transgenic plants will be required to identify the lines that demonstrate stable transmission and expression of the inserted genes.

4.5 SUMMARY AND CONCLUSIONS

Transgenic plants were obtained for two Australian barley cultivars following microprojectile bombardment of isolated scutellar tissues with the selectable marker gene, *bar*, and the reporter gene, *uidA*. This was achieved by using bombardment conditions that did not impair the ability of the scutella to produce embryogenic callus after transformation, and the inclusion of 10 μM copper sulphate in the callus induction medium to improve the frequency of plant regeneration from the bialaphos-resistant callus lines.

The cumulative evidence for the stable transformation of barley included the growth of putative transformed callus and plants on selection medium, the detection of the transgene sequences in the plant genome by Southern analyses, and the transmission of the transgene integration patterns to the T_1 progeny plants in a Mendelian manner. The work described here also demonstrates that the fusion of a truncated (1→3,1→4)-β-glucanase isoenzyme EII gene promoter to the maize *Adh-1* intron could direct constitutive expression of the reporter gene in barley, but not expression patterns normally seen with the endogenous gene. Although higher transformation frequencies could be obtained with further refinement of the technology, the current transformation frequency was considered sufficient to utilise this method to insert potentially useful genes into barley.

In Chapter Five, the microprojectile bombardment technique developed in this study and the *Agrobacterium tumefaciens*-mediated transformation procedure reported elsewhere (Tingay *et al.*, 1997; Patel *et al.*, 2000; Trifonova *et al.*, 2001; Wang *et al.*, 2001; Fang *et al.*, 2002) were used to transform barley with a mutated barley (1→3,1→4)-β-glucanase gene.

CHAPTER FIVE

TRANSFORMATION OF BARLEY WITH THE GENE ENCODING THE MUTATED BARLEY (1→3,1→4)-β-GLUCANASE H300P ENZYME

5.1 INTRODUCTION

The enzymatic modification of the endosperm cell walls of germinated barley grain plays an important role in malt quality (Bamforth, 1994; MacGregor, 1996). The endosperm cell walls of the barley grain are mainly composed of high molecular weight (1→3,1→4)-β-glucan (approximately 75% by mass; Fincher and Stone, 1986). During the malting process, endogenous barley (1→3,1→4)-β-glucan endohydrolases (EC 3.2.1.73) hydrolyse the (1→3,1→4)-β-glucan component of the endosperm cells walls. Depending on factors such as malting time, (1→3,1→4)-β-glucan content of the barley and the grain's capacity to rapidly generate high levels of degradative enzymes, varying amounts of (1→3,1→4)-β-glucan will remain in the green malt. Further hydrolysis of the polysaccharide during the malting and brewing processes will be minimal, because malt (1→3,1→4)-β-glucanases are rapidly inactivated during kilning, and at the elevated temperatures used in the brewery, where malt is usually extracted at temperatures of 45-65°C (Loi *et al.*, 1987; Bamforth, 1994). The barley (1→3,1→4)-β-glucanase isoenzymes EI and EII lose more than 50% of their activity after 30 min at 45°C (Loi *et al.*, 1987), while isoenzyme EII is almost completely inactivated after 20 sec at 65°C (Stewart *et al.*, 2001). Isoenzyme EII is slightly more stable at elevated temperatures compared with isoenzyme EI, both in highly purified enzyme preparations (Woodward and Fincher, 1982 b) and in unpurified extracts of germinated grain (Loi *et al.*, 1987). It has been suggested that isoenzyme EII is probably the quantitatively more important isoenzyme for the hydrolysis of cell wall (1→3,1→4)-β-glucan in germinated grain (Loi *et al.*, 1987; Fincher, 1992).

Any undegraded (1→3,1→4)-β-glucan will be extracted from the ground malt during the mashing or lautering processes and subsequently can cause problems with beer filtration, and can produce undesirable hazes and precipitates in the final beer (Bamforth, 1994). To alleviate the problems associated with incomplete degradation of (1→3,1→4)-β-glucans, thermostable fungal or bacterial β-glucanases are frequently used as additives, so that any residual (1→3,1→4)-β-glucan is hydrolysed during mashing or lautering (Simmons, 1994).

In another approach, microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation have been used to insert thermostable β-glucan endohydrolase genes into barley, and thereby to allow continued (1→3,1→4)-β-glucan degradation during mashing. A thermostable (1→3,1→4)-β-glucanase was engineered by intergenic recombination of gene segments from *Bacillus macerans* and *Bacillus amyloliquefaciens* (Olsen *et al.*, 1991). The hybrid gene, under the control of either an α-amylase promoter (Jensen *et al.*, 1996) or a D-hordein promoter (Horvath *et al.*, 2000), was subsequently expressed in germinated transgenic grain. Similarly, a gene encoding a thermotolerant (1→4)-β-glucanase from the fungus *Trichoderma reesei* has been introduced into barley. This gene was expressed in the germinated grain, and the thermostable enzyme was active during the mashing step of the brewing process (Nuutila *et al.*, 1999).

In view of increasing consumer pressure to minimise the use of "artificial" additives during malting and brewing, particularly in Europe, and perceived problems associated with the acceptance of barley engineered with microbial genes, Stewart *et al.* (2001) attempted to engineer improved heat stability into the endogenous barley (1→3,1→4)-β-glucanase isoenzyme EII by site-directed mutagenesis of the protein

coding region of the corresponding cDNA. The mutagenesis was based on the availability of the three-dimensional structure of the enzyme (Varghese *et al.*, 1994), through which rational re-design of the enzyme was theoretically possible. Eight amino acid substitutions were introduced into isoenzyme EII, based on the detailed structure of the isoenzyme and knowledge of factors important for protein stability (Stewart *et al.*, 2001). Several mutated forms of the enzyme were produced, but a histidine to proline substitution at amino acid 300 proved most successful in enhancing the heat stability of the enzyme compared with the wild-type (1→3,1→4)-β-glucanase. The improvement in thermostability was 3.7°C, and the mutated enzyme was designated H300P (Stewart *et al.*, 2001).

In Chapter Four, procedures were described for the microprojectile bombardment of immature barley scutella, the selection of bialaphos-resistant callus, and the regeneration of transformed plants. During the course of this work, technical modifications to the *Agrobacterium tumefaciens*-mediated transformation procedure also resulted in the production of fertile transgenic barley (Tingay *et al.*, 1997; Horvath *et al.*, 2000; Patel *et al.*, 2000; Wang *et al.*, 2001; Trifonova *et al.*, 2001; Fang *et al.*, 2002). In this Chapter, experiments are described through which microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation have been employed to transform barley with the gene encoding the mutated barley (1→3,1→4)-β-glucanase H300P enzyme.

5.2 MATERIALS AND METHODS

5.2.1 Isolation and culture of immature scutella

Donor barley plants (cvs. Sloop and Golden Promise) were grown in 16 cm plastic pots filled with Horsham soil mix (Appendix 1). The barley plants used for microprojectile bombardment and the *Agrobacterium tumefaciens*-mediated transformation experiments were maintained in the glasshouse. The developing spikes were harvested from donor plants when the immature embryos were approximately 1-2 mm in diameter. The procedures for grain sterilisation, and the isolation and culture of immature scutella are described in Section 4.2.1. However, a modified version of the callus induction medium, described in Section 4.2.1, was used here. Sucrose was substituted with maltose (30 g/L), and the medium was solidified with Phytagel™ (3.5 g/L; Sigma Chemicals, St. Louis, MO, USA) rather than agar. The scutella were incubated in the dark at 22-24°C.

Towards the end of this study, a second set of *Agrobacterium tumefaciens*-mediated transformation experiments were performed using the procedure developed by Tingay *et al.* (1997) and modified by Matthews *et al.* (2001). The awns were removed from the spikes, and the grains were rinsed with absolute ethanol and surface-sterilised for 20 min with 20% (v/v) White King® bleach (SaraLee, Victoria, Australia; active ingredients 4.2% w/v sodium hypochlorite and 0.9% w/v sodium hydroxide) that contained two drops of Tween-20. The grains were rinsed thoroughly with sterile water, and immature scutella were isolated as described in Section 4.2.1. The explants were cultured scutellum side-down on the callus induction medium described by Wan and Lemaux (1994). This medium was composed of MS macro- and micro-nutrients, supplemented with 30 g/L maltose, 1 mg/L thiamine-HCl, 0.25

FIGURE 5.1

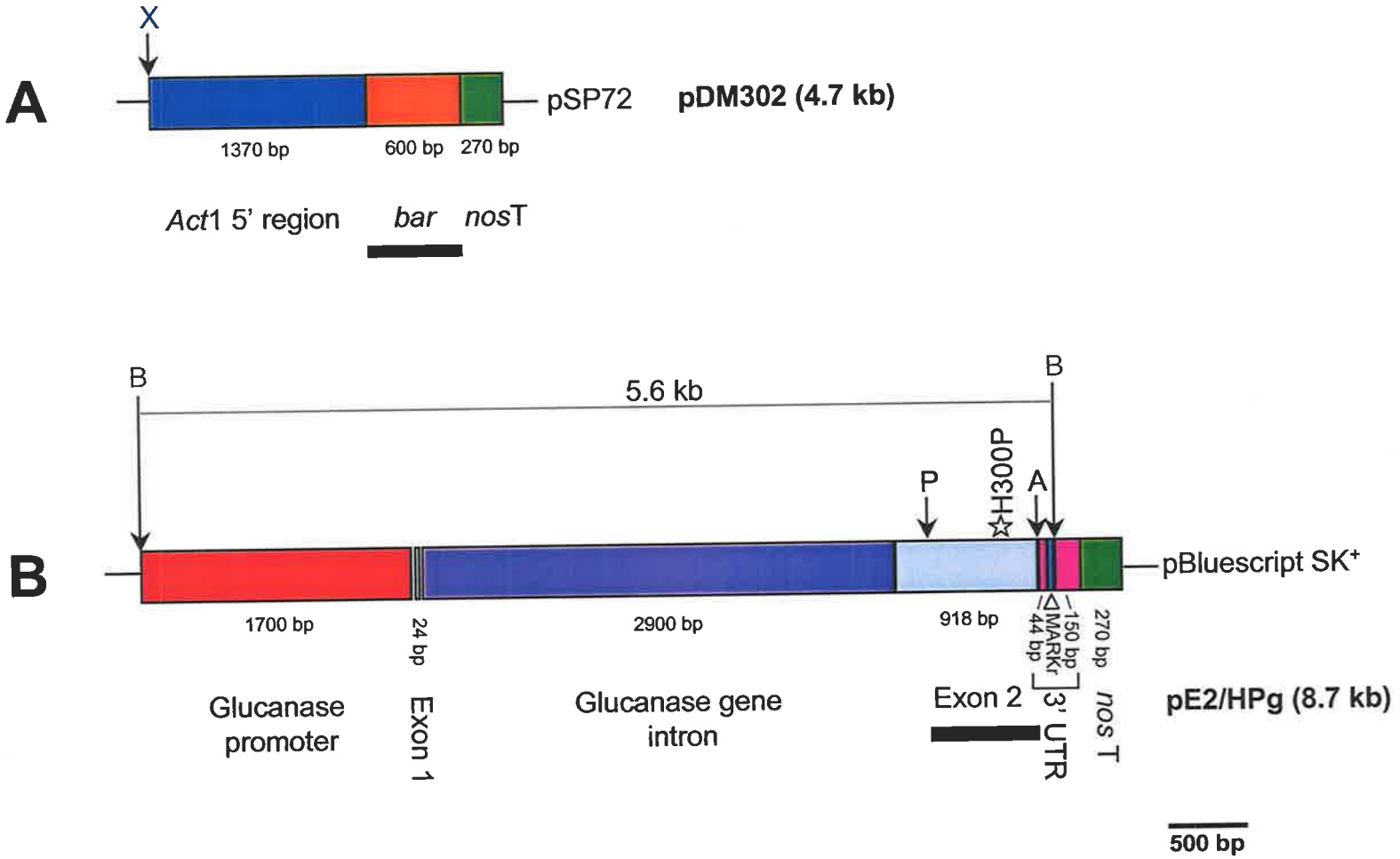
Schematic representation of the gene constructs used in the microprojectile bombardment experiments, indicating the position of the restriction enzyme sites used for the molecular analyses

A pDM302.

B pE2/HPg.

Abbreviations: *Act*1: promoter, first exon and intron of the rice actin 1 gene; *bar:* coding region of the phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus*; *nos*T: transcript termination region of the nopaline synthase gene from *Agrobacterium tumefaciens*; UTR: untranslated region; MARKr: a 35 bp transgene "marker" sequence inserted in the 3' UTR to assist with the analysis of transgene expression; H300P: mutation in the (1→3,1→4)-β-glucanase gene that resulted in a histidine to proline substitution at amino acid 300 of the mutated enzyme.

Restriction enzyme sites used for DNA analyses are shown in coloured letters. Solid coloured lines indicate the DNA restriction fragments expected from the digests performed. Abbreviations of the restriction enzyme sites: A: *Acc*I; B: *Bam*HI; P: *Pst*I; X: *Xho*I. The *bar* containing DNA fragment isolated from pDM302 and the *Pst*I/*Acc*I DNA fragment excised from pE2/HPg (indicated by solid black lines) were used as gene probes in Southern analyses.


A
X
pSP72
pDM302 (4.7 kb)
1370 bp
600 bp
270 bp
Act1 5' region
bar
nosT
B
B
5.6 kb
B
P
H300P
A
pBluescript SK+
1700 bp
24 bp
2900 bp
918 bp
~150 bp
ΔMARKr
44 bp
270 bp
Glucanase promoter
Exon 1
Glucanase gene intron
Exon 2
3' UTR
nos T
pE2/HPg (8.7 kb)
500 bp

g/L myo-inositol, 1 g/L, casein hydrolysate, 0.69 g/L L-proline, 37 mg/L EDTA (ferric-sodium salt), 10 μM $CuSO_4$, 2.5 mg/L 3,6-dichloro-o-anisic acid (Dicamba), and was solidified with 3.5 g/L Phytagel™. The callus induction medium was prepared according to the procedure of Bregitzer *et al.* (1998). The components of the medium were prepared as three separate solutions. Solution 1 contained maltose, solution 2 contained EDTA (ferric-sodium salt) and KH_2PO_4, and solution 3 contained the remaining ingredients. The solutions were autoclaved, cooled to approximately 70°C, and combined prior to pouring into 90 mm x 10 mm plastic Petri dishes. The freshly isolated scutella were immediately used for co-cultivation with *Agrobacterium tumefaciens*.

5.2.2 Microprojectile bombardment

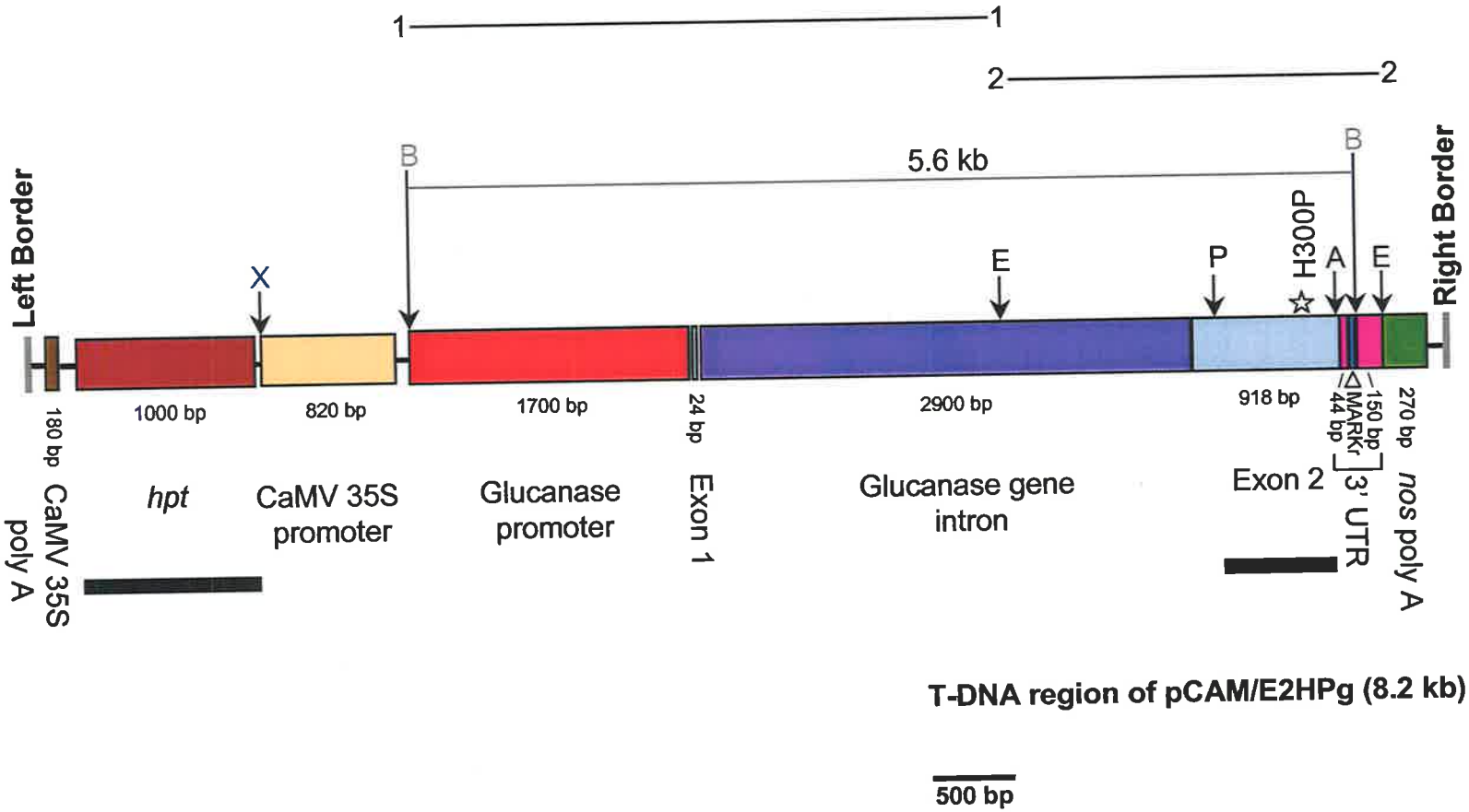
Plasmids pDM302 (Cao *et al.*, 1992) and pE2/HPg (Stewart, 1999) were used for microprojectile bombardment. Schematic maps of these plasmids are shown in Figure 5.1. The details of plasmid pDM302 are described in Section 3.2.2. Plasmid pE2/HPg contains the entire barley (1→3,1→4)-β-glucanase isoenzyme EII gene (Wolf, 1991), which includes the endogenous promoter region, the open reading frame, the single intron, the H300P amino acid substitution, and a transgene "marker" sequence (MARKr). The 35 bp DNA "marker" oligonucleotide sequence was inserted in the 3' untranslated region (UTR) of the gene to distinguish the transgene from the endogenous gene during subsequent PCR analyses. Plasmid DNA of the two gene constructs was prepared using the procedure described in Section 3.2.2, except that the overnight culture of pE2/HPg contained 50 mg/L kanamycin sulphate.

FIGURE 5.2

Schematic map of the T-DNA region of pCAM/E2HPg, indicating the position of the restriction enzyme sites used for the molecular analyses

Two DNA fragments were excised from pE2/HPg (labelled 1 and 2) and were inserted into pCAMBIA1390 to create pCAM/E2HPg. Abbreviations: CaMV: cauliflower mosaic virus; *hpt:* the coding region of the hygromycin phosphotransferase gene from *E. coli;* CaMV 35S poly A: the polyadenylation sequence of the CaMV 35S gene; *nos* poly A: the polyadenylation sequence of the nopaline synthase gene from *Agrobacterium tumefaciens*; UTR: untranslated region; MARKr: a 35 bp transgene "marker" sequence inserted in the 3' UTR to assist with the analysis of transgene expression; H300P: mutation in the (1→3,1→4)-β-glucanase gene that resulted in a histidine to proline substitution at amino acid 300 of the mutated enzyme.

Restriction enzyme sites used for DNA analyses are shown in coloured letters. Solid coloured lines indicate the DNA restriction fragments expected from the digests performed. Abbreviations of the restriction enzyme sites: A: *Acc*I; B: *Bam*HI; E: *Eco*RI; P: *Pst*I; X: *Xho*I. The *hpt* containing DNA fragment isolated from pCAMBIA1390 and the *Pst*I/*Acc*I DNA fragment excised from pE2/HPg (indicated by solid black lines) were used as gene probes in Southern analyses.


1
1
2
2
B
5.6 kb
B
Left Border
X
E
P
H300P
A
E
Right Border
180 bp
1000 bp
820 bp
1700 bp
24 bp
2900 bp
918 bp
44 bp
150 bp
270 bp
CaMV 35S poly A
hpt
CaMV 35S promoter
Glucanase promoter
Exon 1
Glucanase gene intron
Exon 2
3' UTR
nos poly A
T-DNA region of pCAM/E2HPg (8.2 kb)
500 bp

The gold microcarriers (0.6 μm diameter) were prepared for microprojectile bombardment using the procedure described in Section 3.2.3. The DNA-coated microcarriers were prepared according to the protocol reported in Section 4.2.3, except that the molar ratio of the mutated (1→3,1→4)-β-glucanase gene to the selectable marker gene, *bar*, was increased from 1:1 to 2.5:1. For microprojectile bombardment, pre-cultured explants (one to eight day old) were arranged scutellum side-up in the centre of a 55 mm x 15 mm plastic Petri dish containing osmoticum medium (Section 4.2.3). The osmoticum treatment and the bombardment conditions are described in Section 4.2.3.

5.2.3 Selection and plant regeneration for microprojectile bombardment

The procedures employed to produce bialaphos-resistant callus lines, capable of plant regeneration, are described in Section 4.2.4.

5.2.4 Binary vector pCAM/E2HPg

Plasmid pCAM/E2HPg was constructed to deliver the gene encoding the mutated (1→3,1→4)-β-glucanase H300P enzyme into barley by *Agrobacterium tumefaciens*-mediated transformation (Stewart, 1999). To produce this DNA construct, the barley (1→3,1→4)-β-glucanase isoenzyme EII gene was excised as two fragments (a 3.7 kb *Bam*HI/*Eco*RI fragment and a 2.1 kb *Eco*RI fragment; labelled 1 and 2 in Figure 5.2) from plasmid pE2/HPg (Section 5.2.2) and inserted into plasmid pCAMBIA1390 (CAMBIA: Centre for the Application of Molecular Biology to International Agriculture, Canberra, ACT, Australia). Plasmid pCAMBIA1390 contains the hygromycin phosphotransferase (*hpt*) gene under the

control of the cauliflower mosaic virus (CaMV) 35S promoter. The schematic map of the T-DNA region of plasmid pCAM/E2HPg is shown in Figure 5.2.

5.2.5 *Agrobacterium tumefaciens* growth conditions

Agrobacterium tumefaciens strain AGL1 (Lazo *et al.*, 1991) containing binary vector pCAM/E2HPg was grown for two days at 27°C on solidified Luria-Bertani medium (Section 3.2.2) supplemented with 25 mg/L rifampicin and 50 mg/L kanamycin sulphate. A single colony of the *Agrobacterium* culture was inoculated in 5 ml MG/L liquid medium (Garfinkel and Nester, 1980) that contained 25 mg/L rifampicin and 50 mg/L kanamycin sulphate. The liquid culture was shaken vigorously at 27°C for 40 h. The standard *Agrobacterium* inoculum was prepared by aliquotting 200 µl liquid culture into sterile 1.5 ml Eppendorf tubes that contained 200 µl sterile 15% (v/v) aqueous glycerol. The tubes were inverted and incubated at room temperature for 6 h before storage at -80°C.

To prepare the *Agrobacterium* culture for transformation, 200 µl standard inoculum was added to 10 ml MG/L liquid medium, supplemented with 50 mg/L kanamycin sulphate and grown overnight with vigorous shaking at 27°C. Towards the end of this study, a revised procedure was used to prepare the *Agrobacterium* culture for transformation. Two hundred µl glycerol stock was added to 5 ml MG/L medium, and the culture was grown overnight with vigorous shaking at 27°C. The optical density of the *Agrobacterium* culture was measured at 600 nm at the end of the incubation period and adjusted to 1 prior to the co-cultivation step.

5.2.6 Transformation with *Agrobacterium tumefaciens*

In initial experiments, approximately 75 six to eight day old scutella were arranged scutellum side-up in the centre of a 90 mm x 10 mm plastic Petri dish that contained modified callus induction medium, described in Section 5.2.1. The scutella were covered with 150 µl *Agrobacterium* suspension, and the Petri dish was held at a 45° angle to drain away excess bacterial suspension. The scutella were turned over (cut side-up) and dragged across the surface of the medium to the edge of the Petri dish. Twenty-five scutella were transferred to a fresh plate of callus induction medium and cultured scutellum side-down for two days in the dark at 22-24°C.

After two days of co-cultivation, the explants were transferred to callus induction medium that contained 150 mg/L Timentin® (SmithKline Beecham, Pty. Ltd., Melbourne, Australia) and cultured for two to three weeks in the dark at 22-24°C to inhibit bacterial growth. The callus derived from an individual explant was broken into small pieces and transferred to the modified callus induction medium, which contained 95 µM hygromycin B (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA). Every fortnight for a further six weeks, necrotic callus pieces were discarded while callus pieces that grew were again broken into small pieces, transferred to the same selection medium, and returned to the dark to produce hygromycin-resistant callus lines.

To induce plant regeneration, embryogenic callus sectors were transferred to hormone-free MS medium that contained 1 µM $CuSO_4$, 150 mg/L Timentin® and either 47.5 or 57 µM hygromycin B and incubated in the light (16 h day/8 h night photo-period) at 22-24°C. The regenerated plants were removed from the selection medium and transferred to culture boxes (Magenta Corporation, Chicago, IL, USA)

that contained hormone-free MS medium supplemented with 1 μM $CuSO_4$ to accelerate plant growth. When the plants had grown to the lid of the box, they were transplanted to 8 cm plastic pots filled with Horsham soil mix and handled according to the procedure described in Section 2.2.2.

In later experiments, 25 freshly isolated scutella were cultured cut side-up in the centre of a 90 mm x 10 mm plastic Petri dish that contained callus induction medium, based on the recipe of Wan and Lemaux (1994), which was described in Section 5.2.1. *Agrobacterium* suspension (50 μl) was aliquotted onto the scutella, and the infection process was undertaken as described above. The explants were co-cultivated for three days in the dark at 22-24°C. Following co-cultivation, the scutella were removed to fresh callus induction medium containing 150 mg/L Timentin® and 95 μM hygromycin B and cultured in the dark. The entire callus of an individual scutellum was transferred to fresh selection medium every fortnight for a further six weeks (Matthews *et al.*, 2001). At the end of the callus selection period, the callus derived from a single scutellum was transferred to shoot regeneration medium. This medium was based on the FHG recipe of Wan and Lemaux (1994). It contained FHG macro-nutrients (Hunter, 1988), MS micro-nutrients, 1 mg/L thiamine-HCl, 1 mg/L benzylaminopurine (BAP), 100 mg/L myo-inositol, 0.73 g/L L-glutamine, 62 g/L maltose, 37 mg/L EDTA (ferric-sodium salt), 1 μM $CuSO_4$, 150 mg/L Timentin®, 38 μM hygromycin B, and was solidified with 3 g/L Phytagel™. This medium was prepared according to the protocol of Bregitzer *et al.* (1998) (Section 5.2.1). The cultures were exposed to light (16 h day/8 h night photo-period) for three to four weeks at 22-24°C. The regenerated shoots were excised from the callus and transferred to culture boxes that contained hormone-free MS medium, supplemented

with 1 μM $CuSO_4$, 150 mg/L Timentin®, and 95 μM hygromycin B to induce root formation. The tissue culture-derived plants that grew vigorously were established in soil using the procedure described in Section 2.2.2.

5.2.7 Southern analyses of putative transformed plants

Genomic DNA was isolated from the leaf tissue collected from putative transformants and their progeny using the procedure described in Section 4.2.7. Genomic DNA (10-15 μg) was digested with either *Bam*HI (20 U/μl) or *Xho*I (10 U/μl) (New England Biolabs, Beverly, MA, USA) at 37°C for 5 h. The DNA fragments were separated on a 1% agarose gel (GIBCO-BRL®, Life Technologies™, Grand Island, NY, USA) at 35 V for 20 h. The digested DNA was transferred to Hybond™-N^+ nylon membrane (Amersham Pharmacia Biotech Ltd., Little Chalfont, Buckinghamshire, UK) with 0.4 M NaOH, according to the manufacturer's instructions. A 600 bp probe, isolated from plasmid pDM302 (Section 3.2.8), was used to detect *bar* hybridising sequences in the genomic DNA of the bialaphos-resistant plants regenerated from microprojectile bombardment. A 1.1 kb *Xho*I DNA fragment, excised from plasmid pCAMBIA1390, was used to detect *hpt* hybridising sequences in the genomic DNA of the hygromycin-resistant plants recovered from the *Agrobacterium tumefaciens*-mediated transformation experiments. The 770 bp *Pst*I/*Acc*I DNA fragment, isolated from plasmid pE2/HPg (Figure 5.1), was employed to identify the endogenous and mutated (1→3,1→4)-β-glucanase genes in the genomic DNA of the plants produced from both transformation technologies. The three DNA probe fragments were isolated from excised gel fragments using the Bresa-Clean™ Nucleic Acid Purification Kit (Bresatec, Adelaide, SA, Australia), according to the manufacturer's instructions.

The probes were labelled by random priming (Feinberg and Vogelstein, 1983) using the MegaPrime™ DNA labelling system (Amersham). Hybridisation was conducted at 65°C using the conditions described in Section 3.2.8. The membrane was washed with 0.1 x SSC, 1% (w/v) SDS for 20 min, air-dried and exposed to X-ray film (RX Fuji Medical X-ray film; RX-U, Tokyo, Japan) at -80°C.

5.2.8 Analysis of transgene transcription

Reverse transcriptase (RT)-PCR (Frohman *et al.*, 1988) was used to analyse mRNA encoding the mutated (1→3,1→4)-β-glucanase enzyme in the germinated T_1 grains. A sample of 12 grains, harvested from the independent primary transformants, was incubated at 37°C for five to seven days. The grains were surface-sterilised with 0.2% (w/v) $AgNO_3$ for 20 min and washed successively with sterile water, 0.5 M sterile NaCl, and six changes of sterile water. The grains were imbibed in antibiotic solution on sterile Whatman filter paper (Maidstone, Kent, UK) in a 90 mm x 10 mm plastic Petri dish. The antibiotic solution consisted of 2% (w/v) KNO_3, 100 mg/L chloramphenicol, 145 mg/L neomycin sulphate, 60 mg/L penicillin and 20 mg/L nystatin. Approximately 2 ml antibiotic solution was aliquotted into a Petri dish, and the grains were arranged on the moistened filter paper. The Petri dish was sealed and stored in the dark for five days at 4°C. Following the cold treatment, the Petri dish was transferred to the light and incubated for three days at 22-24°C to stimulate grain germination. No fungal or bacterial contamination was observed during this incubation period. The coleoptile, roots and scutellum were removed from the germinated grain and discarded. The grains of an individual transformed line

were placed in a sterile 2 ml Eppendorf tube that contained 1.5 ml RNA*later* (Ambion Incorporated, Austin, TX, USA) and stored at -20°C until processed further.

Three grains derived from an individual transformed line were placed in a mortar that contained 1 ml Trizol® (GIBCO-BRL®), and ground to a viscous paste with a pestle. The mortar and pestle had been pre-baked at 180°C for 24 h to destroy RNase contamination. The homogenised tissues were transferred to a sterile 2 ml Eppendorf tube, and the final volume was adjusted to 1 ml with more Trizol®. The tube was incubated for 5 min at room temperature. The sample was centrifuged at 12120 x *g* for 15 min at 4°C, and the supernatant was removed to a new 2 ml Eppendorf tube that contained 200 µl chloroform. The sample was shaken vigorously for 15 sec and incubated at room temperature for 5 min. The tube was centrifuged at 12120 x *g* for 15 min at 4°C. The colourless, upper aqueous phase was transferred to a fresh 2 ml Eppendorf tube that contained 250 µl iso-propanol and 250 µl 1.2 M sterile NaCl. The sample was incubated at room temperature for 10 min to precipitate the RNA and centrifuged at 12120 x *g* for 10 min at 4°C. The supernatant was discarded, and the RNA pellet was washed with 1 ml 75% (v/v) ethanol. The sample was mixed by vortexing and centrifuged at 7555 x *g* for 5 min at 4°C. Following centrifugation, the RNA pellet was briefly air-dried at room temperature and resuspended in 30 µl sterile water.

The Thermoscript™ RT-PCR system (GIBCO-BRL®) was used for cDNA synthesis. Total RNA (1 µg) was combined with 1 µl oligo d (T) primer (50 µg/µl), and the volume was adjusted to 10 µl with sterile water. The sample was heated at 65°C for 5 min and chilled on ice. The 5 x cDNA synthesis buffer (4 µl), 1 µl 0.1 M dithiothreitol, 1 µl RNAseIN (40 U/µl), 2 µl 10 mM dNTPs, 0.5 µl Thermoscript™ RT

(15 U/μl), and 1.5 μl sterile water were added to the sample. The mixture was incubated at 52°C for 1 h and heated at 85°C for 5 min to terminate the reaction. RNAse H (1 μl; 2 U/μl) was added to the reaction, and the mixture was incubated at 37°C for 30 min. The reaction volume was adjusted to 50 μl with sterile water. The cDNA synthesis reaction was stored at -20°C until used for PCR analysis.

For PCR amplification, the two primers E2f (5'-CCATCCTCGGCGTGTTCAGCC-3') and MARKr (5'-GGGTCAGGGCTTCGGATCCACCTTGCGCCACG-3') were used as the forward and reverse primers, respectively. The reverse primer, MARKr, was complementary to the transgene "marker" sequence in the 3' UTR of the mutated (1→3,1→4)-β-glucanase gene and therefore would not amplify endogenous barley (1→3,1→4)-β-glucanase genes (Stewart, 1999). The PCR reaction contained 1 μl cDNA synthesis reaction, 2.5 μl 10 x PCR buffer (GIBCO-BRL®), 0.75 μl 50 mM $MgCl_2$, 1 μl 5 mM dNTPs, 1 μl each primer (0.4 nmoles), 0.5 μl Taq DNA polymerase (GIBCO-BRL®), ± 2.5 μl dimethyl sulphoxide, and the volume was adjusted to 25 μl with sterile water. The PCR temperature program was 35 cycles of 94°C for 40 sec, 50°C for 40 sec, and 72°C for 60 sec (Stewart, 1999). The resulting fragments were analysed by electrophoresis on a 1.6% agarose gel. The expected size of the transgene PCR product was approximately 690 bp.

To ensure that an equivalent amount of cDNA was used for each amplification (Stewart, 1999), a control PCR reaction was performed for each sample using the same conditions as described above, except that two primers specific for the mRNA encoding the constitutive actin enzyme of barley were used. The forward and reverse primers were

5'-GTCTTTCCCAGCATTGTAGG-3' and

5'-CGACACGGAGCTCATATAGAA-3', respectively. The expected size of the actin PCR fragment was approximately 215 bp.

5.2.9 Enzyme extraction

Barley grains of a transformed line homozygous for the selectable marker gene, *hpt*, and the mutated (1→3,1→4)-β-glucanase gene (AGROSL1.$T_{2.2}$), as well as a homozygous non-transgenic line produced in the segregating T_1 generation (AGROSL1.$T_{1.8}$) were used for this experiment. Grain surface-sterilisation and germination were performed using the procedures described in Section 5.2.8. Three-day germinated grain was homogenised in 50 mM sodium acetate buffer, pH 5.0, containing 10 mM sodium azide, 10 mM Na_2EDTA, 3 mM 2-mercaptoethanol and 3 mM phenylmethylsulfonyl fluoride at 4°C. All subsequent operations were performed at 4°C. The homogenates were held at 4°C overnight to extract the enzymes, and the insoluble material was removed by centrifugation (3220 x *g*, 20 min). Fractional precipitation of the supernatants with ammonium sulphate was used to recover the proteins of each grain sample at three different saturation levels; 0-40%, 40-80%, and 80-100%. The precipitates were collected by centrifugation (3220 x *g*, 20 min), dissolved in homogenisation buffer, and stored at 4°C.

5.2.10 (1→3,1→4)-β-Glucanase activity assay

(1→3,1→4)-β-Glucanase activity was determined reductometrically (Nelson, 1944; Somoygi, 1952) by monitoring the increase in reducing sugars released from a 0.2% (w/v) solution of barley (1→3,1→4)-β-glucan (Megazyme, Bray, Ireland) at

37°C. The barley (1→3,1→4)-β-glucan was dissolved by boiling the substrate in 50 mM sodium acetate buffer, pH 5.0, for 10 min. Reaction tubes were prepared in quintuplicate and contained 150 µl 50 mM sodium acetate buffer, pH 5.0, 50 µl 1% (w/v) barley (1→3,1→4)-β-glucan and 50 µl diluted extract. The activity assays were performed at 37°C for 15 min. The reactions were terminated by adding 250 µl alkaline copper reagent (Somoygi, 1952; Appendix 3), and the reaction tubes were heated at 100°C for 10 min. The reaction tubes were cooled to room temperature, and 250 µl arsenomolybdate reagent (Nelson, 1944; Appendix 3) was added to develop the blue colour that indicated the production of reducing sugars. The reaction tubes were briefly vortexed, centrifuged (3220 x *g*, 10 min), and the absorbance was measured at 660 nm against barley (1→3,1→4)-β-glucan and enzyme-only blanks. The reducing sugar production was calculated by comparing absorbance values to glucose standards (0, 10 and 20 nmol). Errors of ±10% were typically observed between the quintuplicate data values. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol glucose equivalents per minute from the substrate (Stewart *et al.*, 2001). The specific activity is expressed as units per milligram of protein in the grain extract (Slakeski and Fincher, 1992) to overcome differences in the amount of soluble protein extracted from the different grain samples (Loi *et al.*, 1987). The protein concentration of each grain extract was measured with the Coomassie® protein assay reagent (Pierce, Rockford, IL, USA; Bradford, 1976), using BSA as a standard.

5.2.11 Heat stability assays for (1→3,1→4)-β-glucanase activity

Two assay procedures were used to measure the thermostability of the (1→3,1→4)-β-glucanases extracted from the transgenic and non-transgenic grain. Aliquots of the diluted extracts were heated at a range of temperatures for 15 min, and residual activity was measured at 37°C as described in Section 5.2.10. In the second procedure, samples were held at a constant temperature of 48°C and the progressive decline of enzyme activity was monitored over time at 37°C using the reductometric assay (Section 5.2.10). At least three independent experiments were conducted for each assay procedure. All incubations for the thermostability assays were performed in a Julabo VC F10 water-bath (Seelbach, Germany), and the accuracy of the water-bath's thermostat was measured with a standard mercury thermometer (Stewart, 1999).

The specific activity was calculated for each treatment, and diagnostic plots were produced to determine whether the data sets generated for the two different assay procedures were normally distributed, with constant variance. From the diagnostic plots, these assumptions held true for the assay that measured progressive loss of enzyme activity at 48°C. However, the interpretation of the fitted vs. residual plots for the data obtained from the incubation of the extracts at different temperatures for 15 min necessitated that the data be transformed by adding a constant and taking the natural logarithm of the specific activity. The analysis of variance for data generated from both types of thermostability assays was performed using the GENSTAT, Version 6 statistical program. The significance of treatments and their interactions on specific activity were tested at the 5% significance level in a hierarchical manner with the interaction tested before the main effects, provided

there was no significance in the earlier test. The main effects on specific activity were grain type and time for the extracts incubated at 48°C, and grain type and temperature for the extracts incubated at different temperatures for 15 min. An F-test (Moore and McCabe, 1993) was used to determine the statistical significance of terms. If the F probability was ≤ 0.05, it was concluded that the relevant term had a statistically significant effect on specific activity at the 5% level. If the F-test detected an interaction or a main effect to be statistically significant, a Fisher's Least Significant Difference (LSD) test was performed to determine where significant differences were occurring in the assay procedure.

FIGURE 5.3

Selection and regeneration of putative transformants following microprojectile bombardment of barley scutella

A Emergence of bialaphos-resistant callus.

B Growth of bialaphos-resistant callus line on callus induction medium containing 16 μM bialaphos.

C Regeneration of putative transformed plants on hormone-free culture medium containing 3.2 μM bialaphos.

D Mature putative transformants established in soil.

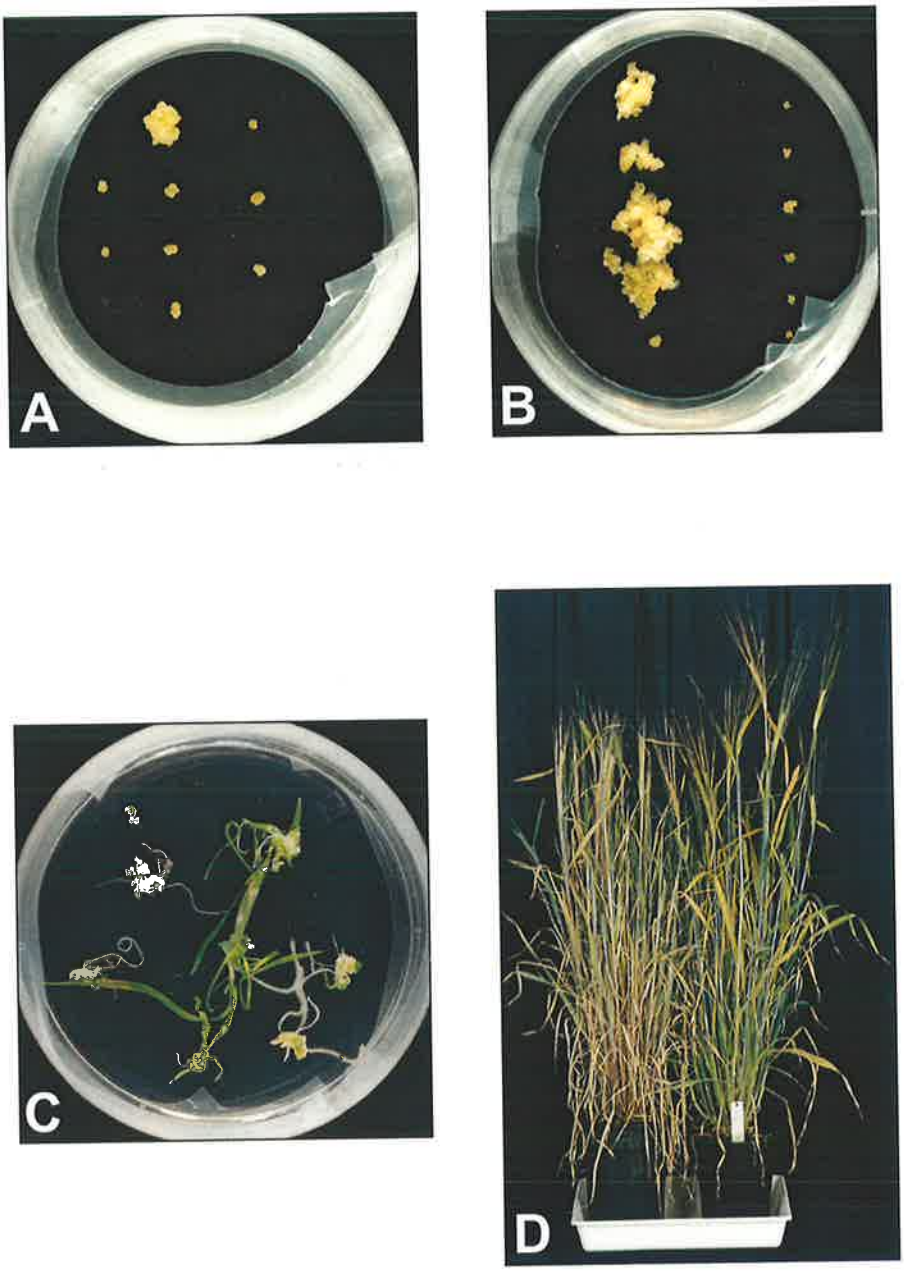

A
B
C
D

FIGURE 5.4

Selection and regeneration of putative transformants from the first batch of *Agrobacterium tumefaciens*-mediated transformation experiments

A Development of hygromycin-resistant callus.

B Growth of a hygromycin-resistant callus line on culture medium containing 95 μM hygromycin B.

C Regeneration of putative transformants on hormone-free culture medium containing 47 μM hygromycin B.

D Mature putative transformants established in soil.

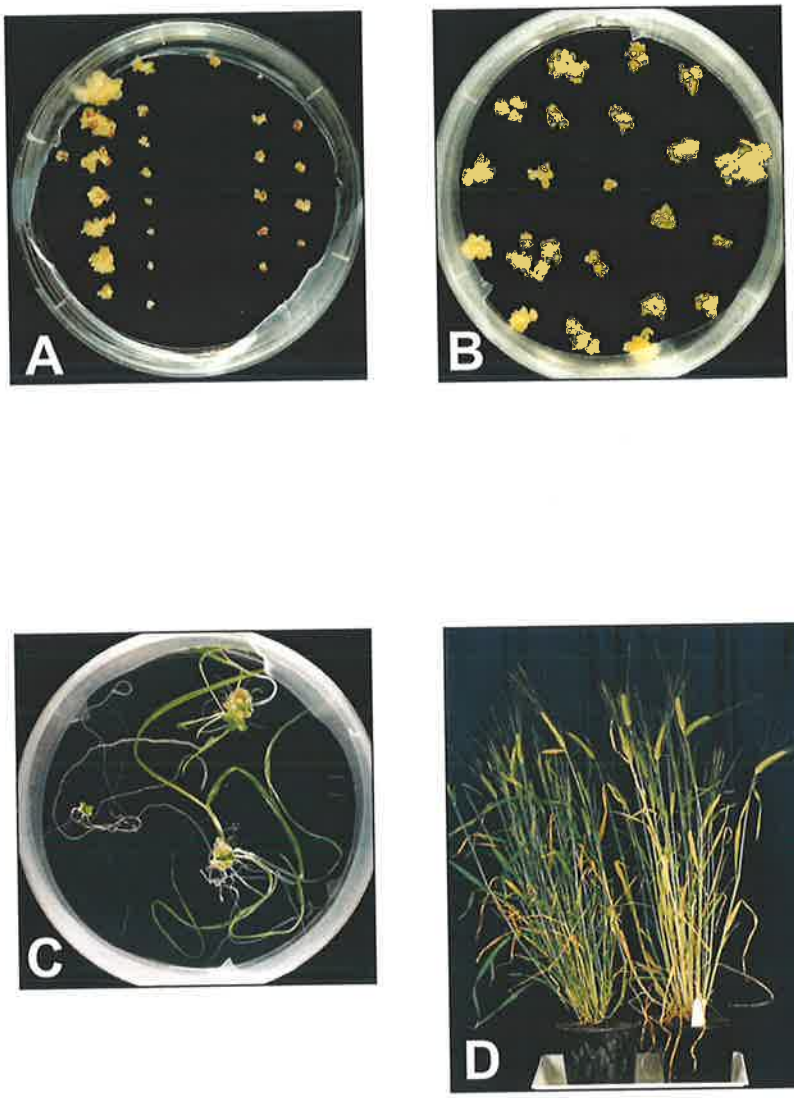

A
B
C
D

FIGURE 5.5

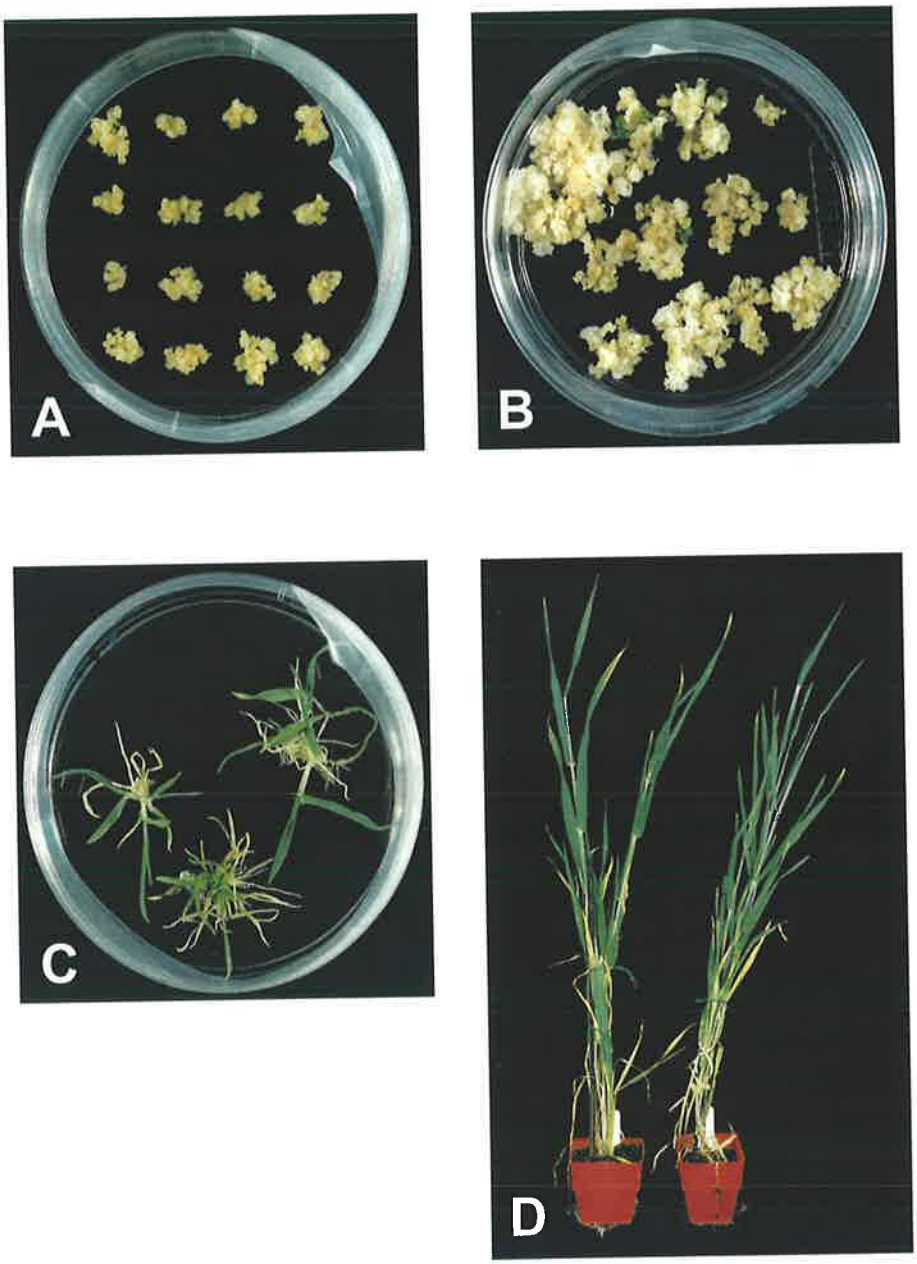
Selection and regeneration of putative transformants using the *Agrobacterium tumefaciens*-mediated transformation procedure developed by Tingay *et al.* (1997) and modified by Matthews *et al.* (2001)

A The callus produced by individual scutella after six weeks culture on selection medium containing 95 µM hygromycin B.

B Shoot regeneration from callus cultured on FHG medium containing 38 µM hygromycin B.

C Regeneration of putative transformants on hormone-free culture medium containing 95 µM hygromycin B.

D Putative transformants established in soil.


A
B
C
D

5.3 RESULTS

5.3.1 Transformation by microprojectile bombardment

The steps involved in the selection and multiplication of bialaphos-resistant callus, and the regeneration of putative transformed plants following microprojectile bombardment are shown in Figure 5.3.

During the course of this work, four experiments were performed using pre-cultured immature scutella of Sloop as target tissues for microprojectile bombardment. A total of 736 scutella were bombarded to produce 3 bialaphos-resistant callus lines (GUNSL1-3) from which plants were regenerated.

5.3.2 *Agrobacterium tumefaciens*-mediated transformation

The initial batch of *Agrobacterium tumefaciens*-mediated transformation experiments relied on the selection and multiplication of hygromyin-resistant callus to yield putative transformed lines capable of plant regeneration (Figure 5.4). A total of 3225 pre-cultured immature Sloop scutella were treated with *Agrobacterium tumefaciens*, and three regenerable, hygromycin-resistant callus lines were recovered.

Freshly isolated immature scutella were used for the second set of *Agrobacterium tumefaciens*-mediated transformation experiments. Following co-cultivation, the scutella were transferred to selection medium, where they produced callus. The callus continued to grow during the subsequent transfers to fresh selection medium, and at the end of this incubation period, the callus of an individual scutellum (hereafter referred to as a callus line) was composed of brown/discoloured sectors and pale-yellow, fast-growing sectors (Figure 5.5 A). Fewer Sloop scutella

produced hygromycin-resistant callus, and the callus tissue developed less vigorously in culture compared with the explants of Golden Promise. All of the callus lines produced on selection medium were transferred to shoot differentiation medium. After two to three weeks culture, regenerated shoots were excised from the callus (Figure 5.5 B) and transferred to hormone-free MS medium that contained the selection agent to induce root formation (Figure 5.5 C). Sometimes, more than one shoot was recovered from a single callus line. In this case, multiple shoots were transferred to hormone-free plant regeneration medium and treated as independent putative transformants until proven otherwise by Southern analysis. After three weeks, some of the regenerated shoots remained green, grew vigorously, and developed strong roots; some grew slowly and produced short roots, while the remainder turned yellow and failed to produce roots. For this transformation procedure, the most reliable indicator of the integration of the selectable marker gene, *hpt*, into the barley genome was the ability of the regenerated shoots to produce strong root systems in hormone-free MS medium that contained the selection agent. The putative transformants that survived selection were transferred to soil and grown to maturity (Figure 5.5 D).

Non-transformed scutella were also grown under selection conditions. The control scutella produced callus on the selection medium and regenerated shoots on the differentiation medium. However, plant regeneration was completely inhibited when the shoots recovered from the control callus were transferred to the hormone-free MS medium that contained the selection agent.

In these experiments, a total of 232 Golden Promise scutella were co-cultivated with *Agrobacterium tumefaciens* to recover 20 hygromycin-resistant plants,

FIGURE 5.6

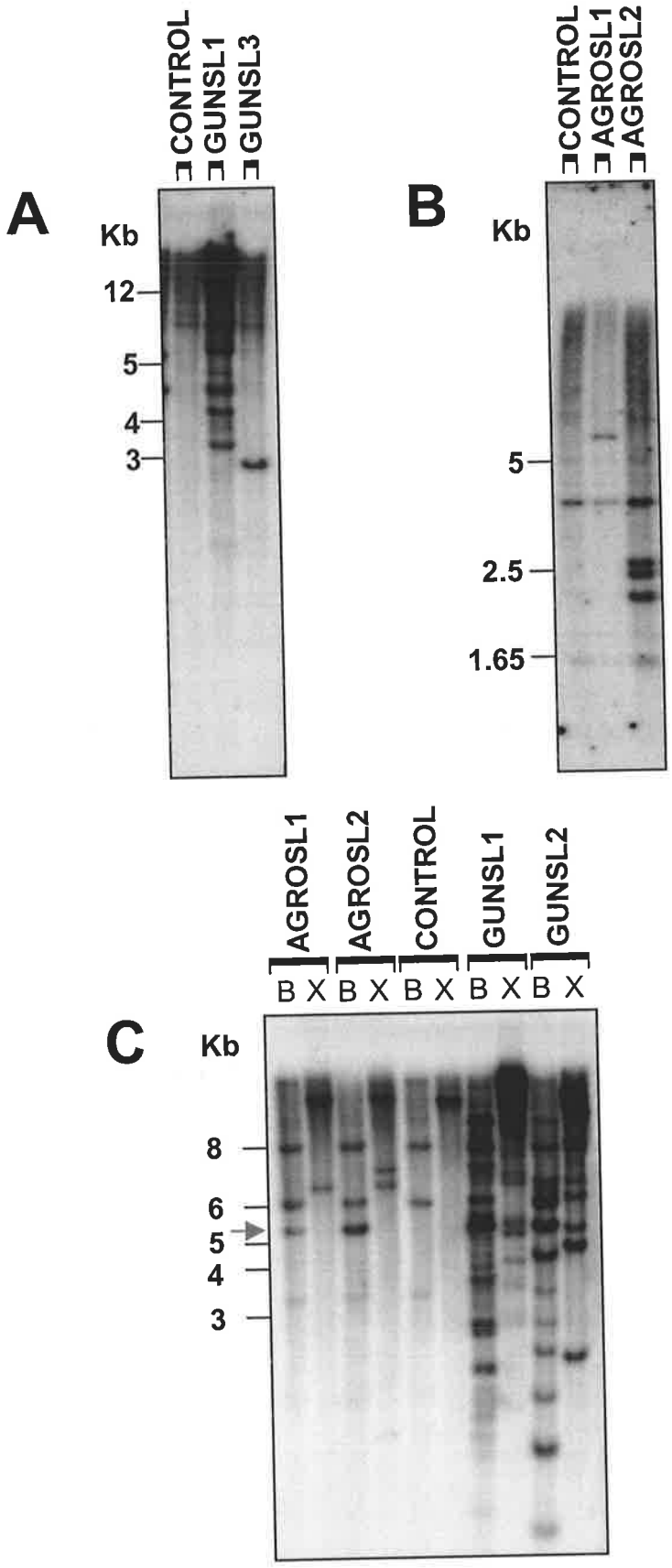
Southern analyses of putative transformed plants recovered from microprojectile bombardment and the first batch of *Agrobacterium tumefaciens*-mediated transformation experiments

A Genomic DNA of two bialaphos-resistant plants (GUNSL1 and GUNSL3) and a non-transformed barley plant (Control) was digested with *Xho*I and hybridised with a ^{32}P-labelled *bar* gene probe.

B Genomic DNA from two hygromycin-resistant plants (AGROSL1 and AGROSL2) and a non-transformed barley plant (Control) was digested with *Bam*HI and hybridised with a ^{32}P-labelled *hpt* gene probe.

C Genomic DNA of putative transformants (AGROSL1, AGROSL2, GUNSL1 and GUNSL2) and a non-transformed barley plant (Control) was digested with *Bam*HI (B) and *Xho*I (X) and hybridised with a ^{32}P-labelled *Pst*I/*Acc*I fragment of plasmid pE2/HPg. The arrow indicates the *Bam*HI DNA restriction fragment (5.6 kb) that corresponds to the insertion of an intact (1→3,1→4)-β-glucanase transgene.

The mobilities of the DNA marker fragments are indicated on the left-hand side of the autoradiograms.


A
CONTROL
GUNSL1
GUNSL3
Kb
12
5
4
3
B
CONTROL
AGROSL1
AGROSL2
Kb
5
2.5
1.65
C
AGROSL1
AGROSL2
CONTROL
GUNSL1
GUNSL2
B X B X B X B X B X
Kb
8
6
5
4
3

while 552 Sloop scutella were co-cultivated with *Agrobacterium tumefaciens* to regenerate 11 putative transformants.

5.3.3 Molecular analyses of putative transformed plants

Genomic DNA isolated from the bialaphos-resistant plants (GUNSL1-3) was digested with *Xho*I and probed with the coding region of the selectable marker gene, *bar*. Plasmid pDM302 contains a single *Xho*I restriction site (Figure 5.1 A). The other *Xho*I site must therefore reside in the plant genome flanking the transgene insertion site. Each *Xho*I restriction fragment that hybridised to the *bar* probe represented a unique insertion of the coding region of the selectable marker gene into the barley genome. The number of *bar* coding region insertions ranged from one for plant GUNSL3 to at least six for transformant GUNSL1 (Figure 5.6 A). Two *bar* gene insertions were detected in the *Xho*I-digested genomic DNA of plant GUNSL2 (data not shown).

Genomic DNA isolated from the hygromycin-resistant plants was digested with *Bam*HI and probed with the coding region of the selectable marker gene, *hpt*. There are two *Bam*HI restriction sites located in the T-DNA region of plasmid pCAM/E2HPg (Figure 5.2). However, the *Bam*HI DNA fragments detected with the *hpt* probe were bordered at one end by the site present in the promoter region of the barley (1→3,1→4)-β-glucanase gene insert (Figure 5.2) and at the other end by a *Bam*HI site located in the barley genome flanking the integrated T-DNA region. Each *hpt*-hybridising restriction fragment therefore corresponded to a unique insertion of the coding region of the selectable marker gene into the plant genome. All of the *Bam*HI-digested DNA fragments detected with the *hpt* probe were larger than 2 kb (Figures

FIGURE 5.7

Southern analyses of hygromycin-resistant plants recovered from *Agrobacterium tumefaciens*-mediated transformation using the procedure developed by Tingay *et al.* (1997) and modified by Matthews *et al.* (2001)

A Genomic DNA of the putative transformants (AGRO) and a non-transformed barley plant (Control) was digested with *Bam*HI and hybridised with a ^{32}P-labelled *hpt* probe. A hyphenated number (1-4) at the end of the transformed line was used to identify plants derived from the same hygromycin-resistant callus.

B Genomic DNA of the putative transformants (AGRO) and a non-transformed barley plant (Control) was digested with *Xho*I and hybridised with a ^{32}P-labelled *Pst*I/*Acc*I fragment of pE2/HPg.

The mobilities of the DNA marker fragments are indicated on the left-hand side of the autoradiograms.

A
Kb
2
3
4
5
6
7
AGROSL4
AGROGP5-1
AGROGP6
AGROSL7-1
AGROSL7-2
AGROSL7-3
AGROGP8
AGROGP9
AGROGP10
CONTROL
AGROSL7-4
AGROGP11
CONTROL
AGROSL12
AGROGP5-2
AGROGP13
AGROGP14
AGROSL15
CONTROL
AGROGP16

B
Kb
5
6
8
9
10
12
AGROSL4
AGROGP5-1
AGROGP6
AGROSL7-3
AGROGP8
AGROGP9
AGROGP10
CONTROL
AGROGP11
CONTROL
AGROSL12
AGROGP13
AGROGP14
AGROSL15
CONTROL
AGROGP16
AGROGP17
AGROGP18
AGROGP19
AGROGP21
AGROSL20

5.6 B and 5.7 A), which is the minimum size of the hybridising fragment expected from the restriction map of plasmid pCAM/E2HPg (Figure 5.2). The detection of a single hybridising fragment provided strong evidence for the insertion of one *hpt* coding region into the genomic DNA of transformants AGROSL1, AGROGP9, AGROSL12 and AGROSL15 (Figures 5.6 B and 5.7 A). The detection of greater than 10 hybridising bands in the digested DNA of transformant AGROGP10 suggested the insertion of more than 10 *hpt* coding regions (Figure 5.7 A). A cross-hybridising *Bam*HI restriction fragment, approximate size 4 kb, was detected in the genomic DNA of transformants AGROSL1 and AGROSL2 and a non-transformed plant following hybridisation with the coding region of the *hpt* gene (Figure 5.6 B).

To detect rearrangements or fragmentation in the mutated (1→3,1→4)-β-glucanase gene, genomic DNA of the putative transformants was digested with *Bam*HI, which was expected to release an intact transgene from the introduced DNA. It was anticipated that the *Pst*I/*Acc*I probe of plasmid pE2/HPg would hybridise to a 5.6 kb *Bam*HI restriction fragment (Figures 5.1 and 5.2). This hybridising fragment contains the promoter region, the open reading frame, the single intron, the H300P amino acid substitution and the transgene "marker" sequence. Southern analysis detected this diagnostic *Bam*HI restriction fragment in the genomic DNA of two different plants regenerated from both *Agrobacterium tumefaciens*-mediated transformation and microprojectile bombardment (Figure 5.6 C). The extra *Bam*HI hybridising DNA fragments observed in the genomic DNA of the two plants recovered from microprojectile bombardment indicated that the transgene sequences had undergone extensive molecular rearrangement or fragmentation prior to or during integration. The *Pst*I/*Acc*I fragment of plasmid pE2HPg also detected two *Bam*HI

restriction fragments (approximate sizes, 8 kb and 6 kb) in the genomic DNA isolated from the putative transformants and a non-transformed plant (Figure 5.6 C). These hybridising fragments represent the native barley (1→3,1→4)-β-glucanase isoenzyme EI and EII genes.

Genomic DNA of the putative transformants was digested with *Xho*I and hybridised with the *Pst*I/*Acc*I DNA fragment from plasmid pE2/HPg to estimate the number of insertion events for the mutated (1→3,1→4)-β-glucanase gene. The single *Xho*I site for plasmid pE2/HPg is located in the multiple-cloning site of the vector pBluescript (SK^+). The number of transgene integration events ranged from six to at least eight among the plants obtained from microprojectile bombardment (Figure 5.6 C). For the T-DNA region of plasmid pCAM/E2HPg, the unique *Xho*I site resides in the 3' region of the CaMV35S promoter (Figure 5.2). Among the plants recovered from *Agrobacterium tumefaciens*-mediated transformation, the number of mutated (1→3,1→4)-β-glucanase gene insertions varied from one to six (Figures 5.6 C and 5.7 B). The *Pst*I/*Acc*I probe also detected two high molecular weight *Xho*I restriction fragments larger than 12 kb in the genomic DNA of the transformed and non-transformed plants (Figures 5.6 C and 5.7 B). These hybridising fragments correspond to the wild-type barley (1→3,1→4)-β-glucanase isoenzyme EI and EII genes.

As mentioned earlier (Section 5.3.2), plant regeneration from the callus lines produced by *Agrobacterium tumefaciens*-mediated transformation frequently resulted in the recovery of multiple plants from the same callus line. To investigate whether the plants regenerated from the same scutellum were derived from the same or different transformation event(s), five sets of hygromycin-resistant plants,

FIGURE 5.8

Southern analyses of multiple plants regenerated from the same hygromycin-resistant callus line

A Genomic DNA of multiple plants (1-3) regenerated from different hygromycin-resistant callus (A-E) and a non-transformed barley plant (Control) was digested with *Bam*HI and hybridised with a ^{32}P-labelled *hpt* gene probe.

B Genomic DNA of the same plants in **A** was digested with *Xho*I and hybridised with a ^{32}P-labelled *Pst*I/*Acc*I fragment of plasmid pE2/HPg.

The mobilities of the DNA marker fragments are indicated on the left-hand side of the autoradiograms.

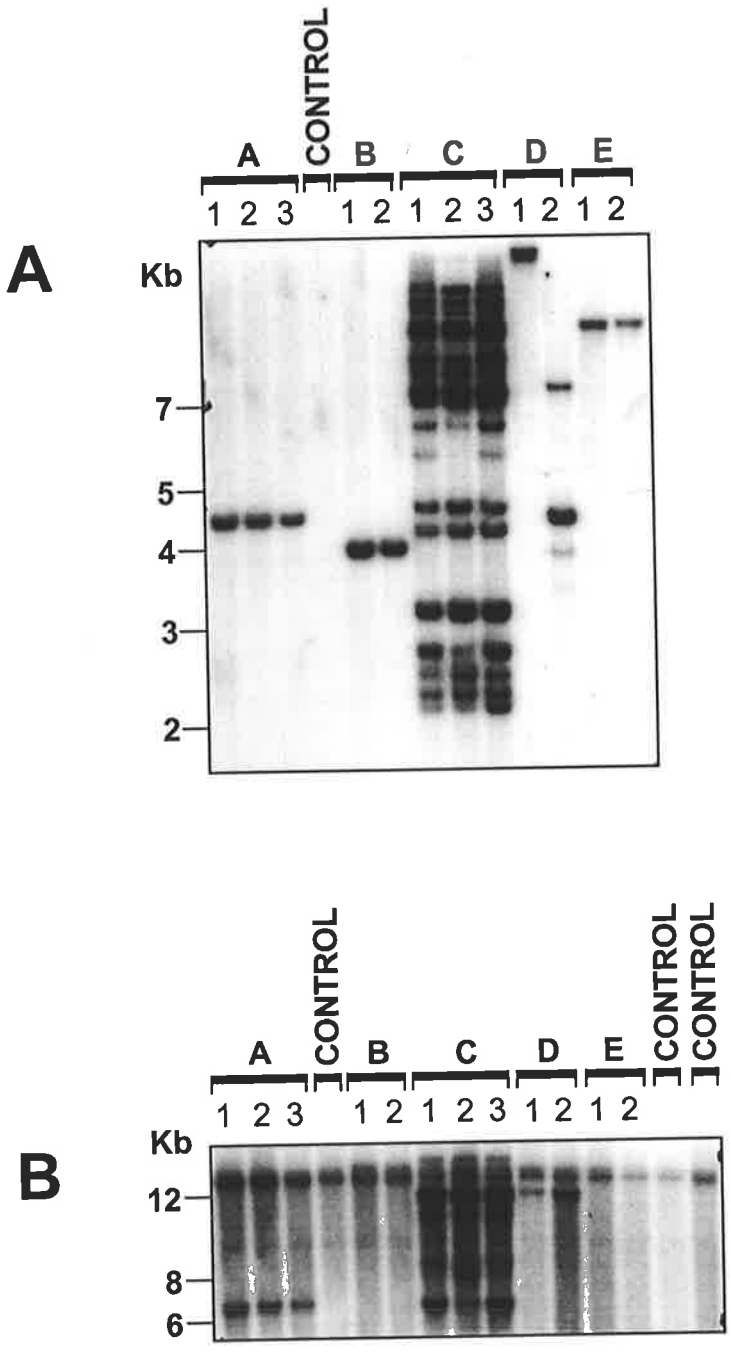

A
CONTROL
A
B
C
D
E
1 2 3
1 2
1 2 3
1 2
1 2
Kb
7
5
4
3
2
B
CONTROL
CONTROL
CONTROL
A
B
C
D
E
1 2 3
1 2
1 2 3
1 2
1 2
Kb
12
8
6

TABLE 5.1

Estimated number of transgene insertion sites in barley transformants produced in this study

Transformant line	*bar*	*hpt*	(1→3,1→4)-β-glucanase transgene
GUNSL1	4		8
GUNSL2	2		6
GUNSL3	1		3
AGROSL1 [1]		1	1
AGROSL2 [1]		3	2
AGROSL3 [1]		ND	2
AGROSL4		2	1
AGROGP5		4	3
AGROGP6		6	2
AGROSL7		2	3
AGROGP8		7	3
AGROGP9		1	1
AGROGP10		greater than 10	4
AGROGP11		6	4
AGROSL12		1	1
AGROGP13		2	1
AGROGP14		3	3
AGROSL15		1	2
AGROGP16		2	1
AGROGP17		ND	2
AGROGP18		ND	6
AGROGP19		ND	1
AGROSL20		ND	3
AGROGP21		ND	1
AGROSL22		1	1
AGROGP23		6	3
AGROSL24		2	2
AGROGP25		1	2
AGROGP26		4	1
AGROGP27		2	3
AGROGP28		5	3
AGROSL29		1	1
AGROSL30		1	1
AGROSL31		3	1
AGROGP32		1	1
AGROSL33		1	1
AGROGP34		2	3

ND: not determined.

[1] Transformants recovered from the first batch of *Agrobacterium tumefaciens*-mediated transformation experiments.

regenerated from five different callus lines, were subjected to Southern analyses to detect differences in the transgene integration patterns (Figures 5.8 A and B). Southern analyses revealed that the plants regenerated from three callus lines were identical, indicating that the transformants were recovered from the same transformed cell (Figures 5.8 A and B; callus lines A, B and E). For callus line C, however, separate plants regenerated from the same callus showed a single band difference in an otherwise identical hybridisation pattern, implying that molecular rearrangement occurred after transgene integration (Figures 5.8 A and B). A pair of plants with completely different transgene integration patterns were recovered from the remaining callus line, suggesting plant regeneration from independently transformed cells located on the same scutellum (Figures 5.8 A and B; callus line D).

Based on the results of the Southern analyses, 37 independently transformed plants composed of 17 Sloop transformants and 20 Golden Promise transformants were produced during this study. The estimated numbers of insertion events for both transgenes in each independent transformed line are presented in Table 5.1. Among the plants that were recovered from the *Agrobacterium tumefaciens*-mediated transformation procedures, the mean number of *hpt* insertion events was three, and the mean number of mutated (1→3,1→4)-β-glucanase gene insertions was two. Although a small population of transformed plants was recovered from microprojectile bombardment, the mean number of insertion events for the selectable marker gene, *bar*, and the mutated (1→3,1→4)-β-glucanase gene were two and six, respectively. Eight Sloop transformants carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene (Table 5.1; AGROSL1, AGROSL4, AGROSL12, AGROSL22, AGROSL29, AGROSL30, AGROSL31 and AGROSL33), and they were all

TABLE 5.2

Summary of barley transformation experiments performed with microprojectile bombardment and *Agrobacterium tumefaciens*

Cultivar	Total number of bombarded [A] or infected [B] scutella	Number of transformation events [3]	Number of fertile lines	Transformation frequency (%)
Sloop [A]	736	3	2	0.4
Sloop [B,1]	3225	3	3	0.001
Sloop [B,2]	552	11	11	2.0
Golden Promise [B,2]	232	20	18	8.6

[1] Transformants recovered from the first batch of *Agrobacterium tumefaciens*-mediated transformation experiments.
[2] Plants regenerated from *Agrobacterium tumefaciens*-mediated transformation experiments using the protocol developed by Tingay *et al*. (1997) and modified by Matthews *et al*. (2001).
[3] Determined by Southern hybridisation to the coding region of the selectable marker gene (*hpt/bar*) and/or the *Pst*I/*Acc*I fragment of plasmid pE2/HPg.

regenerated from *Agrobacterium tumefaciens*-mediated transformation. Thirty-four primary transformants were fertile and demonstrated variable seed set. The remaining three transformed plants (GUNSL1, AGROGP23 and AGROGP26) were morphologically abnormal and sterile.

5.3.4 Summary of the transformation experiments

The results from the different transformation experiments are summarised in Table 5.2. For each procedure, the transformation frequency (%) was expressed as the number of independent transgenic lines (determined by Southern analyses) divided by the total number of treated scutella, multiplied by 100.

Three transformants were obtained for Sloop following microprojectile bombardment of 736 immature scutella (Table 5.2). This corresponded to a transformation frequency of 0.4%. For the first set of *Agrobacterium tumefaciens*-mediated transformation experiments, three transformants were regenerated from 3225 co-cultivated Sloop scutella (Table 5.2). In this case, the transformation frequency was 0.001%. The second batch of *Agrobacterium tumefaciens*-mediated transformation experiments employed the procedures developed by Tingay *et al.* (1997) and modified by Matthews *et al.* (2001) to obtain 11 Sloop transformants from 552 treated scutella (transformation frequency 2%) and 20 Golden Promise transformants from 232 transformed scutella (transformation frequency 8.6%) (Table 5.2).

FIGURE 5.9

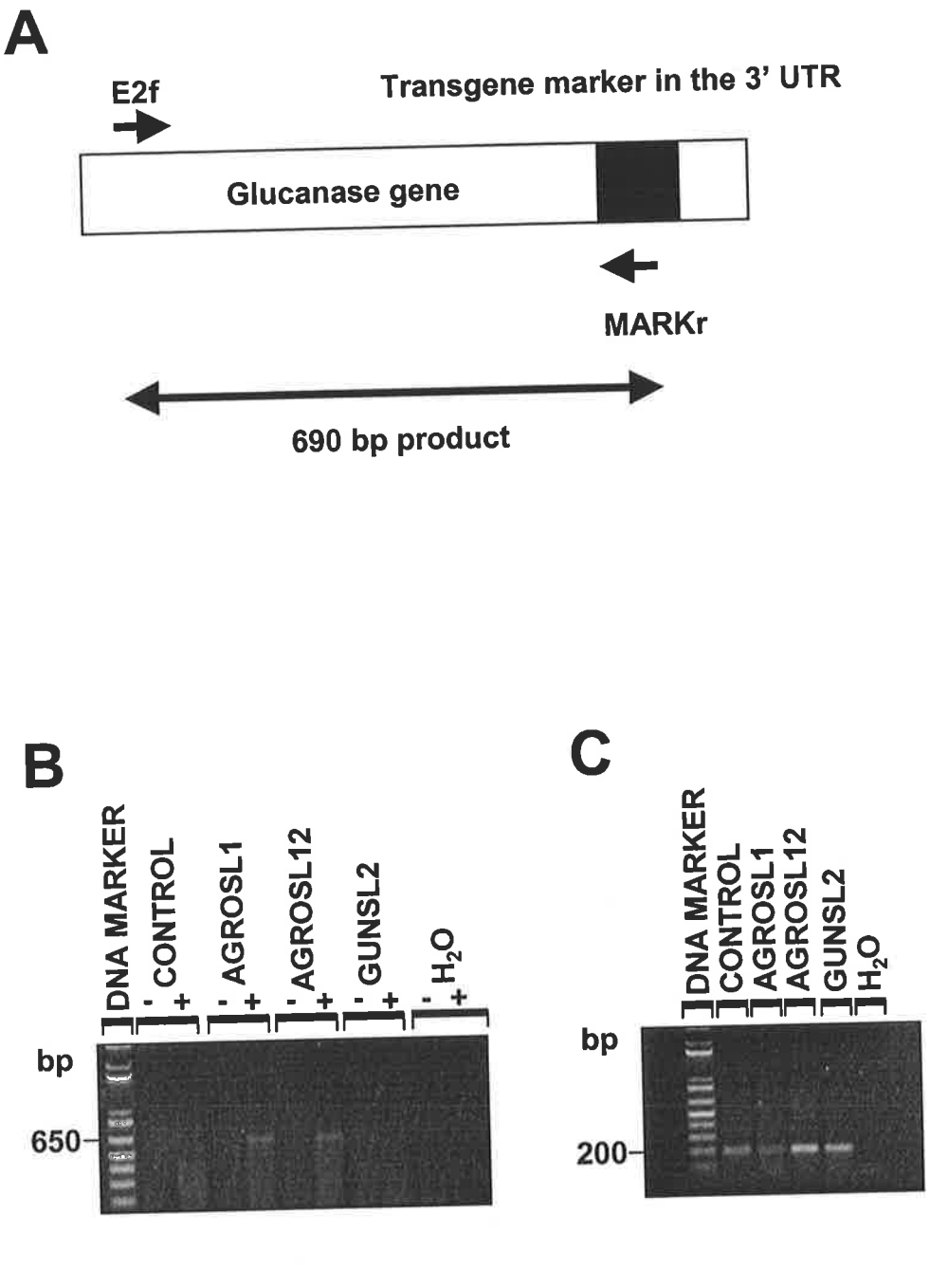
Reverse Transcriptase (RT)-PCR analysis of the RNA extracted from germinated T_1 grain of transformed lines AGROSL1, AGROSL12, and GUNSL2

A Diagrammatic representation of the PCR product amplified with the primers E2f and MARKr (Stewart, 1999). The MARKr primer is specific for the mutated (1→3,1→4)-β-glucanase gene.

B Products amplified with primer pairing E2f and MARKr.

C Products amplified with the actin specific primers.

Control: germinated Sloop grain; +/-: reaction volume contained +/- 10% (v/v) dimethyl sulphoxide.


A
E2f
Transgene marker in the 3' UTR
Glucanase gene
MARKr
690 bp product
B
DNA MARKER
CONTROL
AGROSL1
AGROSL12
GUNSL2
H2O
bp
650
C
DNA MARKER
CONTROL
AGROSL1
AGROSL12
GUNSL2
H2O
bp
200

5.3.5 Transgene expression and inheritance

The expression of the mutated (1→3,1→4)-β-glucanase gene was examined in the T_1 grain of three independent Sloop transformants. Two transformants were produced by *Agrobacterium tumefaciens*-mediated transformation (AGROSL1 and AGROSL12), and the third transformant was recovered from microprojectile bombardment (GUNSL2). Transformed lines AGROSL1 and AGROSL12 carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene, while line GUNSL2 contained six insertions of this transgene (Table 5.1). Molecular assays also revealed that transformants AGROSL1 and GUNSL2 carried an intact mutated (1→3,1→4)-β-glucanase gene, and that GUNSL2 contained numerous rearranged or fragmented transgene sequences (Figure 5.6 C).

To detect the presence of mRNA that encodes the mutated (1→3,1→4)-β-glucanase H300P enzyme, total RNA was isolated from T_1 grains, and cDNA was synthesised by reverse transcriptase. The forward primer, E2f, and the reverse primer, MARKr, which is unique to the transgene, were used to amplify RNA transcripts that specifically encoded the mutated (1→3,1→4)-β-glucanase enzyme (Figure 5.9 A). A single PCR fragment of the expected size (690 bp) was amplified from the cDNA prepared from the germinated T_1 grains of transformants AGROSL1 and AGROSL11 (Figure 5.9 B). The cDNA prepared from the non-transformed Sloop grains and the T_1 grains of GUNSL2 did not produce a PCR product (Figure 5.9 B). Primers specific for the mRNA encoding the constitutive actin enzyme of barley resulted in a single 215 bp PCR product of comparable abundance in all of the different grain samples and revealed that approximately equivalent amounts of mRNA were used for each amplification reaction (Figure 5.9 C).

FIGURE 5.10

Co-segregation of the selectable marker gene, *hpt*, and the mutated (1→3,1→4)-β-glucanase gene in the T_1 progeny plants of transformed line AGROSL1

A Genomic DNA of the primary transformant AGROSL1, T_1 plant AGROSL1.$T_{1.1}$, eight other T_1 progeny plants and a non-transformed barley plant (Control) was digested with *Bam*HI and hybridised with a ^{32}P-labelled *hpt* gene probe.

B Genomic DNA of the same plants in **A** was digested with *Xho*I and hybridised with a ^{32}P-labelled *Pst*I/*Acc*I fragment of plasmid pE2/HPg. Note that the Control was positioned in a different track.

The mobilities of the DNA marker fragments are indicated on the left-hand side of the autoradiograms.

A
AGROSL1
AGROSL1.T1.1
OTHER T1 PROGENY
CONTROL
OTHER T1 PROGENY
Kb
7
6
4
3
B
AGROSL1
AGROSL1.T1.1
OTHER T1 PROGENY
CONTROL
OTHER T1 PROGENY
Kb
12
8
6

As mentioned in Section 5.3.3, these self-pollinated primary transformants produced variable amounts of T_1 grains. Insufficient amounts of grain were obtained from AGROSL12 and GUNSL2 to allow further analysis of transgene inheritance and expression in the T_1 generation. Furthermore, the transgenic status of the other Sloop lines was either unknown or the confirmed transformants that carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene had not set seed at the time the initial expression experiments commenced (Table 5.1; AGROSL4, AGROSL22, AGROSL29, AGROSL30, AGROSL31 and AGROSL33). As a result, 10 randomly chosen T_1 grains of primary transformant AGROSL1 were used to investigate transgene segregation patterns in the T_1 progeny plants.

Nine of the 10 grains germinated, and the plants reached maturity and yielded as many grains as the seed-derived control plants. The hybridisation patterns for the transgenic T_1 progeny plants were identical to those of the primary transformant, and revealed that all the transgenic T_1 progeny plants carried both the selectable marker gene, *hpt*, and the mutated (1→3,1→4)-β-glucanase gene (Figures 5.10 A and B). The transmission of the *hpt* gene and the mutated (1→3,1→4)-β-glucanase gene from AGROSL1 to its T_1 progeny resulted in six T_1 plants with both transgenes and three with no transgene sequences (Figures 5.10 A and B).

The immature T_2 embryos from one T_1 plant (AGROSL1.$T_{1.1}$) were cultured on hormone-free MS culture medium that contained the selection agent to quickly obtain T_2 plants and to rapidly identify plants homozygous for the transgenes. The embryos from non-transformed Sloop plants did not germinate on the plant regeneration medium that contained the selection agent. After one week in culture, the embryos of AGROSL1.$T_{1.1}$ had germinated, and the plants were healthy and had well-

FIGURE 5.11

Southern analyses of the T_2 progeny plants derived from a single transgenic T_1 plant, AGROSL1.$T_{1.1}$

A Genomic DNA of the primary transformant AGROSL1, the T_1 plant AGROSL1.$T_{1.1}$, ten T_2 progeny plants and a non-transformed barley plant (Control) was digested with *Bam*HI and hybridised with a ^{32}P-labelled *hpt* gene probe.

B Genomic DNA of the same plants in **A** was digested with *Xho*I and hybridised with the *Pst*I/*Acc*I fragment of plasmid pE2/HPg.

The mobilities of the DNA marker fragments are indicated on the left-hand side of the autoradiograms.

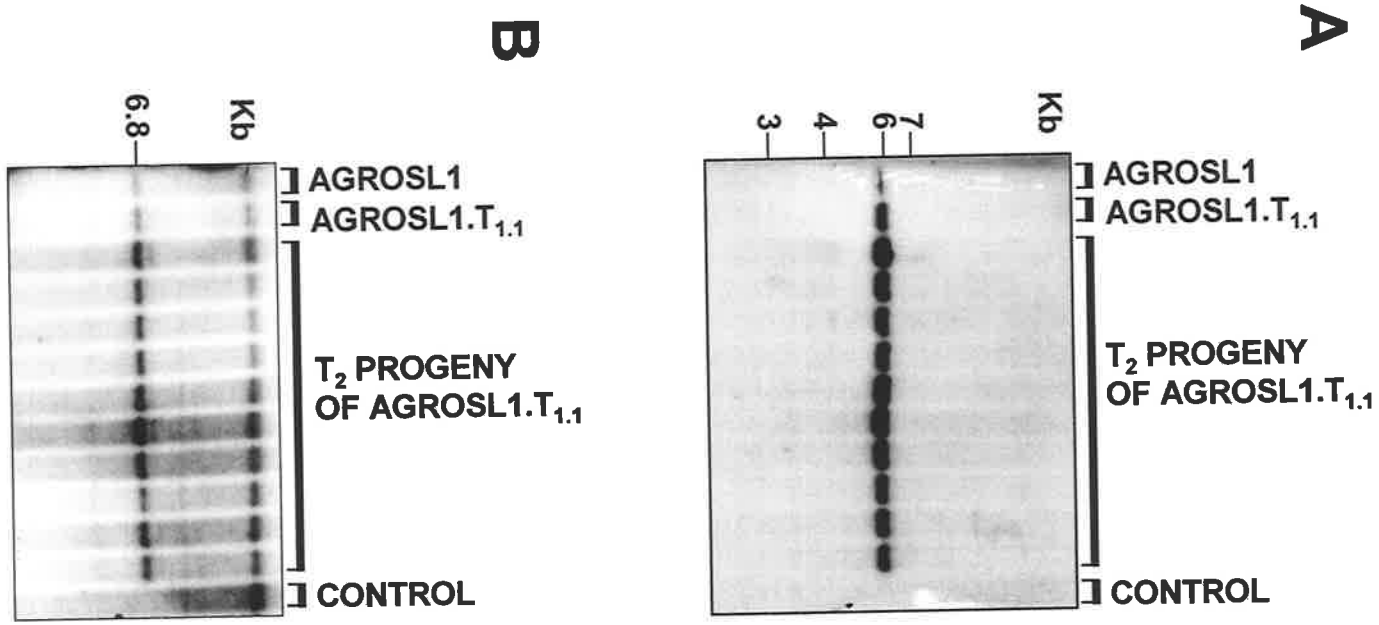
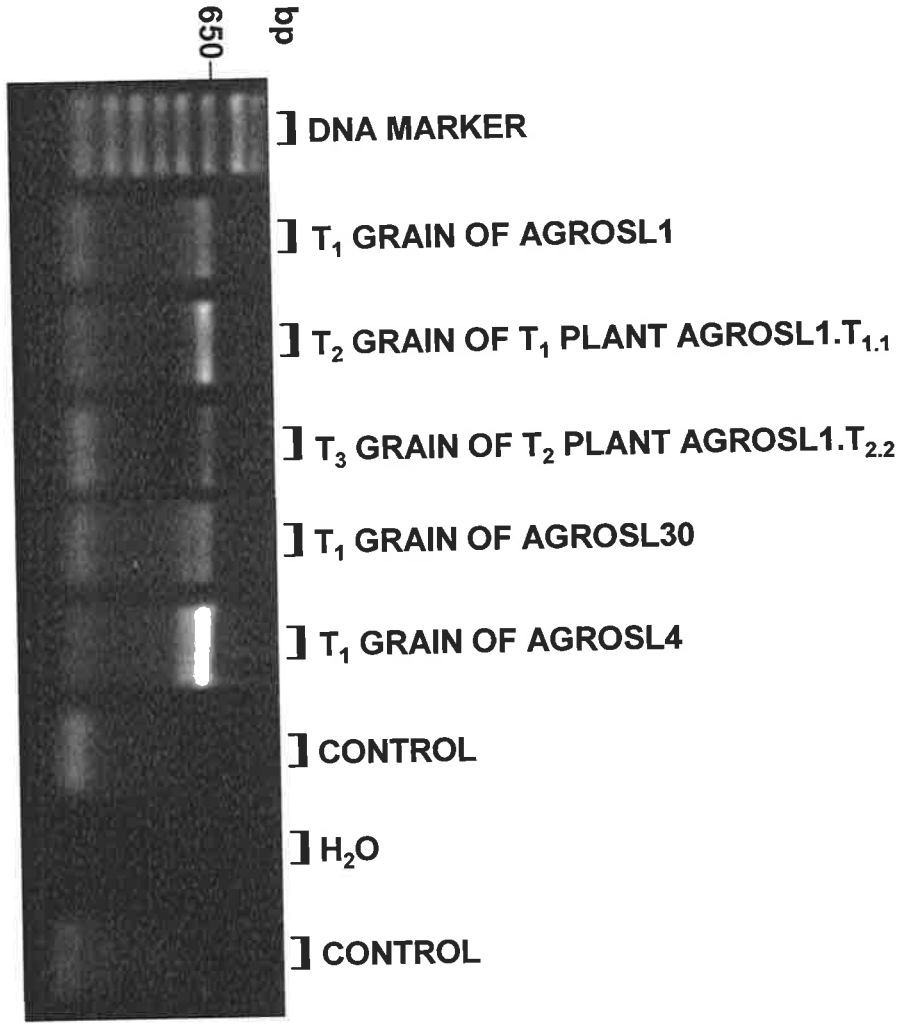

A
Kb
3
4
6
7
AGROSL1
AGROSL1.T1.1
T2 PROGENY
OF AGROSL1.T1.1
CONTROL
B
Kb
6.8
AGROSL1
AGROSL1.T1.1
T2 PROGENY
OF AGROSL1.T1.1
CONTROL

FIGURE 5.12

Reverse transcriptase (RT)-PCR analysis of the RNA extracted from three generations of grain from transformed line AGROSL1, and the T_1 grain of two additional Sloop transformants that carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene

Products amplified with the primers E2f and MARKr. The MARKr primer is specific for the mutated (1→3,1→4)-β-glucanase gene. Reaction volume contained 10% (v/v) dimethyl sulphoxide. Control: germinated Sloop grain.


650
bp
] DNA MARKER
] T_1 GRAIN OF AGROSL1
] T_2 GRAIN OF T_1 PLANT AGROSL1.$T_{1.1}$
] T_3 GRAIN OF T_2 PLANT AGROSL1.$T_{2.2}$
] T_1 GRAIN OF AGROSL30
] T_1 GRAIN OF AGROSL4
] CONTROL
] H_2O
] CONTROL

developed root systems. The data from the germination test indicated that all the progeny plants of AGROSL1.$T_{1.1}$ were resistant to hygromycin selection. Ten T_2 plants derived from the embryo germination test were transferred to soil. Southern analyses revealed that the *hpt* probe and the *PstI/AccI* DNA fragment of plasmid pE2/HPg hybridised to restriction fragments in the genomic DNA of all the T_2 plants (Figures 5.11 A and B).

Total RNA was extracted from the germinated T_1 grain of primary transformant AGROSL1, the T_2 grain of plant AGROSL1.$T_{1.1}$, and T_3 grain of one T_2 plant, AGROSL1.$T_{2.2}$, to determine whether the expression of the mutated (1→3,1→4)-β-glucanase gene was inherited in successive generations. All three generations of grain demonstrated expression of the mutated (1→3,1→4)-β-glucanase gene at the mRNA level (Figure 5.12).

At this time, the T_1 grains became available for two other *Agrobacterium tumefaciens*-derived transformants that contained a single insertion of the mutated (1→3,1→4)-β-glucanase gene (Table 5.1; AGROSL4 and AGROSL30). The results from the RT-PCR analysis detected transgenic mRNA in the germinated T_1 grain of both independent transformed lines (Figure 5.12).

5.3.6 Heat stability of (1→3,1→4)-β-glucanases in transgenic grain

Homozygous transgenic T_3 grain of transformed line AGROSL1 was used to investigate the activity of the (1→3,1→4)-β-glucanases in germinated grain. Fractional precipitation of the enzyme extracts with ammonium sulphate revealed that the majority of the (1→3,1→4)-β-glucanase activity was associated with material precipitated between 40-80% saturated ammonium sulphate (data not shown).

TABLE 5.3

Summary of the repeated measures analysis of variance for the extracts of transgenic and non-transgenic grain incubated at 48°C

Main effect	F probability
Grain type	0.003 *
Time (min)	0.001 *
Grain type x time	0.248 NS

* statistically significant at the 5% level

NS: not statistically significant at the 5% significance level

TABLE 5.4

Summary of the split-plot analysis of variance for the extracts of transgenic and non-transgenic grain following 15 min incubation at a range of different temperatures

Main effect	F probability
Grain type	NA
Temperature (°C)	NA
Grain type x temperature	< 0.001 *

* statistically significant at the 5% level

NA: not applicable to test due to higher order interactions

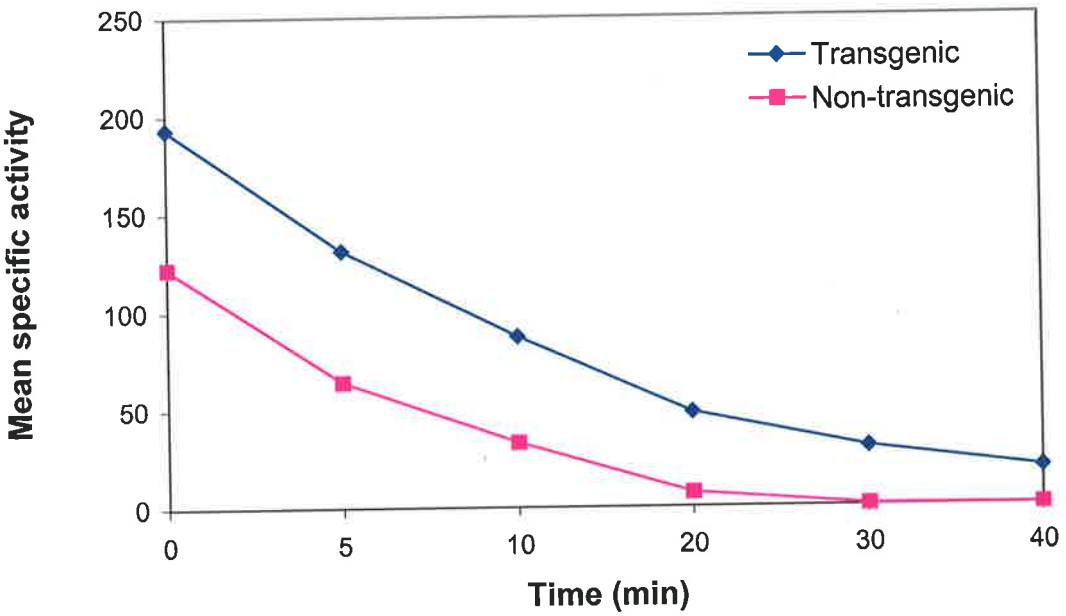
FIGURE 5.13

(1→3,1→4)-β-Glucanase activity in the extracts prepared from transgenic and non-transgenic barley grain

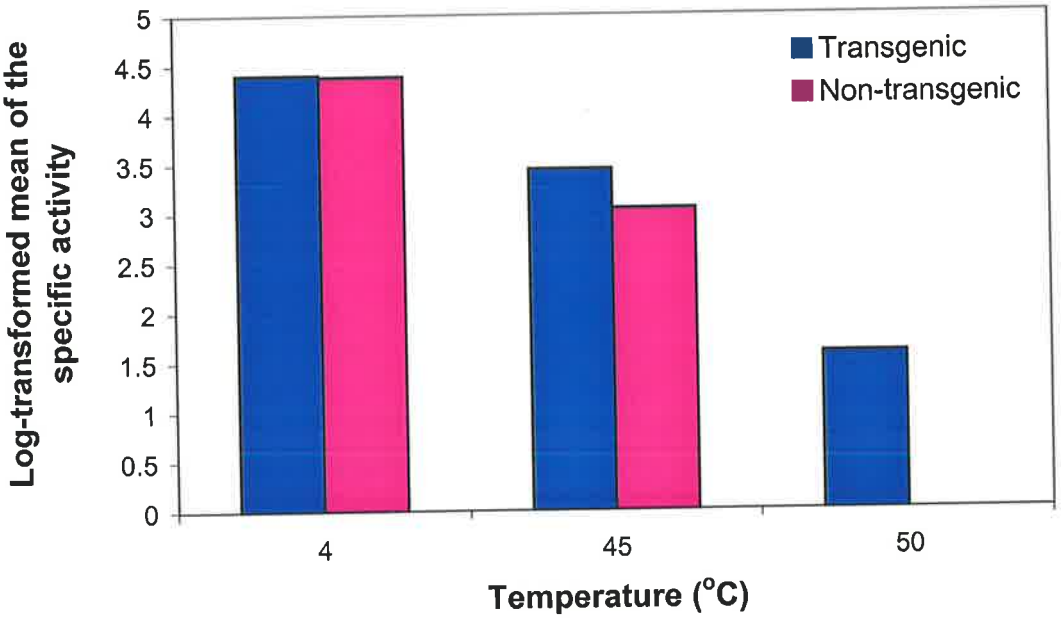
A The mean specific activities for the transgenic and non-transgenic extracts at different time points during incubation at 48°C.

B Log-transformed, mean specific activities for the transgenic and non-transgenic extracts following incubation at different temperatures for 15 min.

A


Mean specific activity
250
200
150
100
50
0
0
5
10
20
30
40
Time (min)
Transgenic
Non-transgenic

B


Log-transformed mean of the specific activity
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
4
45
50
Temperature (°C)
Transgenic
Non-transgenic

Grain type and duration of incubation at 48°C had significant effects on (1→3,1→4)-β-glucanase activity (Table 5.3). The transgenic grain produced a significantly higher amount of assayable (1→3,1→4)-β-glucanase activity than the non-transgenic grain (Table 5.3). The changes in mean specific activities for the enzymes extracted from the transgenic and non-transgenic grain at 48°C are shown graphically in Figure 5.13 A. The LSD test detected differences in the mean specific activities for both extracts at time points t=0, t=5 and t=10 (data not shown). The changes in mean specific activities were small and not significantly different to each other for both extracts after prolonged incubation (t=20, t=30 and t=40) at 48°C (data not shown). No significant interaction exists between grain type and time (Table 5.3), because the plots for the changes in the mean specific activities for both extracts were approximately parallel (Figure 5.13 A). On the other hand, the results from the assay that incubated both extracts at different temperatures for 15 min demonstrated that the interaction between grain type and temperature had a statistically significant effect on specific activity (Table 5.4). Moreover, the LSD test determined that the specific activity detected in the extract of the transgenic grain was significantly different to that measured in the extract of the non-transgenic grain following incubation at 50°C for 15 min (Figure 5.13 B).

5.4 DISCUSSION

Southern hybridisation was used to confirm the integration of the selectable marker gene (*bar* or *hpt*) and the mutated (1→3,1→4)-β-glucanase gene into the genomic DNA of the plants regenerated on selection medium (Figures 5.6 and 5.7). The microprojectile bombardment technique resulted in a 100% co-integration frequency for the two separate gene constructs that carried the selectable marker gene, *bar*, and the mutated (1→3,1→4)-β-glucanase gene. The left and right T-DNA border/plant DNA junction fragments were analysed in the *Agrobacterium tumefaciens*-derived transgenic plants to confirm the integration of both the *hpt* gene and the mutated (1→3,1→4)-β-glucanase gene. Molecular analyses of the left and right T-DNA border fragments of 28 *Agrobacterium tumefaciens*-derived transgenic plants demonstrated that both transgenes were successfully inserted into the plant genome in every case (Table 5.1).

To determine whether the general tendency of *Agrobacterium tumefaciens*-mediated transformation to integrate intact transgene sequences into the plant genome (Bilang *et al.*, 1999) was also valid for barley transformation, the genomic DNA of six transformants was digested with *Bam*HI and probed with the *Pst*I/*Acc*I fragment of plasmid pE2/HPg. It was anticipated that this probe would hybridise to a 5.6 kb *Bam*HI restriction fragment, which includes the promoter, the single intron, the open reading frame, the H300P amino acid substitution and the transgene "marker" sequence (Figures 5.1 and 5.2). The presence of a single hybridising fragment of the correct size in the *Bam*HI-digested genomic DNA of *Agrobacterium tumefaciens*-mediated transformants AGROSL1, AGROSL2 (Figure 5.6 C), and AGROSL3 (data not shown) demonstrated that the inserted T-DNA region contained an essentially

intact mutated (1→3,1→4)-β-glucanase gene. When the genomic DNA of the three transformed plants recovered from microprojectile bombardment (GUNSL1-3) was subjected to the same molecular analysis, only transformants GUNSL1 and GUNSL2 contained the diagnostic *Bam*HI restriction fragment (Figure 5.6 C and data not shown). This fragment corresponded to the insertion of at least one intact mutated (1→3,1→4)-β-glucanase gene into the genomic DNA of these transformants. In addition to this DNA fragment, a mixture of smaller and larger hybridising *Bam*HI restriction fragments were detected in the genomic DNA of the microprojectile bombardment-derived transformants (Figure 5.6 C and data not shown). These hybridising bands represented rearranged or fragmented insertions of the mutated (1→3,1→4)-β-glucanase gene.

Transgenic plants that carry a single insertion of the introduced transgene are generally preferred to those with multiple insertions of the transgene, because the integration of increased numbers of the transgene sequence are frequently associated with heritability and expression instabilities (Finnegan and McElroy, 1994; Alvarez *et al.*, 2000; Srivastava and Ow, 2001). In the present study, Southern analyses revealed that approximately 44% (15/34) of the plants transformed by *Agrobacterium tumefaciens* carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene (Table 5.1). This frequency was close to those observed for the unselected transgene in transformed rice (32%; Hiei *et al.*, 1994) and wheat (35%; Cheng *et al.*, 1997), the same as that in barley (Tingay *et al.*, 1997), but significantly lower than that calculated for maize (70%; Ishida *et al.*, 1996). The differences in the frequencies of *Agrobacterium tumefaciens*-derived transformants that carried a single insertion of the unselected gene in the current study and previous studies could be

attributed to the plant species, the explant type, the promoters used in the gene constructs, the culture medium, or the *Agrobacterium tumefaciens* strain (Cheng *et al.*, 1997; Weir *et al.*, 2001). None of the plants recovered from the co-transformation experiments performed with microprojectile bombardment contained a single insertion of the mutated (1→3,1→4)-β-glucanase gene (Table 5.1). The microprojectile bombardment-derived plants contained high insertion numbers of the mutated (1→3,1→4)-β-glucanase gene, and the number of insertions for the *bar* gene was low (Table 5.1). A similar phenomenon was observed for a large population of transgenic wheat plants produced by microprojectile bombardment with two separate gene constructs (Stoger *et al.*, 1998). In the present study, the inclusion of a higher concentration of the non-selected gene construct compared with the selectable marker gene, *bar*, in the DNA/gold precipitation process could account, in part at least, for the detection of more mutated (1→3,1→4)-β-glucanase gene insertions in the genomic DNA of the plants produced by microprojectile bombardment. The ratio of selectable marker gene and non-selectable gene used to coat the gold particles was 1:2.5 in the present study, which was greater than the 1:1.5 ratio employed by Stoger *et al.* (1998).

In the present study, the two different techniques used for the *Agrobacterium tumefaciens*-mediated transformation experiments had a significant influence on the number of plants regenerated from the immature scutella of Sloop (Table 5.2). The most significant difference between the two sets of *Agrobacterium tumefaciens*-mediated transformation experiments was the composition of the culture media used for the selection and regeneration of putative transformed plants. The early batch of experiments relied on the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and the

media developed for microprojectile bombardment (Section 4.2.4), while the experiments performed towards the end of this study utilised the auxin Dicamba and the media optimised for *Agrobacterium tumefaciens*-mediated transformation of Golden Promise (Tingay *et al.*, 1997). Auxins have been widely employed to stimulate the production of regenerable callus and the formation of somatic embryos in barley tissue cultures (Lührs and Lörz, 1987). In the present study, however, more putative transformants were regenerated from the embryogenic callus grown on selection medium supplemented with Dicamba compared with 2,4-D. Castillo *et al.* (1998) have compared the influence of the auxins, 2,4-D, Dicamba and 4-amino-3,5,6-trichloropicolinic acid (Picloram) on the plant regeneration capacity of three to four month old embryogenic callus cultures of barley. They demonstrated that callus induction medium containing Dicamba improved plant regeneration frequencies from long-term embryogenic callus cultures of barley. A similar effect in the present study could have been enhanced by the addition of BAP to the shoot regeneration medium. This cytokinin has been linked to the stimulation of shoot formation in cereal tissue cultures (Bhaskaran and Smith, 1990), and it is possible that shoot production in Sloop callus cultures improved with the addition of a low concentration of BAP to the differentiation medium. These culture media have been coupled with *Agrobacterium tumefaciens*-mediated transformation of immature barley scutella to successfully regenerate transgenic plants from putative transformed callus (Tingay *et al.*, 1997; Horvath *et al.*, 2000; Patel *et al.*, 2000; Trifonova *et al.*, 2001; Wang *et al.*, 2001; Fang *et al.*, 2002).

The molecular analyses of the Sloop plants, regenerated from the hygromycin-resistant callus lines maintained on Dicamba-containing culture medium, revealed that

the transformation frequency was also improved significantly: 2% compared with 0.001% achieved using callus induction medium supplemented with 2,4-D (Table 5.2). The outcomes reported here for Sloop are in agreement with the observations of Trifonova *et al.* (2001), who demonstrated higher transformation frequencies for bialaphos-resistant callus of Golden Promise maintained on Dicamba-containing culture medium compared with 2,4-D. There is a paucity of published data that assesses the influence of different auxins on cereal transformation efficiency. However, Barro *et al.* (1998) generated transgenic wheat and tritordeum lines using different types and concentrations of auxins. Picloram-containing culture medium induced higher plant regeneration frequencies and resulted in a 4.6 fold increase in transformation frequencies compared with 2,4-D.

Golden Promise was the other cultivar used in the second batch of *Agrobacterium tumefaciens*-mediated transformation experiments. The immature embryos of this tissue culture-responsive cultivar have been utilised in previous studies of *Agrobacterium tumefaciens*-mediated barley transformation (Tingay *et al.*, 1997; Horvath *et al.*, 2000; Patel *et al.*, 2000; Trifonova *et al.*, 2001; Fang *et al.*, 2002), although Wang *et al.* (2001) transformed the scutellar tissues of the Australian cultivar, Schooner. The transformation frequency for Golden Promise was 8.6% in the present study (Table 5.2). Using the same *Agrobacterium tumefaciens* strain and the same Dicamba-containing callus induction medium, lower transformation frequencies have been reported for the intact or bisected immature embryos of Golden Promise (4.2%, Tingay *et al.*, 1997; 4.8%, Trifonova *et al.*, 2001; 3.5%, Fang *et al.*, 2002). Several research groups have demonstrated that the plant regeneration capacity of non-transformed barley callus cultures is improved by increasing the copper sulphate

concentration of the callus induction medium (Dahleen, 1995; Castillo *et al.*, 1998; Bregitzer *et al.*, 1998; Cho *et al.*, 1998). Tingay *et al.* (1997) and Fang *et al.* (2002) added 0.1 μM copper sulphate to their respective callus induction media used for transformation, while Trifonova *et al.* (2001) increased the copper sulphate concentration to 5 μM. In the present study, the inclusion of 10 μM copper sulphate in the callus induction medium could have further enhanced the production of embryogenic structures in the transformed Golden Promise callus cultures, which subsequently resulted in the improved recovery of transformed plants and the higher transformation frequency.

Microprojectile bombardment was also used to transform Sloop with the mutated (1→3,1→4)-β-glucanase gene. Using this procedure, the transformation frequency was 0.4% (Table 5.2), which is very similar to that obtained for the same cultivar with the selectable marker gene, *bar*, and the reporter gene, *uidA* (0.45%; Section 4.4).

The expression of the native barley (1→3,1→4)-β-glucanase isoenzyme EII gene is predominantly restricted to the aleurone layer of the germinated grain (Stuart *et al.*, 1986; Slakeski and Fincher, 1992). It was anticipated therefore that the promoter of the native gene would also drive expression of the mutated (1→3,1→4)-β-glucanase gene in the germinated transgenic grain. RT-PCR analysis was used to examine the expression of the mutated (1→3,1→4)-β-glucanase gene in the germinated T_1 grains of five independent transformants (Figures 5.9 B and 5.12). The results confirmed that the promoter of the native gene directed expression of the mutated (1→3,1→4)-β-glucanase gene in the germinated T_1 grain of the transgenic lines produced by *Agrobacterium tumefaciens*-mediated transformation (Figures 5.9 B and 5.12). The T_1 grain samples were harvested from primary transformants that

carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene (Table 5.1; AGROSL1, AGROSL4, AGROSL12 and AGROSL30). The observed variation in the expression levels could be caused by the position of the integrated gene in the barley genome of the independent transformed lines. The remaining transformation event (GUNSL2) was produced by microprojectile bombardment, and no mRNA of the mutated (1→3,1→4)-β-glucanase gene could be detected (Figure 5.9 B). Additional analyses are required for other tissues of the germinated grain (e.g. coleoptile, root, stem and leaf) to determine whether transgene expression is regulated in a tissue-specific manner in each transformed line.

Although there are numerous reports about transgene activity in cereals following gene transfer (Register *et al.*, 1994; Srivastava *et al.*, 1996; Stoger *et al.*, 1998; Demeke *et al.*, 1999), there is conflicting evidence in regard to the effects that transgene insertion number and structure have on gene expression. In the present study, transgene silencing appeared to be associated with specific features of the transgene integration pattern. All of the analysed transformants derived from *Agrobacterium tumefaciens*-mediated transformation carried a single insertion of the mutated (1→3,1→4)-β-glucanase gene, and there was molecular evidence to confirm the presence of an intact transgene expression unit in one transformed line (Figures 5.6 C and 5.7 B). RT-PCR analysis showed that mRNA encoding the mutated (1→3,1→4)-β-glucanase gene was present in the germinated T_1 grains of these primary transformants (Figures 5.9 B and 5.12). On the other hand, the transgenic plant recovered from microprojectile bombardment carried multiple insertions of the transgene, exhibited extensive rearrangement or fragmentation of the mutated (1→3,1→4)-β-glucanase gene (Figure 5.6 C), and RT-PCR analysis revealed that no

transgenic mRNA was present (Figure 5.9 B). Further analyses of a larger population of *Agrobacterium tumefaciens*-derived transformants are needed to establish whether the tendency of this transformation technology to integrate low numbers of intact transgene sequences into the plant genome and exhibit transgene expression is generally valid for barley.

To further investigate the segregation and transcription levels of the mutated (1→3,1→4)-β-glucanase gene, inheritance and activity patterns of the transgene were monitored in one transformed line, AGROSL1. Southern analyses of the T_1 progeny plants revealed co-segregation of the selectable marker gene, *hpt*, and the mutated (1→3,1→4)-β-glucanase gene (Figures 5.10 A and B). Although the segregation ratio for the transgenic phenotype in the T_1 generation could not be accurately ascertained because of the small population of analysed progeny plants, the data from the Southern analyses suggested that either the transgene insertions were closely linked on the same chromosome or that a single dominant, heterozygous locus controlled the inheritance of the transgenes. In either case, self-pollination of the primary transformant, AGROSL1, would generate transgenic T_1 and non-transgenic T_1 progeny plants in an approximate ratio of 3:1. The T_1 generation would be composed of 25% homozygous transgenic plants, 50% heterozygous transgenic plants, and 25% homozygous non-transgenic plants. The results of the germination test for immature embryos derived from T_1 plant AGROSL1.$T_{1.1}$ implied that this plant was homozygous for the selectable marker gene, *hpt*, because the test did not identify any hygromycin-sensitive progeny plants. The detection of both transgenes in the genomic DNA of all the T_2 plants derived from AGROSL1.$T_{1.1}$ confirmed that the parent plant was homozygous (Figures 5.11 A and B).

Based on the results of the RT-PCR analyses, transcription of the mutated (1→3,1→4)-β-glucanase gene in the germinated grain of this transformed line remained active through to the T_3 generation (Figure 5.12). The stable transcription of the mutated (1→3,1→4)-β-glucanase gene in this transformed line could be attributed to the ability of *Agrobacterium tumefaciens* to insert its T-DNA region into a actively transcribed region of the plant genome as a simple, intact transgenic locus (Pawlowski and Somers, 1996; Birch, 1997).

Through the insertion of the 35 bp MARKr oligonucleotide in the 3' UTR of the transgene, it was therefore possible to confirm that the gene encoding the mutated (1→3,1→4)-β-glucanase was stably inherited in transformed line AGROSL1, and that mRNA encoding the transgene was transcribed. However, it was not possible to confirm that the transgenic mRNA was correctly translated into an active enzyme, without prolonged purification procedures and amino acid sequence analysis to detect the H300P mutated form of the enzyme. Furthermore, no antibodies capable of distinguishing the wild-type and mutated forms were available. As an initial test for the presence of active H300P enzyme, the heat stability of extracts of germinated grain from transgenic and non-transgenic barley lines were compared, in the expectation that significant levels of the H300P mutated enzyme would be reflected in higher thermostability of (1→3,1→4)-β-glucanase activity in germinated transgenic grain. The (1→3,1→4)-β-glucanases in the extracts of transgenic and non transgenic grain were partially purified by fractional precipitation with ammonium sulphate, prior to the measurement of activity.

The heat stability of the barley (1→3,1→4)-β-glucanase isoenzymes EI and EII has been the subject of a number of previous studies (Woodward and Fincher

1982 b; Brunswick *et al.*, 1987; Loi *et al.*, 1987; Chen *et al.*, 1995; Stewart *et al.*, 2001). The wild-type enzymes are very unstable at temperatures above 45°C, and the purified forms of both isoenzymes are completely inactivated following heat treatment at 50°C for 15 min (Woodward and Fincher 1982 b). In the current study, preliminary results from the assays for enzyme activity indicated that the addition of the mutated (1→3,1→4)-β-glucanase gene to the barley genome significantly increased the amount of enzyme produced in the transgenic grain (Table 5.3). The most likely explanation for this observed increase in (1→3,1→4)-β-glucanase activity is that the mutated gene produced an active form of the mutated enzyme in the germinated grain of the homozygous transgenic line. Reductometric analyses demonstrated that this significant difference in the specific activity of the enzymes extracted from the transgenic and non-transgenic grain was maintained at the different time points in the assay performed at 48°C (Figure 5.13 A). Furthermore, (1→3,1→4)-β-glucanase activity was completely inactivated in the extract of non-transgenic grain following incubation at 50°C for 15 min, while the transgenic extract retained a higher and statistically significant level of enzyme activity under similar conditions (Figure 5.13 B). Additional analyses are required to accurately monitor the decay of (1→3,1→4)-β-glucanase activity in the extracts of the transgenic and non-transgenic grain over temperature points within the range of 45-55°C.

Therefore, the important remaining questions relate to whether or not the data collected from the molecular and biochemical assays accurately reflect the expression of the actual H300P enzyme, encoded by the transgene, and whether the expressed enzyme is more thermostable than the wild-type enzyme under simulated malt extraction conditions.

To demonstrate unequivocally that the increased (1→3,1→4)-β-glucanase activity detected in the transgenic extract is due to the synthesis of the mutated H300P enzyme, it will now be necessary to provide direct evidence that the mutated (1→3,1→4)-β-glucanase isoenzyme EII is present in germinated transgenic grain. This could be accomplished by rigorous purification of the enzymes from the extracts of germinated grain (Woodward and Fincher, 1982 a). Trypsin digestion of the purified enzyme would generate a series of peptide fragments, which would be purified by High Performance Liquid Chromatography prior to amino acid sequencing. The sequence information would be compared with the previously determined amino acid sequence of the wild-type (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986) to identify the histidine to proline substitution at amino acid 300 of the mutated enzyme. The relative proportions of peptides with H300 and P300 would give an indication of the amount of transgene product in the (1→3,1→4)-β-glucanase preparation.

Ultimately, it will be important to demonstrate that the thermostability of the mutated (1→3,1→4)-β-glucanase in extracts of transgenic grain is also observed under mashing conditions commonly used in the brewery. Stewart (1999) tested the thermostability of the mutated (1→3,1→4)-β-glucanase H300P enzyme *in vitro*, under simulated mashing conditions at 65°C. In this study, kiln-dried non-transformed malt was mashed and incubated at 65°C for 20 min to inactivate endogenous (1→3,1→4)-β-glucanase activity. Following the heat-inactivation treatment, mutated (1→3,1→4)-β-glucanase H300P enzyme was added to the mash, and the decay of (1→3,1→4)-β-glucanase activity was monitored at 65°C. Although these experiments demonstrated that the mutated enzyme retained activity at least fives times longer

than the wild-type enzyme under similar experimental conditions, the capacity of the mutated enzyme to hydrolyse residual (1→3,1→4)-β-glucan was minimal compared with the bacterial and fungal β-glucanases currently employed in the brewing industry. If a similar result was observed for the transgenic grain produced in the current study, further improvement in enzyme thermostability would be required to ensure that mutated barley (1→3,1→4)-β-glucanases have useful commercial applications. It is estimated that a mutated barley (1→3,1→4)-β-glucanase with an improvement in thermostability of 10-12°C would be required to alleviate problems with residual (1→3,1→4)-β-glucans during mashing or lautering.

5.5 SUMMARY AND CONCLUSIONS

Microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation procedures were employed to transform barley with a mutated (1→3,1→4)-β-glucanase gene in this study. The selectable marker gene, *bar*, in combination with the selection agent, bialaphos, was used for microprojectile bombardment, and *Agrobacterium tumefaciens*-mediated transformation relied on the selectable marker gene, *hpt*, and the antibiotic hygromycin B to regenerate putative transformants of Sloop and Golden Promise. Molecular analyses confirmed the integration of the different selectable marker genes into the plant genome of the putative transformants, and identified transformed Sloop plants that possessed a single insertion of the mutated (1→3,1→4)-β-glucanase gene.

Transcription of the mutated (1→3,1→4)-β-glucanase gene was examined in the transformed Sloop lines recovered from both transformation technologies. Based on the outcomes of the RT-PCR analyses, four independent transformed lines derived from *Agrobacterium tumefaciens*-mediated transformation demonstrated detectable levels of transgenic mRNA in the germinated T_1 grains. On the other hand, no mRNA for the mutated (1→3,1→4)-β-glucanase gene was detected in a transformed line produced by microprojectile bombardment. The apparent absence of transgene transcription in this line, compared with the transformants obtained by *Agrobacterium tumefaciens*-mediated transformation, could be explained by differences in the number and structure of mutated (1→3,1→4)-β-glucanase gene insertions and/or the transgene integration site.

Southern analyses confirmed the stable integration and transmission of both transgenes to the T_3 generation for one transformed Sloop line produced by

Agrobacterium tumefaciens-mediated transformation. RT-PCR analysis demonstrated that the transgene was transcribed in the grain that was homozygous for the mutated (1→3,1→4)-β-glucanase gene. Statistical analyses revealed that grain homozygous for the mutated (1→3,1→4)-β-glucanase gene produced a significantly higher amount of enzyme activity than the non-transgenic grain. In addition, residual enzyme activity detected in the transgenic extract was significantly greater than the non-transgenic extract following incubation at 50°C for 15 min. Taken together, these results demonstrated that the mutated (1→3,1→4)-β-glucanase gene was inherited in a Mendelian fashion, expressed and correctly processed in barley cells, and indicated that the mutated enzyme was functional in the extract of the transgenic grain.

The heat lability of the barley (1→3,1→4)-β-glucanases, especially at the temperatures employed during the kilning and mashing stages of the malting process, has provoked considerable interest in the development of techniques that improve the thermostability of these enzymes (Fincher, 1994). One approach that has been investigated involved the use of site-directed mutagenesis to introduce a histidine to proline substitution at amino acid 300 of the barley (1→3,1→4)-β-glucanase isoenzyme EII, which led to increased enzyme stability at elevated temperatures (Stewart *et al.*, 2001). In the current study, a transgenic approach was used to insert this mutated (1→3,1→4)-β-glucanase gene into the barley genome of an elite Australian cultivar and improve enzyme activity in the extract of the transgenic grain. The success of this approach provides "proof-of-concept" for the use of genetic manipulation in the improvement of malting quality in barley and means that other useful genes can be inserted into barley with the reasonable

expectation that the transgenic lines will assist breeders to release cultivars with desirable quality traits.

CHAPTER SIX

SUMMARY AND FUTURE DIRECTIONS

6.1 SUMMARY OF EXPERIMENTAL RESULTS

The primary objective of the work described in this thesis was to develop plant regeneration and transformation protocols for elite Australian barley cultivars. The establishment of this technology was critical in allowing the activities of a variety of selectable marker and reporter genes and a thermostable barley (1→3,1→4)-β-glucanase gene to be studied in a cereal background. The scutellar region of the immature embryo was identified as the target tissue most likely to produce fertile plants following transformation, because the scutellum-derived callus possessed a higher capacity for plant regeneration compared with cell suspension and protoplast cultures. Microprojectile bombardment-mediated transformation was subsequently used to produce six independent transformed barley lines that carried the *bar* and *uidA* genes. A thermostable barley (1→3,1→4)-β-glucanase gene was inserted into the barley genome using microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation procedures.

Barley (1→3,1→4)-β-glucanases are key enzymes in the malting and brewing industries, where their primary responsibility is to initiate degradation of the (1→3,1→4)-β-glucans located in the endosperm cell walls of the germinated grain. However, the barley (1→3,1→4)-β-glucanases are rapidly denatured at elevated temperatures employed during the kilning and mashing processes. Site-directed mutagenesis of the gene encoding the barley (1→3,1→4)-β-glucanase isoenzyme EII was used to introduce a histidine to proline substitution at amino acid 300 of the enzyme, which improved the thermostability of the mutated form of the enzyme compared with the wild-type enzyme (Stewart *et al.*, 2001).

Thirty-seven barley lines transformed with this thermostable barley (1→3,1→4)-β-glucanase were produced in the current study. Southern analyses confirmed the stable integration of the mutated barley (1→3,1→4)-β-glucanase gene in the barley genome and demonstrated that *Agrobacterium tumefaciens*-mediated transformed plants tended to carry low numbers of transgene insertions and exhibit predictable patterns of integration compared with plants recovered from microprojectile bombardment. The results of the RT-PCR analyses demonstrated that the native promoter of the barley (1→3,1→4)-β-glucanase isoenzyme EII gene directed expression of the mutated (1→3,1→4)-β-glucanase at the mRNA level in the T_1 grains of four transformants obtained from *Agrobacterium tumefaciens*-mediated transformation.

Finally, one of these lines was analysed for the segregation of the thermostable (1→3,1→4)-β-glucanase gene. The distribution of the thermostable (1→3,1→4)-β-glucanase gene in the T_1 grains of this transformed line was examined by Southern analysis, and the results indicated that this line carried a single insertion of the transgene that behaved as a dominant transgenic locus. The T_3 grain of this line, which was homozygous for the thermostable (1→3,1→4)-β-glucanase gene, was used to investigate enzyme thermostability. The transgenic barley grain produced a significantly higher amount of (1→3,1→4)-β-glucanase activity compared with extract of the non-transgenic grain. In addition, the crude extract of the transgenic grain retained a statistically significant amount of enzyme activity compared with the extract of the non-transgenic grain following incubation at 50°C for 15 min. These observations could be attributed to the expression of the thermostable (1→3,1→4)-β-glucanase gene at the protein level.

6.2 FUTURE DIRECTIONS

A number of experiments could be conducted in the future to complete and expand on work described in this thesis. As described earlier (Section 5.4), these experiments would initially endeavour to elucidate the nature of the increased enzyme activity in the transgenic extract and assess the performance of the mutated (1→3,1→4)-β-glucanase enzyme under mashing conditions encountered at the brewery.

In the long-term, additional studies could be initiated to determine the factors that influence the efficiency of *Agrobacterium tumefaciens*-mediated transformation for different barley cultivars. In the current study, the transformation frequency for Sloop (2%) was low compared with Golden Promise (8.6%) (Table 5.2). Thus, a future possibility for this research could be to investigate methods that improve plant regeneration from embryogenic callus of elite Australian cultivars. The current study relied on the use of an elevated concentration of copper sulphate in the callus induction medium and the inclusion of benzylaminopurine (BAP) in the shoot regeneration medium to regenerate Sloop plants from putative transformed callus cultures. Further improvement in the plant regeneration frequency for Sloop could be achieved through modification of additional components of the tissue culture media, including boric acid and $FeSO_4$ concentrations (Dahleen and Bregitzer, 2002), and the ratio of inorganic:organic nitrogen (Nuutila *et al.*, 2000). Furthermore, the reduction in the transformation frequency for Sloop could relate to the low frequency of explant survival following co-cultivation with *Agrobacterium tumefaciens*. Non-responsive scutella became brown, and callus induction was completely inhibited. This "hypersensitive-type" response could be mediated by an oxidative

burst mechanism in which a rapid and transient production of reactive oxygen species occurs as a result of an incompatible interaction between *Agrobacterium tumefaciens* and the cells of the scutellum (Frame *et al.*, 2002). In the future, the inclusion of compounds with known antioxidant activity (e.g. ascorbic acid and L-cysteine) in the co-cultivation medium could improve the viability of barley scutellum cells following *Agrobacterium tumefaciens* infection. As a consequence, more cells capable of plant regeneration would survive the infection process, resulting in higher transformation frequencies. The addition of these compounds to the tissue culture media used for *Agrobacterium tumefaciens*-mediated transformation of rice and maize resulted in improved transformation frequencies for these cereal species (Enríquez-Obregón *et al.*, 1999; Frame *et al.*, 2002). In general, techniques that help to preserve the regeneration capacity of the scutellar cells that can produce plants following *Agrobacterium tumefaciens*-mediated transformation will also be critical in improving barley transformation frequencies. These methods could include using a shorter *in vitro* culture phase to select transformed cells (Fang *et al.*, 2002) and employing a "resting" step prior to selection to reduce the impact that the combined stress of *Agrobacterium tumefaciens* and selection has on plant regeneration from transformed cells (Zhao *et al.*, 2001).

Finally, the transformation technologies developed in the current study will be employed to study gene function in transgenic barley. The morphological, agronomic, and biochemical changes in the transformed plants could help to elucidate roles for genes discovered in functional genomics programs conducted at the Universities of Adelaide and Melbourne (Professor GB Fincher, personal communication). When the function of a gene has been correctly identified in

transgenic Golden Promise, the work described here will allow the selected gene to be inserted into an elite cultivar (e.g. Sloop) and facilitate the rapid incorporation of the useful gene into national barley breeding programs.

APPENDICES

APPENDIX 1

Composition of Horsham soil mix

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

pH was approximately 6.0-6.5.

The mix was not heat-treated.

APPENDIX 2

Culture media

	MSB3D	L1D2	L2M	L7D0.5
Macro-nutrient mg/L				
NH_4NO_3	1520	750	200	250
KNO_3	2224	1750	1750	1500
$CaCl_2.2H_2O$	441	450	450	450
KH_2PO_4	370	200	200	200
$MgSO_4.7H_2O$	170	350	350	350
Micro-nutrient mg/L				
H_3BO_3	6.2	5	5	5
$MnSO_4.H_2O$	16.9	15	15	15
$ZnSO_4.7H_2O$	8.6	7.5	7.5	7.5
KI	0.83	0.75	0.75	0.75
$CoCl_2.6H_2O$	0.024	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$Na_2MoO_4.2H_2O$	0.24	0.25	0.25	0.25
Iron solution mg/L				
Na_2EDTA	37	37	37	37
$FeSO_4.7H_2O$	28	28	28	28
Amino acids mg/L				
L-PROLINE	-	150	150	300
L-GLUTAMINE	-	750	750	1500
L-ASPARAGINE	-	100	100	200
Vitamins mg/L				
ASCORBIC ACID	-	1	1	1
D-BIOTIN	-	0.005	-	0.005
Ca-PANTOTHENATE	-	0.5	0.5	0.5
CHOLINE CHLORIDE	-	0.5	-	0.5
FOLIC ACID	-	0.2	-	0.2
NICOTINIC ACID	-	1	1	1
p-AMINOBENZOIC ACID	-	1	-	1
PYRIDOXINE HCL	-	1	1	1
RIBOFLAVIN	-	0.1	-	0.1
THIAMINE HCL	1	10	10	10
MYO-INOSITOL	250	100	200	100
Sugar g/L				
SUCROSE	30	-	-	-
MALTOSE	-	50	30	180
Other mg/L				
2,4-D	3	2	0.5	0.5
BAP	-	-	1	-
CASEIN HYDROLYSATE	1000	-	-	-
pH	5.8	5.7	5.7	5.7

Hormone-free MS medium was the same as MSB3D, except that 2,4-D was omitted. With the exception of the hormone-free MS medium, all media were sterilised by ultrafiltration. Solid media were prepared as 2 x concetrates and mixed 1:1 with double strength Phytagel™ (Sigma Chemical Company, St Louis, MO, USA; standard concentration, 2.5 g/L).

APPENDIX 3

Complex reagents used for the reductometric (1→3,1→4)-β-glucanase assay

Somogyi Solution 1 A	
KNa tartrate	15 g
Na_2CO_3	30 g
Volume (H_2O)	300 ml
Dissolve completely before adding 20 g $NaHCO_3$	
Somogyi Solution 1 B	
Na_2SO_4	180 g
Volume (H_2O)	500 ml
Heat to degas and cool the solution to room temperature. Add A to B and make volume up to 1 L	
Somogyi Solution 2	
$CuSO_4.5H_2O$	5 g
Na_2SO_4	45 g
Volume (H_2O)	250 ml
Mix Solution 1 and 2 in a 4:1 ratio just before use	
Nelson Solution A	
NH_4 molybdate	25 g
Concentrated H_2SO_4 (18 M)	21 ml
Volume (H_2O)	450 ml
Dissolve completely	
Nelson Solution B	
Na arsenate	3 g
Volume (H_2O)	25 ml
Mix solution B with A and incubate at 37°C for 24-48 h to stabilise the colour	

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