

GENETIC MODIFICATION IN *PINUS PATULA*
USING TRANSGENIC TECHNOLOGY

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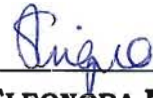
"All men have stars," he answered, "but they are not the same things for different people. For some, who are travellers, the stars are guides. For others they are no more than little lights in the sky. For others, who are scholars, they are problems. For my businessman they are wealth. But all these stars are silent. You-you alone-will have the stars as no one else has them -...You-only you-will have stars that can laugh!"

The Little Prince – Antoine De Saint-Exupéry

DECLARATION

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal (formerly University of Natal), Pietermaritzburg, from January 2000 to August 2006 under the supervision of Professor J. van Staden and the co-supervision of Dr N.P. Makunga and Dr N.B. Jones.

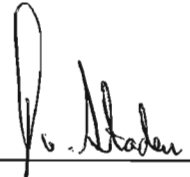
This study represents original work and has not been submitted in any form for another degree or diploma to any other university and is the result of my own investigation. The work of others has been duly acknowledged in the text.



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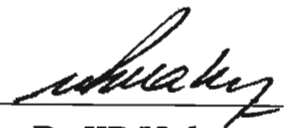
2006

We certify that the above statement is correct.



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ABSTRACT

Progress in tree biotechnology initially trailed behind agricultural crops due to their long life cycle, difficult tissue culture and regeneration protocols, and their abundance in natural forests. However, rapid global deforestation rates, together with an increased world demand for pulp, paper and timber products, have prompted scientific and commercial focus to improve genetic timber stocks.

South Africa, a tree-poor country (where indigenous forests are protected), has relied almost solely on exotic plantations to meet its demand for timber. A pioneer study investigating the feasibility of using direct (biolistic) and indirect (*Agrobacterium*-mediated) methods for gene transfer was undertaken in *Pinus patula* Schiede et Deppe, a Mexican softwood and a forerunner for saw timber, pulpwood and paper in the South African forest industries. The aim of the transformation methods was to impart herbicide resistance to the trees. This was achieved via the introduction of a *bar*-GUS pAHC25 cassette under the control of the *ubiquitin* promoter.

To provide target material for transformation, two *in vitro* micropropagation pathways were used: somatic embryogenesis and organogenesis. Both embryonal suspensor masses (ESM) and somatic embryos at various stages of development were initially used as target explants for the biolistic study using an established *in vitro* protocol. A stepwise selection was implemented in order to allow transformed (particularly bombarded) cultures the opportunity to regenerate under selection pressure using MSG3 maintenance medium supplemented with BASTA® herbicide at 1 mg l⁻¹ followed by 3 mg l⁻¹ active ingredient at the next subculture. Biolistic transgene delivery was more efficient when sorbitol was included in the pre-bombardment medium enabling use of higher vacuum and shooting pressures, without lowering the regeneration potential of ESM significantly. Bombarded material from two genotypes (Lines 2 and 3) was regenerated to produce mature somatic embryos using an optimized regeneration regimen. The indirect study with *Agrobacterium tumefaciens* (LBA4404), transformed with the pAHC25 vector via triparental mating or heat shock, used a

variety of target tissues including: mature somatic embryos, ESM and mature zygotic embryos (MZE's) - a novel *in vitro* system for *P. patula*. The *Agrobacterium*-mediated method resulted in optimized decontamination conditions using a combination of liquid MSG3 (or sterile dH₂O for mature embryos) supplemented with 500 mg l⁻¹ cefotaxime, with rotation, and sterile 65 mm Whatman No. 3 filter paper stacks, which avoided excess filtering and stress to transformation material. Further efforts to aid regeneration during the indirect study included L-proline post-transformation, though no mature somatic embryos were regenerated at the conclusion of the *Agrobacterium*-mediated study. Recovery of transformed ESM in both studies was best during the active growth phase 4-6 d after subculture. Regeneration with good somatic embryo potential was an exigent aspect in both transformation studies.

Expression of positive histochemical GUS activity in all transformed material was confirmed by polymerase chain reaction (PCR) analysis indicating that *Pinus patula* tissue was amenable to transformation. A new *bar* PCR regime was implemented in *P. patula*. In the biolistic study, a higher transformation efficiency of *bar* amplicons (53%) than GUS amplicons (45%) was observed, reflecting their non-linked status on the pAHC25 transformation vector.

This is the first report of biolistic transformation of *P. patula* that will allow for the production of transgenic ESM. The production of transgenic *P. patula* holds great promise for commercial development in the South African forestry industry. The application of transgenic trees in the timber industry is numerous but the aims most relevant to *P. patula* include wood modification and disease resistance to pathogens like pitch canker fungus.

PUBLICATIONS FROM THIS THESIS

Nigro SA, Makunga NP, Jones NB and Van Staden J (2004) A biolistic approach towards *Pinus patula* Scheide et Deppe embryonal suspensor masses (ESM). *Plant Growth Regulation* 44: 187-197.

Nigro SA, Makunga NP, Jones NB and Van Staden J (**In preparation**) Short communication: Report on *Agrobacterium*-mediated transfer in *Pinus patula*. *South African Journal of Botany*.

Nigro SA, Makunga NP, Jones NB and Van Staden J (**In preparation**) Mini review: Gene transfer in Conifers: A decade in review. *South African Journal of Botany*.

BOOK CHAPTER

Ford CS, Fisher LJ, Jones NB, **Nigro SA**, Makunga NP and Van Staden J (2005) Somatic embryogenesis in *Pinus patula*. In: SM Jain and P Gupta (Eds) Protocol for Somatic Embryogenesis in - Woody Plants. Springer, Dordrecht, Netherlands. Pp 121-139. ISBN 10-14020-2984-5.

CONFERENCE CONTRIBUTIONS

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PAPERS AND POSTERS PRESENTED AT INTERNATIONAL CONFERENCES

Nigro SA, Makunga NP, Jones NB and Van Staden J (2002). Generation of transgenic *Pinus patula* embryogenic tissue via particle bombardment transfer (POSTER). 17–20 Nov 2002: 8th International Pacific Rim Biotechnology Conference, Auckland, New Zealand.

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ABBREVIATIONS

| | | |
|--------------------|---|--|
| A ₂₆₀ | - | Absorbance at 260 nm |
| A ₂₈₀ | - | Absorbance at 280 nm |
| ABA | - | Abscisic acid |
| ANOVA | - | Analysis of variance |
| AS | - | Acetosyringone (3'5'-Dimethoxy-4'-hydroxy-acetophenone) |
| BA | - | N ⁶ -benzyladenine |
| <i>bar</i> | - | Phosphinothricin acetyl transferase gene of <i>Streptomyces hygroscopicus</i> ATCC 21705 (THOMPSON <i>et al.</i> 1987) that confers bialaphos resistance |
| bp | - | Base pair |
| CaMV35S | - | Promoter of Cauliflower mosaic virus gene encoding 35S rRNA |
| Ci | - | Curie |
| dATP | - | Deoxyadenosine triphosphate |
| dCTP | - | Deoxycytidine triphosphate |
| ddH ₂ O | - | Deionized distilled water |
| dGTP | - | Deoxyguanosine triphosphate |
| dH ₂ O | - | Distilled water |
| DCR | - | Douglas fir cotyledon revised medium (GUPTA and DURZAN 1985) |
| DMSO | - | Dimethyl sulfoxide |
| DNA | - | Deoxyribonucleic acid |
| DNase | - | Deoxyribonuclease |
| dNTP | - | Deoxynucleoside triphosphate |
| dTTP | - | Deoxythymidine triphosphate |
| EDTA | - | Ethylenediamene tetraacetic acid |
| ESM | - | Embryonal suspensor mass |
| EtBr | - | Ethidium bromide |
| GUS | - | Hydrolase β-glucuronidase enzyme (JEFFERSON <i>et al.</i> 1987) |
| HPLC | - | High-performance liquid chromatography |
| LB | - | Luria-Bertani (medium) |
| MSG ₃ | - | Maintenance medium (BECWAR <i>et al.</i> 1990) |
| MIII | - | Molecular Weight Marker III (Roche, Germany): 13-fragment mixture |

| | | |
|-------------|---|--|
| | | from cleavage of λ -DNA with <i>EcoRI</i> and <i>HindIII</i> |
| MUG | - | 4-methylumbelliferyl β -D-glucuronide |
| MXIV | - | Molecular Weight Marker MXIV (Roche): 15-fragment mixture prepared through cleavage of a specially constructed Roche plasmid with specific restriction enzymes |
| MZE | - | Mature zygotic embryo |
| OD | - | Optical density |
| PCR | - | Polymerase chain reaction |
| PEG | - | Polyethelene glycol |
| PPT | - | Phosphinothricin |
| PVPP | - | Polyvinylpolypyrriollidone |
| RNase | - | Ribonuclease |
| Sarkosyl | - | <i>N</i> -laurosarcosine |
| SDS | - | Sodium dodecyl sulphate |
| SM | - | Suspension medium |
| TAE | - | Tris-acetic acid-EDTA buffer |
| Tris-Cl | - | Tris hydrochloride |
| <i>Taq</i> | - | <i>Thermus aquaticus</i> enzyme |
| TDZ | - | Thidiazuron |
| TE | - | Tris-EDTA buffer |
| <i>uidA</i> | - | Gene encoding β -glucuronidase from <i>Escherichia coli</i> |
| v/v | - | Volume per volume |
| w/v | - | Weight per volume |
| X-gluc | - | 5-bromo-4-chloro-3-indolyl- β -D-glucuronide |

CHAPTER 1

GENERAL INTRODUCTION

Pinus patula Schiede et Deppe (in Schlechtendal et Chamisso, Linnaea VI, 354) is a woody conifer that has become one of the most important softwood species in the South African forestry industry. It is the most popular pine for timber and pulp production as demonstrated by its extensive growth in southern African plantations. This gymnosperm is also known by the vernacular names of Mexican weeping pine, jelicote pine, pinho patula, patula pine (trade name) or spreading-leaved pine (WORMALD 1975; POYNTON 1984). It was first introduced into South Africa by recommendation of Sir David Hutchins from the high mountains of tropical Mexico in 1907, by means of seed at Tokai in the Western Cape (LEGAT 1930; LOOCK 1950; POYNTON 1979; BURGESS and WINGFIELD 2001), as part of the many softwoods imported from northern America. Areas of indigenous forests were not sufficient to meet the needs of the expanding population motivating the introduction of this exotic species.

Pinus of the family Pinaceae was placed in the section *Pinus* subsection *Oocarpae* by LITTLE and CRITCHFIELD (1969) replacing that of SHAW (1914) (WORMALD 1975). The subsection *Oocarpae* also includes *P. radiata* Don, *P. attenuata* Lemm, *P. muricata* Don, *P. greggii* Engelm. ex Parl. *P. pringlei* Shaw and *P. oocarpa* Scheide. The natural distribution of these species ranges from Mexico to Central and North America to northern Nicaragua. The South African introduction of *P. patula* spurred a widespread use of this species under plantation conditions throughout Africa. *Pinus patula's* fast growth, good silvicultural features and availability of seed from South African sources promoted its use in Angola, Lesotho, Kenya, Malawi, Mozambique, Zambia, Zimbabwe, Swaziland and Tanzania (POYNTON 1984).

The *Pinus* genus is well adapted to dry climates and to growth in soils that are normally too poor for agriculture, though somewhat susceptible to drought

(POYNTON 1984). *Pinus patula* thrives in humid, temperate regions of the summer rainfall areas particularly where mist belts occur, in South Africa, where it is extensively used for afforestation. This species is both frost and snowfall tolerant, and grows best at higher altitudes (MORRIS and PALLETT 2000).

This evergreen genus is fast-growing and can reach between 20-40 m in height when mature. It has a spreading crown. Male and female flowering occurs in September (POYNTON 1979). Pine needles are slender secondary leaves that have replaced the single primary leaves and are the principal reservoir of chlorophyll. *Pinus* needles develop meristematic cells at their base known as short shoots or dwarf shoots. The long-conical cones that ripen in December are hard, apex-tapering, serotinous and have a yellow to nut-brown colouring.

The bark of soft pines is generally smooth and has colouring characteristic to the species (MIROV and HASBROUCK 1976). Patula pine bark moves from dark brown to grey, rough, thick, scaly and deeply fissured in lower stems; to reddish to yellowish-brown, scaly; and, to thin bark on upper portions of the stem (LOOCK 1950). The species yields a white to yellowish-white sapwood containing pink heartwood.

Softwoods are preferentially used for paper packing materials such as corrugated cardboard boxes due to their pliable nature. Pines are planted for pulpwood or structural timber (Forest Owners Association, FOA 1998). Young *P. patula* is mainly used for the manufacture of boxes, newsprint and pulping. The uses of mature patula pine lumber are extensive: being utilised for light constructional work, fencing posts, B-grade poles (preservatives are required), furniture (cabinet work), ceiling, weatherboarding, panelling, pulpwood, food containers and flooring (POYNTON 1979; POYNTON 1984). Commercial or exported products include pulp and paper (POYNTON 1984). *Pinus patula* plantations are suitable for donga reclamation as the thick mat of fallen branchlets and needles retard surface runoff, limiting the advance of soil erosion (POYNTON 1984).

1.1 Forestry in the South African Context

The first exotic plantations, using *Eucalyptus globulus*, were established in South Africa in 1876 with the aim of preserving natural woodlands by intensive timber production. Public perception on the role of forestry has since darkened to be one of environmental destruction and not one of financial and economic impetus to the Country. Timber plantations are vigorously regulated by obligatory application for planting permits which are only issued based on results from impact studies, particularly the effect of tree planting on surface water resources (ZWOLINSKI and BAYLEY 2001).

Less than 1.4% of the total land base of South Africa is covered by timber plantations, 375 000 ha making up *Pinus patula*, that produce about 18 million cubic metres of timber each year (FOA 1998; OWEN and VAN DER ZEL 2000; FOA 2001; FOA 2002). In 1999/2000 forestry contributed a gross output of R 2 574 million with forestry products adding 8.6% to the gross value of agricultural output of R 25.9 billion (FOA 2002). In addition to small grower schemes, 60 000 people are directly employed by the forestry industry with thousands more being employed indirectly through forestry contracting operations (FORESTRY SOUTH AFRICA 2002). FOA (1998) has indicated that *P. patula* and *P. elliottii* dominate the South African softwood industry (BURGESS and WINGFIELD 2001). Pines are usually planted, on 50% of the total afforested area (FOA 1998). *Pinus patula* being the most popular species planted at 24% followed by *P. elliottii* at 15%, and then *P. radiata* and *P. taeda* both planted at 5% (ZWOLINSKI and BAYLEY 2001).

In South Africa, the efficiency of industrial development is largely dependent on innovation rather than on the exploitation of natural resources. This is particularly due to the low precipitation, where only about 7% of South Africa's land mass receives sufficient rainfall (800 mm/annum or more) for intensive forestry (DENISON and KIETZKA 1993). South Africa's average annual rainfall is only 450 mm rendering it a water-scarce country. *Pinus patula* requires a mean annual rainfall of 700 mm at high altitudes and 950 mm elsewhere (MORRIS and PALLETT 2000). Product-based selection now formulates the silvicultural systems

and the breeding strategies that are applied (DENISON and KIETZKA 1993). Forestry presently integrates breeding and nursery technology, site selection, protection against pests and diseases, and enhanced tolerance of trees to frost and drought (ZWOLINSKI and BAYLEY 2001). The integration of biotechnology has the potential to assist not only in addressing commercial forestry issues such as plantation site constraints, pests and diseases and the requirement for novel or improved product quality, but also in making mill processes more efficient and environmentally-friendly.

1.2 Developments in Forest Biotechnology

Timber production goals have shifted from volume production to value output (ZWOLINSKI and BAYLEY 2001) in response to a worldwide decline of natural resources, which is most acute in developing countries where existing plantation improvement programmes are not always as advanced as in the industrialised world (WATT *et al.* 1997). Genetic gain in plantation forestry was predominantly achieved through conventional breeding, recognizing the importance of tree parentage combined with forest management (ZOBEL and TALBERT 1984). These breeding programmes traditionally identified superior traits for the production of elite genotypes, and helped to secure superior planting stock for commercial growers (WALTER *et al.* 2002). Conventional tree breeding and silviculture programmes are limited by the large areas required for screening through progeny testing and breeding, long life cycles needed to reach maturity (20 years for pulpwood rotations) of forest trees (GUPTA 1988); as well as the impact on genetic gain from genotype by environmental interaction (GEI) evident in *P. patula* (KANZLER 2004). Further biological constraints include the large size of trees and delayed sexual processes common to woody species (WATT *et al.* 1997).

Softwood seedling forestry relies on the production of large quantities of usually, open-pollinated seed, from a range of optimum families established in a clonal seed orchard. In order to further improve genetic gain, a selection of only the best performing families can be bulked, or controlled pollination techniques can be used to cross top performing families. In either case realisation of genetic gain

can be limited by reduced seed yields. The development of improved vegetative propagation techniques to produce cuttings from a limited supply of seed, however, allows the selective deployment of a few, highly productive families (KANZLER 2004). Cutting propagation can be restricted by the decline of rooting ability with ortet age and the limited number of shoots produced from each donor plant (HÖGBERG *et al.* 1998). Clonal forestry, the ability to propagate a single elite individual within a selected family offers the opportunity to further reap the benefits of genetic gain. It is a technique that has been successfully implemented for some forestry species, including both pine (TRUEMAN and PARK 2005) and eucalypt hybrids (DE ASSIS 2001). For *P. patula*, however, the rapid initiation of ontogenetic aging in the hedge plants, typified by the onset of reproductive maturity (LEAKEY *et al.* 1992), can result in variation in rooting of cuttings, in their growth habit, flowering and leaf morphology (DE JAGER 2000; MITCHELL *et al.* 2004). These constraints effectively eliminate the use of conventional propagation techniques, such as cuttings for the clonal propagation of *P. patula*.

Tissue culture provides an alternative for mass propagation as well as providing the potential to propagate tissues from mature trees. Mature conifers facilitate better selection of desirable genotypes. The production of trees via *in vitro* methods, makes use of buds (terminal, axillary, fascicle), somatic embryogenesis (Section 2.1) and organogenesis (Section 2.2) (WALTER *et al.* 1998a). The development of cryopreservation protocols, as described for *P. patula* (FORD *et al.* 2000b) allows for the storage of embryogenic tissue of possible elite genotypes in a juvenile state while trials are conducted in the field. Tissue culture-based technologies therefore offer an opportunity to overcome the drawbacks of the lengthy testing periods required for conifers. It is during the extended testing phase that ontogenetic effects become apparent in the cuttings taken from older hedges and where an *in vitro* approach associated with technology for cryopreservation to retard the impact of hedge aging would have major benefits. Somatic embryogenesis is such a technique, as it is considered to be a means of maintaining juvenility of stock plant clonal lines.

Tree biotechnology is faced with the challenge of aligning modern DNA-based techniques with conventional breeding methods (WALTER *et al.* 2002). The

increased pace of delivery with regard to tree improvement and mass forest productivity can be achieved with the following techniques (BAJAJ 1986; GUPTA 1988; WATT *et al.* 1997; WALTER *et al.* 1998a; STASOLLA and YEUNG 2003):

- i. Micropropagation and cell culture (somatic embryogenesis, cell suspension culture) of elite and rare lines from explant sources such as tissues, cells and protoplasts. Regeneration from tissue culture-based protocols reduces the time and space needed for selection and propagation of desirable traits. The higher production and labour costs (especially for multiplication) and uncertainty of long-term growth of clonal material are constraints attached to micropropagation. Propagation of pure lines (haploid and homozygous) through anther or pollen culture; early induction of flowering by application of growth regulators to shorten the breeding cycle;
- ii. Propagation of virus-free plants using apical meristem culture and micrografting;
- iii. *In vitro* conservation, including germplasm conservation, and cryopreservation of elite genotypes;
- iv. Generation of new germplasm by the production of haploids, triploids, polyploids and the isolation of mutants and variants;
- v. Molecular analysis with elegant techniques such as genetic fingerprinting, mapping, *in vitro* genotypic selection using marker aided selection (MAS), functional genomics, differential display and expressed sequence tags (EST's), which allow the use of microarray technology to analyze transcript gene levels during a physiological process. DNA markers indicate the differences in DNA sequences of genomic regions that affect important traits among individual trees and so help to identify genotypes of economic interest. Use of DNA markers in tree enhancement programmes help to optimize selection for genetic improvement through fingerprinting of genotypes and parentage testing of elite seed pollination techniques. Advances in gene regulation, particularly in pine somatic embryo development, have been made possible with differential display that is able to track hundreds of genes concomitantly during development. Unravelling gene expression patterns helps to identify useful candidate genes that assist towards our understanding of physiological processes; and,

- vi. Genetic engineering – the introduction of traits not readily available in breeding populations using recombinant DNA, direct and indirect gene transfer and protoplast fusion amongst others (Table 2.1).

An efficient and reliable transformation method will be an important tool for future applications in forestry. The risks and benefits of using genetic engineering in forestry are discussed in Section 2.9. Tree improvement programmes are aimed at alteration of tree form, performance or productivity and increased vigour through modification of root systems and leaf performance (MATHEWS and CAMPBELL 2000). Other aims include: increased biotic and abiotic stress tolerance, pest and pathogen resistance (such as virus resistance), herbicide resistance, and insect resistance through introduction of a gene encoding a *Bt* (*Bacillus thuringiensis*) toxin which inhibits the insect's digestive tract. Research into wood modification for improved timber quality has increased including decreasing lignin content and increased cellulose content (which would make it easier to process pulp and paper products), improving durability, hardness and dimensional stability and hardness (TANG and NEWTON 2003). The analysis and manipulation of the flowering pathway would achieve accelerated flowering for the benefit of reproduction in conifers (WALTER *et al.* 1998a; TANG and NEWTON 2003). More molecular biological research into promoters and genes from a variety of backgrounds has widened the scope to include changes in reproductive development and production of novel secondary metabolites (WALTER *et al.* 2002).

CHAPTER 2

LITERATURE REVIEW

2.1 Somatic Embryogenesis

The first report of somatic embryo formation in *Daucus carota* suspensions by STEWARD *et al.* (1958) and REINERT (1958) awakened the potential for somatic embryogenesis in other species. The list of methods for various species is ever-growing and despite being intractable *in vitro* as compared with angiosperms, much progress has been made with coniferous species over the last 20 years.

Somatic embryogenesis is defined as a process in which a single cell or small group of asexual (or somatic cells) are induced to form embryos in culture without any connection with pre-existing vascular tissue (TAUTORUS *et al.* 1991; MERKLE and DEAN 2000; VON ARNOLD *et al.* 2002; STASOLLA and YEUNG 2003). This process provides an ideal model system for the investigation of plant differentiation, cell totipotency, embryological studies (TAUTORUS *et al.* 1991; VON ARNOLD *et al.* 2002) and accelerates the genetic gain of a commercial crop species (STASOLLA and YEUNG 2003). Somatic embryogenesis has been used to study the regulation of embryo development and is an important tool for large scale vegetative propagation (TAUTORUS *et al.* 1991; VON ARNOLD *et al.* 2002).

Generally, the embryogenic process is only successful in conifers when induced from juvenile explants such as microspores (microsporogenesis), ovules, zygotic and somatic embryos and seedlings (VON ARNOLD *et al.* 2002; STASOLLA and YEUNG 2003). Although little is known about the origin of somatic embryogenesis in conifers, three mechanisms of embryogenic tissue growth are proposed: cleavage polyembryony by multiplication of embryonal heads, formation of somatic embryos through divisions of meristematic cells within the suspensor, and formation of somatic embryos from asymmetric divisions of single cells (VON ARNOLD and HAKMAN 1988). Induction of embryogenic tissue is significantly

affected by culture media and conditions resulting in species specific compositions (TAUTORUS *et al.* 1991). These components are: plant growth regulators, pH, light regimes, concentrations of basal medium and carbohydrate sources, nitrogen level and composition, mineral elements, and type of gelling agent.

The procedure of conifer plant regeneration has been succinctly outlined as: initiation, proliferation, prematuration or partial drying as in the case of *P. patula*, maturation, germination, and *ex vitro* acclimatization (VON ARNOLD *et al.* 2002). Embryogenic cultures in the Pinaceae are translucent to white. They appear mucilaginous when cultured on solid medium supplemented with benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic cultures of coniferous species are typically maintained on a medium supplemented with lower amounts of growth regulators (auxin and cytokinin), and a low concentration of sucrose (STASOLLA *et al.* 2002).

Developmental processes leading to somatic embryo production correlate to early and late zygotic embryogeny (FILONOVA *et al.* 2000a). Somatic embryos stop prolific cleavage polyembryogenesis, start to accumulate storage material such as starch, proteins and lipids and show a size increase (FEIRER *et al.* 1989; SKRIVER and MUNDY 1990).

Proliferating cultures consist primarily of immature somatic embryos. FILONOVA *et al.* (2000a) revealed through time-lapse tracking techniques that proliferating proembryogenic masses (PEMs) undergo three distinct stages (PEM I, II, III) which proliferate in the presence of exogenous auxin and cytokinin. Stage I PEMs continuously multiply by unequal division of embryogenic cells with dense cytoplasm. PEMs have high interclonal variation of shape and cellular constitution, in contrast to somatic embryos which have morphologically conservative structures. Somatic embryos also have a distinct protoderm-like cell layer, embryonal tube cells and suspensor. The stages of PEMs present in embryogenic cultures are characterised as follows (FILONOVA *et al.* 2000a): PEM I cell aggregates are a small compact group of densely cytoplasmic cells subtended by a vacuolated usually elongated cell; PEM stage II has more than one vacuolated cell; PEM stage III present an enlarged group of densely

cytoplasmic cells with disturbed polarity and appear loose rather than compact. The withdrawal of auxins and cytokinins induces somatic embryo formation that develop *de novo* from PEM III in two waves of programmed cell death (PCD) (FILONOVA *et al.* 2000b). Proembryogenic masses degrade during the first wave of programmed cell death giving rise to somatic embryos. The second wave of cell autolysis disposes of embryo-suspensor cells in subsequent early embryo formation (FILONOVA *et al.* 2000b). Finally, PEM and embryo-suspensor structures exhibit progressive cell suicide manifested by DNA fragmentation and autolysis of the cytoplasm, resulting in a large central vacuole. The PEM-to-somatic embryo transition represents an important developmental switch that determines the yield and quality of mature somatic embryos and plant production (BOZHKOVA *et al.* 2002). Early events for plant development are critical as it is during this process that the protodermal cell layer, primary apical meristems and embryo polarity axis are initiated (BOZHKOVA *et al.* 2002). In the last few years the research focus has shifted from manipulation of culture conditions, required for mature structures, towards understanding early events in embryogenesis and improving those *in vitro* conditions in order to improve the number and quality of mature somatic embryos (STASOLLA and YEUNG 2003).

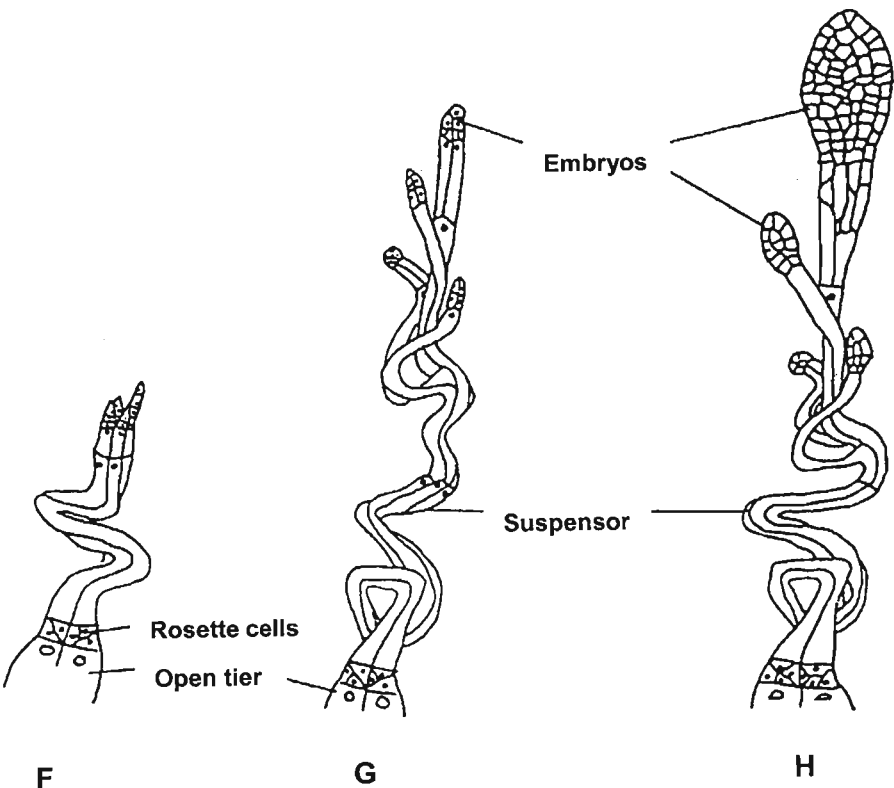
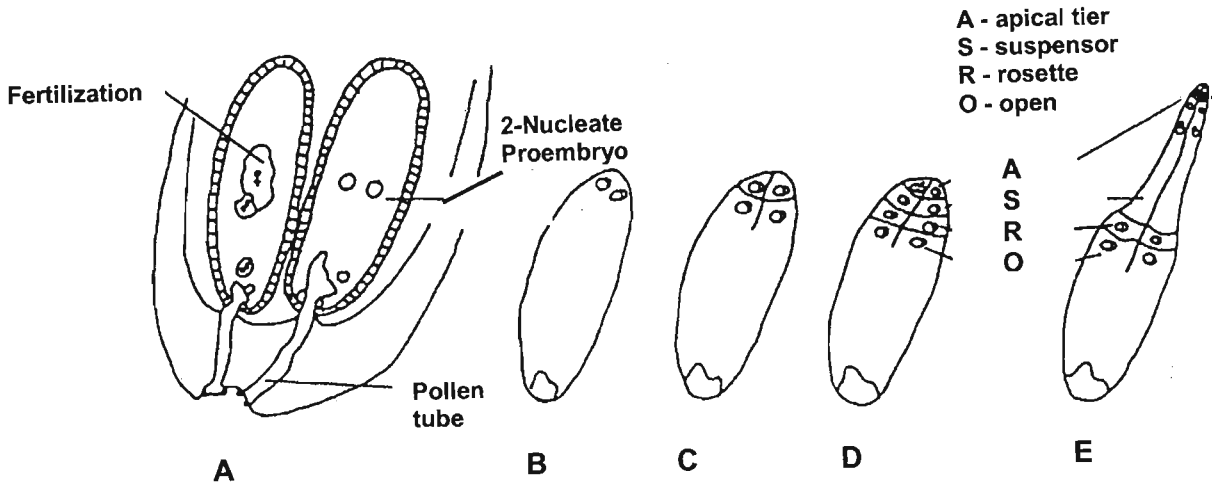
Somatic embryos are organized into two cell types: an embryonic region with rounded densely cytoplasmic cells that stain red with acetocarmine; subtended by elongated suspensor cells that are highly vacuolated and are permeable to Evan's blue (ATTREE and FOWKE 1993; FILONOVA *et al.* 2000b; STASOLLA and YEUNG 2003). Somatic embryos have the ability to produce bipolar structures with the potential for both root and shoot production. Two natural processes, characteristic to conifer embryony, may occur, simple or cleavage polyembryony. Simple polyembryony occurs in both *Picea* and *Pinus* species where fertilization of more than one egg per ovule occurs (TAUTORUS *et al.* 1991). The *Pinus* species undergoes an additional step of cleavage polyembryony. Embryogenic tissue is continuously initiated by cleavage polyembryogenesis in pines, resulting in a mass of immature somatic embryos (Figure 2.1). Here the apical tier cells divide into four genetically identical files of cells, all of which have equal opportunity to develop into a separate embryo. Only one becomes dominant, leaving the rest to degenerate and become redundant. This state of constant embryo division and

ability to provide large quantities of tissue for the regeneration of trees via somatic embryo production makes the use of embryogenic tissue attractive for gene transfer (MERKLE and TRIGIANO 1992; ELLIS 1995; SUTTON 2002; VON ARNOLD *et al.* 2002).

In general the maturation process is carried out on solidified media. ATTREE *et al.* (1991) showed that somatic embryo maturation required a low sucrose concentration as well as elevated osmotic levels using high molecular weight compounds such as polyethylene glycol (PEG). Maltose is a widely used carbon source during the maturation of conifer somatic embryos, its superiority attributed to its slow metabolism when compared to sucrose (RAMAROSANDRATANA *et al.* 2001a).

The production of mature developmental stages is encouraged by the removal of auxins and cytokinins, addition of the plant hormone abscisic acid (ABA) and an environment of elevated osmotic concentration, ideally using myo-inositol (ATTREE and FOWKE 1993; STASOLLA and YEUNG 2003). This effectively inhibits PEM or cell proliferation. ABA cannot induce the transition of PEM-to-embryo, which could explain continued proliferation of embryogenic cultures even when transferred from auxin and/or cytokinin-containing medium to ABA-containing medium (BOZHKOVA *et al.* 2002). The PEM-to-embryo stage occurs shortly after the withdrawal of plant growth regulators (PGR's). However, no maturation is possible if embryogenic heads within the early filamentous embryos are undeveloped (loosely aggregated and interspersed with vacuolated cells) prior to ABA treatment (JALONEN and VON ARNOLD 1991). Newly formed somatic embryos can develop in a PGR-free environment for up to 7 days before transfer to ABA-supplemented medium and still maintain their ability to respond to ABA (BOZHKOVA *et al.* 2002). Nonetheless, prolonged contact with ABA retards growth of plants derived from somatic embryos (BOZHKOVA *et al.* 2002).

Figure 2.1 Fertilization and cleavage polyembryogenesis in *Pinus*. The fertilized egg divides into two (**A**) then four, free nuclei in the egg cell centre giving impetus to proembryo development. The nuclei orientate themselves into a single tier (**B**) which progressively divides to form an eight-celled (**C**) then 16-celled proembryo consisting of four tiers of four cells each (**D**). Cell wall formation begins at the eight-celled stage. The third (suspensor) tier of cells elongates and pushes the distal apical tier into the female gametophyte tissue (**E**), thereby ending the proembryo stage. Cleavage polyembryony begins with the separation of apical tier cells into four files of cells (**F**). The development of the suspensor region results from continued division and elongation of the basal cells (**G**). Despite the genetic identity, one of the embryos becomes dominant and the others degenerate (G, H). The club-shaped dominant embryo enlarges rapidly (**H**). Adapted from OWENS and BLAKE (1985)



As part of its role in the maturation process, abscisic acid (ABA) promotes the deposition of storage reserves, inhibits precocious germination of zygotic embryos and inhibits PEM proliferation (KERMODE 1990; ROBERTS *et al.* 1990; FILONOVA *et al.* 2000a). *Picea abies* PEMs I and II were shown to die in response to ABA, with most of PEMs III transdifferentiating to somatic embryos followed by individual development to mature forms (FILONOVA *et al.* 2000a). SKRIVER and MUNDY (1990) postulated that the *rab* or ABA genes lead to the assembly of mRNA's that control the accumulation of storage products and late embryogenesis-abundant (LEA) proteins. ABA has become one of the most effective plant growth regulators for the stimulation of the maturation process, due in part to its accumulation in the seed during mid-to-late stages of development of the embryo (FELLENBERG 1982). Exogenous ABA applications may be necessary to replace the endogenous supply provided by embryo megagametophytic tissue *in ovulo* embryogenesis (reviewed by STASOLLA *et al.* 2002). In nature, ABA is involved in various physiological and developmental processes in plants (ZEEVAART and CREELMAN 1988). It has a significant role in plant stress responses such as cold and drought, seed development, dormancy and germination (discussed in ROCK 2000; XIONG and ZHU 2003). In developing seeds endogenous ABA levels peak twice (KARSSSEN *et al.* 1983): the first peak occurs 10 d after pollination (maternal origin) and the second peak as embryonic ABA accumulates later. The peaks promote embryo maturation, synthesis of late LEA proteins (which provide desiccation tolerance in the seed), synthesis of storage proteins and nutrition reserves, initiation of seed dormancy, and then decline during the desiccation processes (ROCK and QUATRANO 1985; XIONG and ZHU 2003). The exact role of ABA in stimulating embryo maturation is not known due to multiple effects observed: reduction of cell proliferation and initiation of embryo development possibly by affecting nucleotide biosynthesis; changes in nitrogen metabolism (NH_4^+ is taken up preferentially to NO_3^-) followed by storage protein accumulation; and changes in amino acid metabolism (JOY *et al.* 1997; STASOLLA *et al.* 2002). ABA may also provide desiccation tolerance in low moisture conditions (FINKELSTEIN and ROCK 2002) as is found during the maturation of somatic embryos.

Factors other than ABA are responsible for maturation of somatic embryos (reviewed by STASOLLA *et al.* 2002), such as osmoticum. ATTREE *et al.* (1991) showed that simple salts and sugars are not as effective as the high molecular weight compounds, polyethylene glycol (PEG) or dextrans, for maturation. Low molecular weight compounds, such as sucrose, initially result in plasmolysis. Drought induced-stress can be activated by high molecular weight or non-permeating compounds as well as high concentrations of gelling agents (KLIMASZEWSKA *et al.* 2000), conditions that stimulate somatic embryos. After prolonged culture, osmotic recovery occurs with the simultaneous movement of both water and sucrose into the cells by osmosis (ATTREE *et al.* 1991). The effects of ABA and osmoticum appear to be additive (STASOLLA *et al.* 2002).

Further considerations are the use of anti-auxins, such as 2-(*p*-chlorophenoxy)2-methylpropionic acid (PCIB), which have been shown to improve the transition from proliferating embryogenic tissue to mature somatic embryos. However, overexposure to PCIB resulted in a reduced number of cotyledons (FIND *et al.* 2002). Reduced proliferation but no significant effect on maturation quality was achieved with 2,3,5-tri-iodobenzoic acid (TIBA). Lowered concentrations of boron only had a positive effect on maturation whilst phloroglucinol (an auxin synergist) induced the opposite effect of stimulating increased proliferation but no maturation (FIND *et al.* 2002).

HAKMAN and VON ARNOLD (1988) defined various stages of mature somatic embryo development (Figure 3.5). Firstly, small embryos with clear "zonation" appear (Stage I). These consist of an embryonic region of small densely cytoplasmic cells, subtended by highly vacuolated suspensor cells. The prominent embryonic apex becomes opaque and assumes a smooth and glossy surface (Stage II). Cotyledons become visible at Stage III, where they show a likeness to zygotic embryos in mature seeds (HAKMAN and VON ARNOLD 1988).

Differentiation of the root and shoot apical meristems is a defining stage of embryo development. The development of a functional root and shoot system is defined as conversion rate - a useful assessment of embryo quality that is often genotype-dependant (STASOLLA and YEUNG 2003). Imposed desiccation conditions are

often required to convert “morphologically” mature somatic embryos into “physiological” maturity capable of normal functioning (STASOLLA and YEUNG 2003). Loss of water content is vital in seeds during final stages of maturation to indicate cessation of embryo development in preparation for germination (KERMODE 1990), when root elongation occurs. Drying conditions are categorized as full or partial depending on the rate of water loss experienced by the embryos during maturation conditions. Generally, full drying is effective for embryos matured under high osmoticum conditions, whereas embryos developed under low osmoticum conditions respond to partial desiccation treatments (STASOLLA and YEUNG 2003). Conditioning factors that control cell differentiation in somatic embryos may include soluble signal molecules such as extracellular proteins, arabinogalactan proteins (AGP's) and lipochitooligosaccharides (LCO's) (VON ARNOLD *et al.* 2002).

Careful note must be made of culture conditions for early stages of somatic embryogenesis to mimic *in ovulo* conditions as closely as possible thereby producing better quality somatic embryos and eventually plant production (BOZHKOV *et al.* 2002; STASOLLA and YEUNG 2003). The maturation process in *P. patula* has proved to be the most problematic stage of producing somatic embryos, in that not all genotypes respond to the maturation treatment (FORD *et al.* 2005). Somatic embryo maturation and plantlet production has been achieved in *P. patula* (JONES and VAN STADEN 1995; JONES and VAN STADEN 2001). This method is greatly advantageous to further *in vitro* manipulations since it lends itself to the regeneration of putative transformants in a genetic engineering protocol.

2.2 Organogenesis

Organogenesis is a regenerative pathway of *in vitro* vegetative propagation resulting in the formation of monopolar structures that have a connection with pre-existing vascular tissue within maternal callus or explant (CHAWLA 2002), unlike embryogenic structures (Section 2.1). The creation of new forms or differentiation giving way to plant organs such as roots, shoots and bud flowers are products of this process (GEORGE 1993; CHAWLA 2002). Although somatic embryogenesis

has the most potential for large-scale propagation, significant limitations such as low initiation frequencies and genetic specificity require alternative methods (TANG *et al.* 1998).

Plant production through organogenesis is undertaken through one of two ways: *de novo* formation of stem callus (initiation of basal callus followed by shoot bud differentiation) or the emergence of adventitious organs directly from an explant, suitable to herbaceous species (CHAWLA 2002). The term adventitious denotes freshly-originating or new organs from unusual points of origin of an organized explant where a meristem is lacking. Adventitious structures are developed from groups of small, densely cytoplasmic, isodiametric cells (meristemoids) (FLINN *et al.* 1989). Examples may include plantlet production from stem callus cultures, induction of adventitious buds on cultured embryos or cotyledons, adventitious roots, shoots and buds from cultured plant tissues that do not normally produce these organs (GEORGE 1993; CHAWLA 2002). Induction of successful organogenesis requires mitotically active cells (immature explants), increased cell number in larger explants increases probability of cell culture but is also relative to season of year (collection date), intraspecific variation, genotype and physiological conditions of parent plant (CHAWLA 2002).

Commonly, the induction of adventitious buds or callus (usually applied to angiosperms) on cultured embryos or cotyledons (PULIDO *et al.* 1992; HARRY and THORPE 1994; TANG *et al.* 1998; MATHUR and NADGAUDA 1999) is achieved in conifers with the cytokinin BA as the commonly used phytohormone. BA has been applied singly, in combination with other cytokinins or with other exogenous hormones. Organogenesis provides a successful regeneration pathway and has been known to be the most effective way to micropropagate forest tree species. The New Zealand Forest Research Institute (NZ FRI) is an example of an established production laboratory which started producing micropropagated planting stock (through organogenic procedures) for field trials in 1979. Since its expansion, it has become capable of producing between two and three million micropropagated radiata pine per annum (<http://dendrome.ucdavis.edu/Newsletter/walter.html>). The organogenic route for conifer

regeneration involves bud induction and development, shoot elongation and multiplication and rooting (HARRY and THORPE 1994).

Although organogenesis typically had application in micropropagation protocols, genetic transformation protocols have also been applied to organogenic tissues. The *Agrobacterium*-mediated approach has been most commonly used in conifers (refer to Section 2.8 and Table 2.2). Organogenic tissue may have advantages over embryogenic tissue as a transformation explant, as noted in *Pinus radiata*, such as all year round seed availability (CHARITY *et al.* 2002).

2.3 Gene Transfer

Biotechnology combines the conceptual framework of molecular biology and the technical aspects of cell culture systems to develop economically important systems and products. Plant biotechnology has provided the means to manipulate biological systems (CHAWLA 2002). Recombinant DNA technology greatly enhanced the understanding of an organism's genome in the 1970's. This technology allowed for *in vivo* replication of genomic DNA regions that are covalently linked with bacterial plasmid or virus clones (CHAWLA 2002). The first stably transformed higher plant was reported in the early 1980's (FRALEY *et al.* 1983; HORSCH *et al.* 1984). Analysis of transgenic plants has provided insight into fundamental problems in plant and cell biology, moving the horizons of plant breeding from hybrids to transgenics to nutraceuticals.

Gene transfer or DNA uptake refers to the process that inserts foreign DNA (usually ligated to a bacterial plasmid) into protoplasts or whole cells. Assuming an efficient method of transfer was employed, transient gene expression can be determined chromogenically using a reporter gene fusion system such as the β -glucuronidase (GUS) assay (JEFFERSON *et al.* 1987), or is quantified by protein analysis 12-48 h after transfer. Genetic transformation, however, refers to the stable integration of a foreign gene into the genome of a plant, now termed transgenic, regenerated from DNA-treated protoplasts or intact cells (JÉNÉS *et al.* 1993).

CHRISTOU (1995) believes one of the greatest milestones of plant biotechnology to be the realisation of *Agrobacterium spp.* ability to transfer genetic material between organisms without sexual crossing (BARTON *et al.* 1983; CAPLAN *et al.* 1983; HERRERA-ESTRELLA *et al.* 1983). Traditional breeding methods alone, restrict the existing gene pool due to sexual incompatibility of many interspecific and intergeneric crosses (NISBET and WEBB 1990). Ironically, in the past often the elite cultivars of greater agronomic and industrial value were not the first to be incorporated into a biotechnology programme this having resulted in greater understanding of the molecular biology of potato, petunia, tomato and tobacco (CHRISTOU 1996).

Recombinant DNA technology can overcome limiting species-barriers between genes that can be encountered in conventional plant breeding (GRIERSON and COVEY 1984). The role of transgenic plants for crop improvement initially rested predominately with dicotyledons, notably from the Solanaceae as these are natural hosts of *Agrobacterium*, and only a few monocotyledons and woody plants. Genetic improvement through transformation was slow to produce transgenic trees, in comparison to agricultural crops, due to long rotation cycles and the recalcitrance to *Agrobacterium spp.* that most conifers initially displayed to infection (WALTER and SMITH 1999). The list of genetically transformed species (for economically important crops used industrially) has grown exponentially; rendering tabulation dated but searches on the web provide the latest reports (BIRCH 1997).

The most obvious application for plant genetic engineering is crop improvement. The changes in oil and protein contents of crops, vitamin enrichment, herbicide resistance, disease and pest resistance including insect resistance, and virus tolerance are some of these applications. Potential in transgenic trees (described in detail in Section 1.2) include increased pest and pathogen resistance (such as virus resistance), herbicide resistance, and insect resistance, improved timber quality, the analysis and manipulation of the flowering pathway and production of novel secondary metabolites.

The methods for gene transfer vary for different species within both dicotyledons and monocotyledons. Gene transfer is mediated by indirect or direct gene transfer into protoplasts or intact cells. Indirect transfer employs plant viral vectors (they contain DNA rather than the usual RNA genome) and *Agrobacterium spp.* for the carrier of the gene of interest. Direct methods introduce naked DNA immediately into the genome (Table 2.1).

Direct gene targeting, is a straightforward physical process which does not require biological vectors, avoiding host range problems. In direct transformations of protoplasts, a wound response is elicited which often increases the proportion of cells competent for transformation.

Table 2.1: Available methods for gene transfer

| DIRECT GENE TRANSFER | |
|---|---|
| - INTO PROTOPLASTS | - INTO INTACT CELLS |
| <ul style="list-style-type: none"> • Chemical treatment: PEG, CaCl₂ • Electroporation • Physico-chemico • Sonication • Microinjection | <ul style="list-style-type: none"> • Macroinjection into cell aggregates • Dry embryo incubation in DNA • Pollen tube pathway • Micro-whiskers • Biolistic-mediated transfer |

A curious phenomenon of inactivation of the transgene was observed as the commercialization of transgenic crops became more pervasive. Desirable new phenotypes created after transfer of foreign DNA were found to be genetically unstable during propagation (FINNEGAN and McELROY 1994). Loss of expression of the introduced gene did not correlate with the loss of the transgene, but instead its inactivation. The past decade produced studies unravelling these disturbing events and implicated double stranded RNA (dsRNA) for silencing of genes. Homology dependant gene silencing (HDGS) results from a redundancy of information from multiple copies of transgenes or when additional copies of an endogenous gene are expressed ectopically. HDGS is carried out through one of two mechanisms: post-transcriptional gene silencing or transcriptional gene silencing (REDDY *et al.* 2003). RNA silencing, termed post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in animals, does not restrain transcription of a gene locus but rather initiates sequence-specific

degradation of targeted homologous mRNA's in the cytoplasm (YU and KUMAR 2003). On the other hand, transcriptional gene silencing (TGS) is thought to repress transcription by methylation of homologous DNA sequences (MATZKE *et al.* 2001). Both of these mechanisms are thought to be triggered by the presence of double stranded RNA, which is further cleaved into small RNAs to function in epigenetic gene-silencing processes. RNA silencing is thought to be effective as part of a defence system used against transposable elements and viral infection as well as in the regulation of endogenous gene expression. The advantages of RNA silencing have been explored practically as a reverse genetics approach in plant functional genomics (discussed in YU and KUMAR 2003).

2.4 The Biolistic Gene Transfer System

Biolistic gene transfer, coined to describe biological ballistics, is a process which employs high-velocity acceleration of microprojectiles coated with foreign DNA, often resulting in stable transformation of the target tissue (CHRISTOU 1996). Foreign DNA is "bombarded" into target cells or tissue. The DNA-coated microparticles penetrate the tissue cell wall and membrane, delivering the gene of interest into the plant genome (Figure 2.2). The biolistic method has been described with a variety of names such as the particle gun method, the microprojectile method or bombardment, biolistic transfer, particle bombardment, the gene gun method, the bio-blaster method or the particle acceleration or transfer method. Biological ballistics describes both the process and associated apparatus used to shoot biological materials into living targets (SANFORD 1988).

Genetic evolution was breached in 1984, as the invention of the biolistic process promised the universal ability to deliver genes into any cell, tissue or organelle which previously may have been recalcitrant to other transformation methods (SANFORD 1988; BATTY and EVANS 1992, KLEIN *et al.* 1992). The inventors J.C. SANFORD, E.D. WOLF and N.K. ALLEN outlined three goals for an ideal particle delivery system. Firstly, the transformation of existing transformable cells, tissues and species would be achieved more directly, simpler and rapidly. Secondly, recalcitrant cells, tissue and species would be readily transformed where previous methods failed. The third aim would allow researchers to use a

uniform apparatus, and subsequently the same protocol, for all gene transfers – from prokaryote to eukaryote (SANFORD 1988).

SANFORD (1988) had been trying for several years to introduce DNA into pollen, thereby transforming a whole plant. Eventually the feasibility of using ion beams in place of micro-lasers for creating holes in the cell wall was discussed with Ed Wolf, a director of the National Nanofabrication Facility at Cornell University. This seemed impractical and so focus was turned towards “shooting DNA”. However, the low density and fragile nature of naked DNA would not withstand a shooting event unless the DNA was associated with solid particles. The first prototype was a simple air blast device to accelerate 4 µm diameter spheres at high velocity to infiltrate onion (*Allium cepa*) epidermis. The onion epidermis provided an ideal model target as it provided a large-celled monolayer in which the penetration-efficiency as well as direct evaluation of cell viability could easily be observed using light microscopy (SANFORD 1988). Penetrated cells survived despite the large size of microparticles used though the air blast device was highly disruptive to target tissue, particularly near the epicentre of the blast, and could not generate sufficient velocity for use of smaller microparticles suitable for smaller cells. Biological material such as ether-dried *Escherichia coli* and *Bacillus thuringiensis* penetrated the onion cells with poor efficiency in large aggregated clusters. The ballistic particle device attempted to implement higher velocities and less tissue damage with the inclusion of a “macroparticle” or macro-carrier accelerator, with microparticles on its surface, housed in a vacuum bombardment chamber (SANFORD 1988). RNA, DNA, viral RNA, fluorescent-stained nucleic acids and soon after plasmid DNA with the chloramphenicol acetyl transferase (CAT) marker gene were used during transfer experiments. The arena for target tissue has ranged from both dicotyledonous and monocotyledonous species with meristematic tissue and embryogenic suspension cultures being amenable to transformation and regeneration of plants. Other “target tissue” included mitochondria in yeast, chloroplasts in *Chlamydomonas* as well as plant organelles.

The basic principles of the macroparticle concept or biolistic gene delivery system, such as the Genebooster™ (Figure 2.2), is dependent on high pressured nitrogen gas released by an electric valve and partial vacuum to propel the macroprojectile

loaded with sterile DNA-coated tungsten (or gold) particle suspension, toward the steel mesh, releasing the microcarriers to the target tissue below. The launch velocity of microprojectiles is regulated by the shooting pressure setting or nitrogen pressure, the amount of vacuum in the bombardment chamber, macrocarrier travel distance to the stopping plate and the distance between the stopping plate and the target cells. In the first systems described, upon impact with the stopping plate, the macroprojectile extruded through a small opening that further accelerated the microparticles. Acceleration systems have included shock waves created by electric discharge through a drop of water or the sudden release of compressed air or nitrogen (Genebooster™) (KLEIN *et al.* 1992). A device using a gas impulse has also been designed to directly accelerate the particle-suspension through a capillary tube without the use of a macroprojectile (SAUTTER *et al.* 1991).

Rapid development in the biolistic research front prompted the construction of several biolistic devices. The Biolistic® PDS-1000/He, marketed by Bio-Rad laboratories (KIKKERT 1993), makes use of a shock wave generated by a sudden release of compressed helium to accelerate a thin plastic sheet into a metal screen. The microparticles are dried onto the surface of the carrier sheet from an ethanol suspension. A modified hand-held version, without a vacuum, has been used to deliver genes to the tissues of living animals (KLEIN *et al.* 1992). The “flowing” helium gun was the basis for development for the Particle Inflow Gun (PIG) which makes use of a timer relay-driven solenoid to regulate the amount of helium released (VAIN *et al.* 1993). An early alternative to the original particle gun is the airgun device that incorporates a commercially available airgun in its assembly to accelerate the macrocarrier and tungsten/gold microparticles (OARD 1993). SAUTTER (1993) describes a microtargeting device specifically for use with plant meristems while McCABE and CHRISTOU (1993) defined electric discharge particle bombardment (ACCELL™) technology.

The efficiency of biolistic gene transfer depends on both biological and physical factors. The physiological status of the cells (growth phase and density) as well as composition of the medium (concentration of osmoticum) demand empirical optimization. Incubation conditions such as temperature, photoperiod and

humidity under which bombarded explants are maintained are also important. The physical factors found to influence gene delivery were the momentum of the particles (controlled by distance travelled, acceleration force, vacuum, mass, size and shape), type (must be chemically inert to prevent adverse reactions with the DNA or cell components), agglomeration and dispersion properties, depth of stopping plate aperture, preparation of DNA coating onto tungsten, concentration of DNA: microparticle, nature of DNA (single or double), inclusion of additives such as spermidine and calcium chloride, number of particles per shot, and the number of particles that impact the target per unit area (KLEIN *et al.* 1988; WANG *et al.* 1988; KLEIN *et al.* 1992; CHRISTOU 1995).

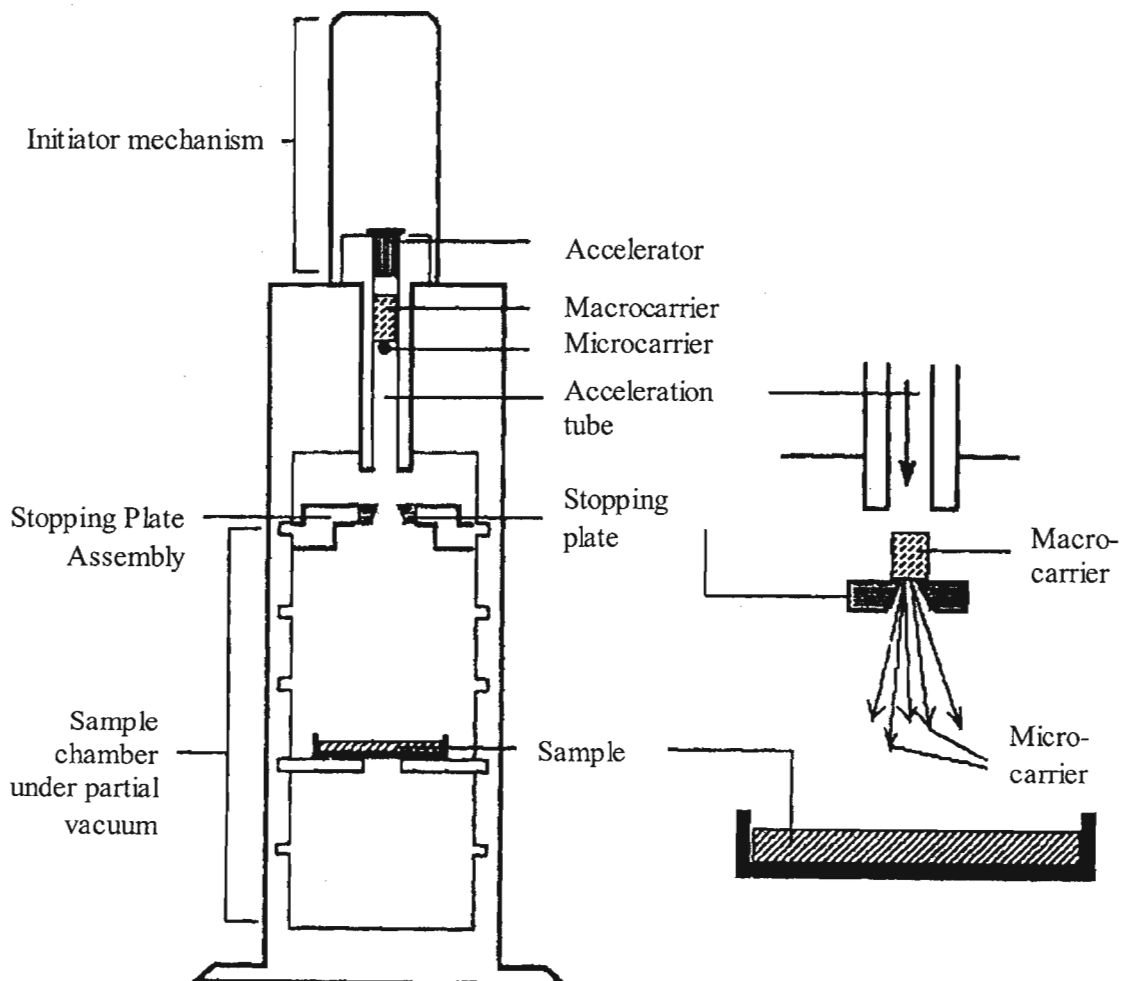


Figure 2.2 Principle of operation for the biolistic device, adapted from the BIOLISTIC Particle Delivery system of Du Pont Company (JÉNÉS *et al.* 1993)

Genetic transformation efficiency achieved by microprojectile bombardment is strongly influenced by biological factors as it is estimated that frequency of gene transfer in transient assay is one out of 10^3 or 10^4 , of which 2-5% are stably transformed. Although transformation efficiency is comparatively low to other established transformation methods, this can be expected to improve with superior sample preparation techniques and advancement of the biolistic process. Fortunately, the use of selectable markers ensures stable transformation in progenies (FINER and McMULLEN 1990; TOMES *et al.* 1990; McCOWAN *et al.* 1991; JÉNÉS *et al.* 1993).

2.5 Overview of *Agrobacterium*-mediated Transformation

The molecular mechanism of *Agrobacterium* was first discovered in the mid 1970's, which enabled successful exploitation of its gene transfer ability for plant transformation of many species in the early 1980's (GELVIN 2000). *Agrobacterium* as a genus can transfer DNA into a wide group of organisms: monocotyledonous and dicotyledonous angiosperm species, as well as interkingdom transfer into eukaryotic cells (only known example) including fungi, yeast, and human cells (reviewed in GELVIN 2000; 2003). Host range is a complex process under the genetic control of factors within the bacterium and plant and varies greatly between species and even between tissues, organs and cell types within a plant (GELVIN 2000; 2003). The *Agrobacterium* genus has been classified into various "biovars" according to their metabolic and growth characteristics: biovar I (*A. tumefaciens*), biovar II (*A. rhizogenes*) and biovar III (*A. vitis*) (KEANE *et al.* 1970); but strains are now being reclassified into true species due to DNA sequencing (reviewed in GELVIN 2003). The molecular mechanism through which *Agrobacterium* genetically transforms its host cells has been intensively studied (for recent reviews and updates, refer to DUMAS *et al.* 2001; TZFIRA and CITOVSKY 2002; GELVIN 2003; HWANG and GELVIN 2004; TZFIRA *et al.* 2004). Biotechnological advances have helped to make *Agrobacterium* more versatile in genetic engineering of conifers (reviewed in TANG and NEWTON 2003) as evidenced by the growing list of transgenic trees (see Table 2.2).

Agrobacterium tumefaciens is a soil-borne Gram-negative phytopathogen that causes the neoplastic crown gall disease in plants (BIRCH 1997) due to the presence of the large tumour-inducing (Ti) plasmid resident in the bacterium or in the case of *A. rhizogenes*, the rhizogenic (Ri) plasmid that elicits hairy root responses. The Ti plasmid is found in only a small percentage of natural populations of *A. tumefaciens* (ZUPAN and ZAMBRYSKI 1995). Crown gall proliferation results from the transfer, integration into the plant genome, and expression of oncogenes encoded on a single stranded (ss) segment of the Ti plasmid, known as transferred DNA (T-DNA) (BIRCH 1997; HWANG and GELVIN 2004). The Ti plasmid varies between 200–800 kb in size with T-DNA, representing less than 10% of the Ti plasmid (GELVIN 2003). T-DNA or T-regions are delimited by highly homologous 25 bp border sequences, left or right T-DNA borders, which are the only required *cis* elements for T-DNA transfer (TZFIRA *et al.* 2004). Therefore, the mobility of the T-DNA is largely determined by these flanking sequences. T-DNA transfer is polar and in particular the deletion or reversing of the orientation of the right border abolishes T-DNA transfer. On the other hand, manipulation of the left border has little or no effect on transfer (ZUPAN and ZAMBRYSKI 1995).

Two major factors located on the Ti plasmid are required for plant transformation: (1) T-DNA, the genetic sequence that gets transferred into the plant genome, and (2) virulence (*vir*) region encoding proteins that mediate T-DNA processing and transfer (GELVIN 2003; HWANG and GELVIN 2004). The *vir* region is the master control of the entire transformation process. This operon is organized into six complementation groups: *virA*, *virB*, *virD*, *virG*, which are essential for transformation, and *virC* and *virE* which enhance the efficiency of the process (ZAMBRYSKI 1988). Genes encoded by the T-DNA are expressed in the plant cells even before integration (HIEI *et al.* 1997). T-DNA does not encode the products that mediate its transfer or transposition (ZUPAN and ZAMBRYSKI 1995). Instead the Ti-plasmid virulence (*vir*) region provides most of the *trans*-acting products for T-DNA transfer and processing. The process of *A. tumefaciens* infection involves several vital steps: (1) bacterial colonisation by recognition of a susceptible (wounded) plant cell, (2) induction of the bacterial *vir* gene expression, (3) T-DNA processing, (4) bacterial attachment, (5) generation of

the T-DNA mobilization complex, (6) T-DNA transfer (through the plant cell membrane) and nuclear targeting, (7) integration of T-DNA (T-strand) into the plant genome (ZAMBRYSKI 1988; TANG and NEWTON 2003).

Release of phenolic compounds from wounded plant cells elicits the production of phenolic compounds, such as acetosyringone (AS) or hydroxy-acetosyringone (OH-AS) and sugar compounds that act as specific inducers for initiation of *vir* gene expression (STACHEL *et al.* 1985). Normally, phenolic compounds are employed during phytoalexin and lignin biosynthesis (GELVIN 2000). The role of cellular responses of the infecting bacterium and plant leading to transformation events was extensively reviewed by BINNS and THOMASHOW (1988) and many other reviews on the biology of *Agrobacterium* have since followed (ZAMBRYSKI 1988, ZAMBRYSKI *et al.* 1989; GELVIN 2000; TZFIRA and CITOVSKY 2002; GELVIN 2003; TZFIRA *et al.* 2004). *Agrobacterium* cells attach to the host cell wall with assistance of binding and attachment proteins ChvA, ChvB, PscA and Att (reviewed by TZFIRA and CITOVSKY 2002). Plant exudates, specifically phenolic compounds and monosaccharides, transcriptionally activate the *Agrobacterium vir* genes when these compounds are recognized by a two-component regulatory signal-transduction system: VirA and VirG proteins (TZFIRA and CITOVSKY 2002; GELVIN 2003). The membrane-bound VirA (detects phenolic production) interacts with the plant exudates and autophosphorylates and subsequently transphosphorylates the VirG protein, a transcriptional regulator. Now phosphorylated, the VirG protein helps to activate the *vir* gene promoters (TZFIRA and CITOVSKY 2002; GELVIN 2003), refer to Figure 2.3.

Vir gene expression results in T-strand production, formation of a bacterium-to-host cell channel, and export of the T-strand and several other Vir proteins into the plant cell (TZFIRA and CITOVSKY 2002), refer to Figure 2.3. Proposed host plant proteins and genes involved in *Agrobacterium*-plant interaction are summarised in TZFIRA and CITOVSKY (2002). T-DNA border sequences act as targets for the VirD1/VirD2 endonuclease that process the T-DNA from the Ti plasmid and also serve as a covalent attachment site for VirD2 protein. T-strand production is initiated by VirD1 and VirD2 proteins that together act as a strand and site-specific nuclease to cut the bottom strand at the T-DNA borders to release a single-

stranded (ss) T-DNA molecular (T-strand) (TZFIRA and CITOVSKY 2002; GELVIN 2003). The T-DNA remains single-stranded and most authors believe that it becomes coated in proteins that protects from nucleolytic degradation and serve as nuclear localization signals (TZFIRA and CITOVSKY 2002; GELVIN 2003; TANG and NEWTON 2003). VirF is thought to be a host-range factor of *Agrobacterium* (TZFIRA and CITOVSKY 2002; GELVIN 2003). The function of VirH remains largely unknown but it has been speculated that it may help to detoxify plant secreted phenolics, that are potentially harmful to the invading *Agrobacterium* (TZFIRA and CITOVSKY 2000).

Four types of secretion systems occur, which share evolutionary related components, when substances are secreted through the bacterial envelope (ZUPAN *et al.* 1998). It is generally accepted that *Agrobacterium* uses a type IV (T4SS) secretion system, comprising of 11 VirB proteins and the VirD4 protein (DUMAS *et al.* 2001), to transport the T-strand and other Vir proteins (including VirE2 and VirF) across the bacterial envelope in a process analogous to bacterial conjugation (GELVIN 2003; HWANG and GELVIN 2004). The T4SS is made up of the filamentous T-pilus appendage (VirB2 is a major component) that is essential for T-DNA transfer and a membrane-associated transporter complex (HWANG and GELVIN 2004). It remains unknown whether the T-pilus is directly or indirectly involved in the transfer of T-DNA and Vir proteins (HWANG and GELVIN 2004).

The large T-complex requires nuclear import from the plant cell into the nucleus, thereby bringing it into closer proximity to the plant genome prior its integration (TZFIRA and CITOVSKY 2002; GELVIN 2003): a process that is facilitated by VirD2 and VirE2, which have nuclear localizing properties (TZFIRA and CITOVSKY 2002). The T-DNA integration step into the plant genome is the least understood but is generally accepted to be through illegitimate recombination and is thought likely to occur at AT-rich regions (BRUNAUD *et al.* 2002). VirD2 and VirE2 may further be involved in T-DNA integration (reviewed in GELVIN 2003). Current T-DNA integration models: double-strand break repair (DSBR) and single-strand-gap repair (SSGR) are outlined in TZFIRA *et al.* (2004).

The T-DNA, in contrast to transposable elements, is stable following its integration into the plant genome. T-DNA expression of the Ti plasmid results in tumours through cell divisions triggered by auxin and cytokinin production by enzymes encoded in the T-DNA. These tumours are now directed to produce and excrete opines, consumed specifically for their carbon or nitrogen source by the invader, which capitalizes on opines (ZUPAN and ZAMBRYSKI 1995).

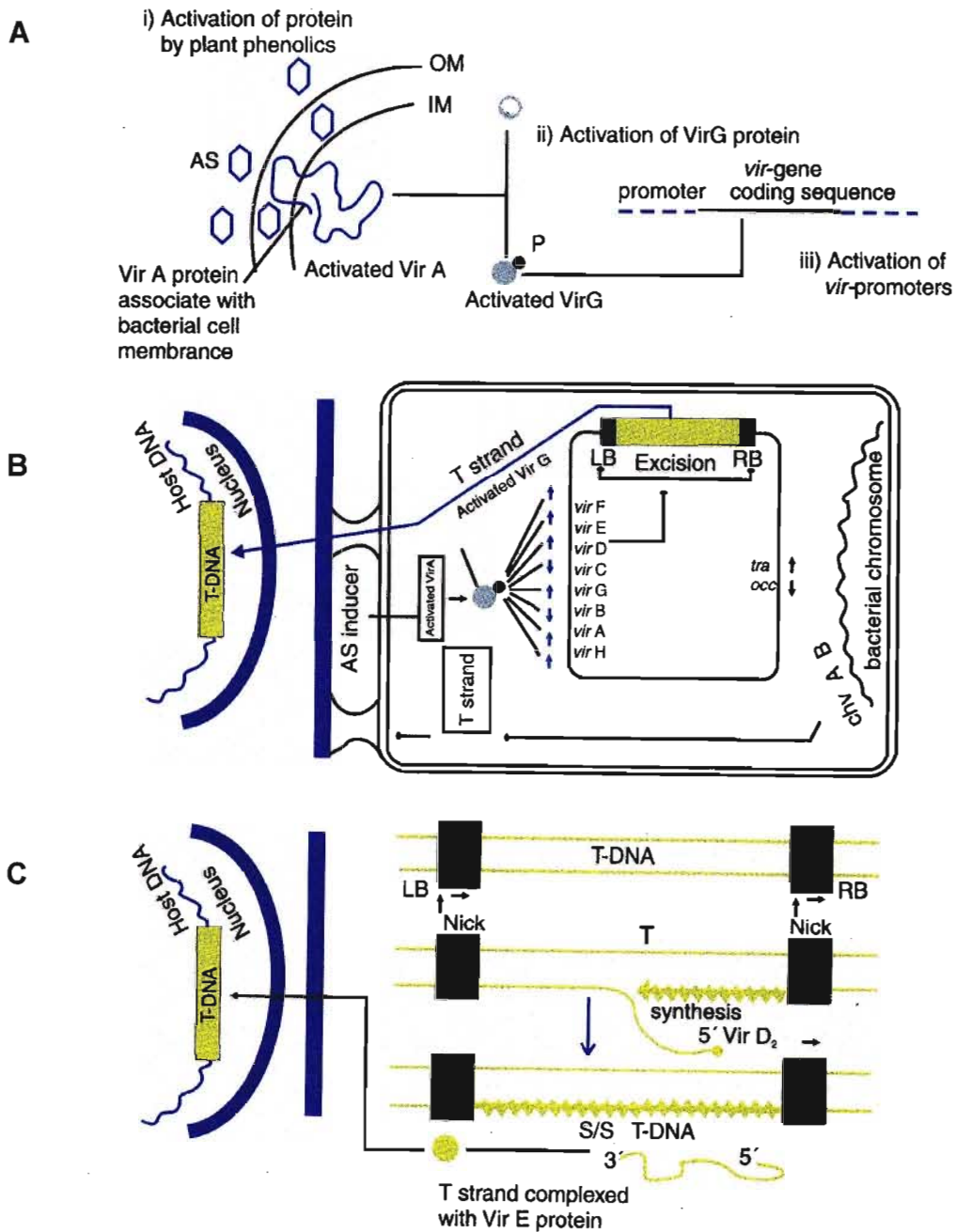


Figure 2.3 Schematic summary of *Agrobacterium*-plant cell interaction (CHAWLA 2002). (A) Activation of the *vir* genes via VirA and VirG, (B) Induction of *vir* genes and T-DNA transfer, (C) transfer of T-DNA to plant cell. OM: Outer membrane; IM: inner membrane; RB: right border; LB: left border

In plant genetic engineering a binary system consisting of a helper Ti-plasmid, with virulence functions and an artificial, disarmed (without oncogenes) T-DNA, is widely used. The binary vector (artificial T-DNA) contains a selectable marker gene and other genes of interest. Improved transformation regimes may be attained through manipulation of the host plant as T-DNA integration in the genome of regenerable cells appears limited. In future it may be possible to over-express endogenous genes involved in the integration process or introduce homologous genes from other species inducing higher rates of transformation (GELVIN 2000).

2.6 Marker Genes

Initial genetic studies in plants were limited by the availability of suitable mutations that served as markers to help determine whether DNA had been successfully transferred into recipient cells. The marker gene is introduced into the plasmid along with the target gene and can be considered as two types: reporter genes or selectable marker genes (CHAWLA 2002). Assayable markers (enzyme, gel activity or immunochemical assays) direct the expression of a readily quantifiable product such as an enzyme or antigen that is not necessarily distinguishable in living cells (BOWEN 1993). Markers should only be expressed inside cells of the target tissue, and not inside contaminating micro-organisms in the particle gun.

Selectable markers are crucial to the development of transformation protocols by allowing the survival of transformed cells, tissue or the expression of a particular phenotype in conditions normally hostile to untransformed tissue. The percentage frequency at which untransformed cells or plants survive at a given level of the toxic selection agent should ideally be at 50% growth inhibition (LD_{50}) for untransformed cells (BOWEN 1993). Available selective marker genes include antibiotic (neomycin phosphotransferase II or *nptII*), antimetabolite genes, hormone biosynthetic genes, and herbicide resistance genes such as the *bar* gene (described in Section 4.1) (CHAWLA 2002). Selective markers must be readily available and soluble in plant material, preferably inexpensive and non-toxic to the researcher (BOWEN 1993).

In contrast with selectable markers, reporter genes do not confer resistance to plant cells against a chemical agent normally inhibitory to plant development. Reporter genes code for products that are directly detectable or catalyse specific reactions whose products in turn are easily detected. They are also able to distinguish the expression of introduced plant genes from background levels of endogenous gene expression (BOWEN 1993). Visible markers include histological, morphological and pigmentation markers (BOWEN 1993). Histological markers confer a distinguishing phenotype in the presence of a substrate or affinity reagent supplied exogenously. The gene fusion construct β -glucuronidase (GUS) reporter gene is the most widely used visual marker in histological studies of transgenic plants (Section 2.7.1). The insertion of a portable plant-derived intron into a reporter gene, such as the GUS gene, ensures more efficient transfer to plants. The intron is spliced efficiently in transgenic plants containing the chimaeric gene construct, giving rise to prominent GUS enzymatic activity. This is usually under the control of a strong promoter such as the Cauliflower Mosaic Virus (CaMV) derived 35S (GUILLEY *et al.* 1982) or maize *ubiquitin* promoter (CORNEJO *et al.* 1993).

2.7 Analysis of Transgenic Material

2.7.1 Transient Gene Expression

One of the desired outcomes of a gene transfer study is the potential expression of the transferred gene of interest, termed transgene, by the recipient cell. The introduced DNA may only be expressed for a short period of time following gene transfer - this is known as transient expression. Transient assays are useful for the analysis of gene expression and rapid monitoring of gene transfer (CHAWLA 2002).

The histochemical GUS assay developed by JEFFERSON *et al.* (1987) has become a popular reporter gene system for transformation of plants. The *Escherichia coli uidA* locus (NOVEL and NOVEL 1973) encodes the hydrolase β -glucuronidase enzyme, which causes an oxidative dimerisation of chromogenic

5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) resulting in a deep blue colour so its presence can be detected *in situ* (Figure 2.4). Blue coloured cells are an indication of transient GUS expression (JEFFERSON *et al.* 1987). β -Glucuronidase, a homotetramer of about 68 kDa mass, which requires an optimum pH 7-8, cleaves a wide variety of β -glucuronides. These are available commercially as spectrophotometric, fluorometric, and histochemical substrates (JEFFERSON *et al.* 1987; CHAWLA 2002).

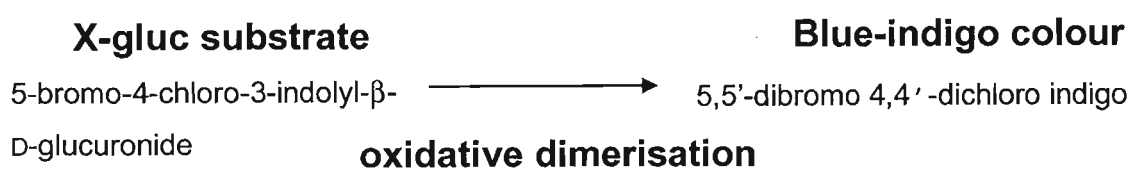


Figure 2.4 Hydrolytic cleavage of the chromogenic substrate X-gluc by β -glucuronidase

This fusion construct has three striking characteristics. Reporter gene activity is unimpaired while fused to other proteins at its amino terminus. Secondly, it is detectable with sensitive histochemical assays to localize gene activity in particular cell types. Thirdly, the reaction catalyzed is sufficiently specific to minimise interference with normal cellular metabolism but still allows the use of a wide variety of substrates (JEFFERSON *et al.* 1987). Another advantage is that the GUS reporter does not require DNA extraction, electrophoresis, or radiography (CHAWLA 2002) and can be quantified though a protein 4-methylumbelliferyl β -D-glucuronide (4-MUG) assay. However, the histochemical procedure used to detect GUS requires destructive harvesting of material (BOWEN 1993), one of the limitations of the assay (Section 7.1).

2.7.2 Stable Integration of the Desired Gene

Stable integration occurs when introduced DNA is integrated into the genome of a plant, expression occurs in the plant and the introduced DNA is inherited in subsequent generations (CHAWLA 2002). The stable integration of DNA fragments into the genome can be verified by screening total transformants for the

respective sequence using the polymerase chain reaction (PCR) or DNA gel blots (Southern hybridization) (FITCH *et al.* 1992; SOLIS *et al.* 1997), which have become popular in transformation studies. PCR and DNA gel blots are primarily used for information on sizes of inserts and homoplasticity.

2.7.2.1 The Polymerase Chain Reaction (PCR)

Kary Mullis from Cetus Corporation, USA, is credited with the idea of the PCR (MULLIS *et al.* 1986) that has helped to simplify recombinant DNA technology. He was later awarded the Nobel Prize in chemistry for his contributions to the development of PCR. A PCR experiment is an *in vitro* method where oligonucleotide (deoxy) primers can amplify a specific segment of genomic DNA or cDNA, using thermostable DNA polymerase. The sequence at the borders of the selected DNA regions must be known in order for the two oligonucleotides to anneal to target DNA for amplification due to the complementary nature of base sequences (CHAWLA 2002). The result is selective amplification of a chosen region of a DNA molecule. The sensitivity of PCR enables *in vitro* isolation of genes from single cells to bones (BROWN 1995). Applications to gene manipulation involve the creation of deletions, short insertions, and chimaeric products, and for mediating site-directed mutagenesis (HIGUCHI 1990 cited in CHO and LEMAUX 1997). Since its invention in the 1980's, PCR has had widespread use in plant transformation studies due to successful analysis of foreign genes in transformed tissues.

Amplification is usually carried out by the *Taq* polymerase DNA enzyme, isolated from *Thermus aquaticus*, that maintains activity after denaturation treatment at 94 °C. The PCR involves two oligonucleotide primers, between 17-30 nucleotides in length, flanking the DNA sequences, effectively delimiting the region to be enzymatically amplified (Figure 2.5). The primers hybridize to opposite strands of the DNA so that the extension reactions create two double-stranded target regions. The PCR method is based on repeated cycles with three steps: template DNA duplex denaturation by heat (94 °C), oligonucleotide primer annealing to target sequences of separated DNA strands (55-65 °C) and DNA synthesis from the 3'-OH end of each primer by DNA polymerase (72 °C). The automated PCR

results in a rapid exponential amplification of the specific DNA fragment as the primer extension products of the previous cycle serve as new templates for the following cycle (OLD and PRIMROSE 1996; CHAWLA 2002).

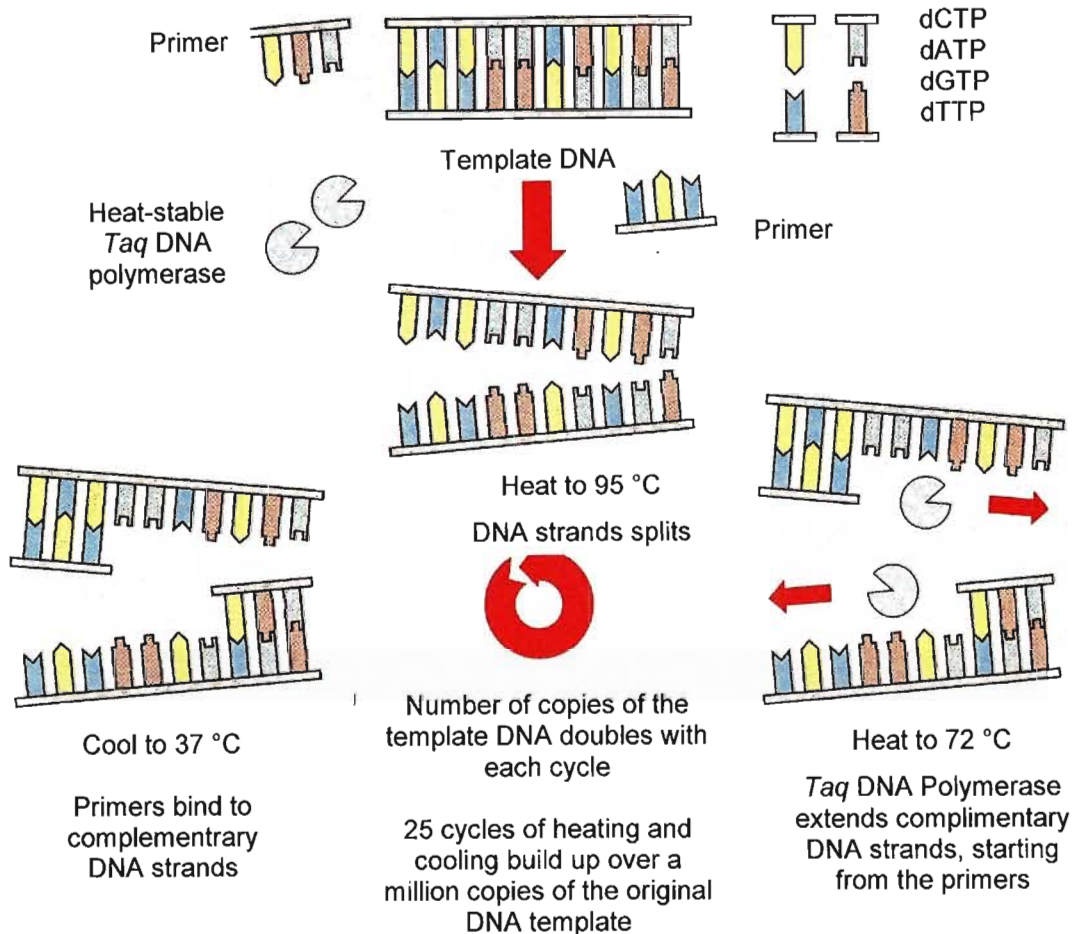


Figure 2.5 A schematic diagram of the polymerase chain reaction (BOEHRINGER MANNHEIM 1995)

2.7.2.2 Southern Hybridization

The original method of DNA blotting was developed by SOUTHERN (1975) for detecting restriction fragments in an agarose gel by blotting onto a nylon or nitrocellulose membrane followed by detection with a probe of complimentary RNA or DNA sequence (CHAWLA 2002). Through Southern hybridization one can detect the presence of one or many copies of the desired sequence such as a transgene after successful genetic transformation. If the probe hybridizes once only, it can be deduced that there is only one copy present. Similarly, multicopies

are observed by the numerous hybridizations: the larger the number of copies, the further the distance the probe will be able to travel in the agarose gel.

For efficient Southern blotting, gel pre-treatment should be performed. This involves exposure of the electrophoresed restricted DNA to a short depurination treatment followed by alkali treatment. This ensures that the DNA fragments are shortened and are in an accessible single stranded state for probing. The gel is further equilibrated in neutralizing solution prior to blotting. In a conventional procedure the agarose gel is mounted on a supported filter paper wick that dips into transfer buffer. The hybridization membrane (nitrocellulose or nylon membrane) is sandwiched between the gel and a stack of paper towels (suitable absorbent material) and the buffer is allowed to soak through by capillary action. The DNA is carried out of the gel by the buffer flow and immobilized onto the solid membrane support. However, this method has the technical drawback of the necessity of a constructed frame around the agarose gel to hold the transfer solution. KOETSIER *et al.* (1993) improved this procedure by using blotting paper to transfer solution from the side, and placed the gel followed by the nylon membrane underneath - resulting in a downward flow. The downward alkaline Southern blotting of DNA was reported to give sharper and 30% stronger signals than conventional methods (KOETSIER *et al.* 1993).

After transfer, nucleic acids are fixed to the membrane by oven baking (80 °C), UV light or incubation in alkaline solutions. Following fixation, the membrane is placed in hybridization solution with labelled RNA, single-stranded DNA, or oligodeoxynucleotides, which are sequence-complimentary to the blot-transferred DNA band to be detected. After hybridization the membrane is washed to remove any unbound radioactivity so regions of hybridization can be detected using autoradiography when the probe is radioactively labelled (OLD and PRIMROSE 1996).

2.8 Gene Transfer to Conifers

While the first genetically engineered tree was a *Populus* species encoding herbicide resistance in the late 1980's (MOFFAT 1996), the first reports of

transgenic conifers, initiated in *Larix decidua* (HUANG *et al.* 1991), also appeared at this time. Much of the earlier transformation work resulted in transient expression following electroporation or *Agrobacterium*-mediated transfer (Table 2.2).

Electroporation is a useful means of gene transfer when used in combination with regenerable protoplasts capable of somatic embryo or plantlet formation. This system avoids host-range limitations of *Agrobacterium*-mediated transformations and can rapidly evaluate plasmid construction, transient expression or stable transformation (TANG and NEWTON 2003). Protoplasts have been isolated for electroporation from embryogenic cultures in *Picea glauca* (BEKKAOUI *et al.* 1988), and *Larix x eurolepis* (CHAREST *et al.* 1991), embryogenic suspension cultures of *Picea mariana* and non-embryogenic cultures of *Pinus banksiana* (TAUTORUS *et al.* 1989). Suspension cultures derived from excised embryos in *Pinus radiata* (CAMPBELL *et al.* 1992) and suspension cultures of somatic embryos in *P. glauca* (BEKKAOUI *et al.* 1990) were also used. However, plant regeneration from conifer protoplasts is difficult, so this method of gene transfer is mainly used to examine transgene expression and any influencing factors (TANG and NEWTON 2003).

Reports of regenerated stably transformed plantlets after bombardment of somatic embryos were soon to follow in *Picea glauca* (ELLIS *et al.* 1993), *Picea mariana* (CHAREST *et al.* 1996; TIAN *et al.* 2000), and *Larix laricina* (KLIMASZEWSKA *et al.* 1997). Regeneration of embryogenic tissue following biolistic transfer was achieved in *Pinus radiata* (WALTER *et al.* 1998b; GRACE *et al.* 2005), *Picea abies* (CLAPHAM *et al.* 2000) and *Pinus pinaster* (TRONTIN *et al.* 2002). More recently, stably transformed chir pine plants were regenerated after biolistic transfer of mature zygotic embryos (PARASHARAMI *et al.* 2006).

The use of microprojectile-mediated transformation in gene transfer studies increased in popularity as did the range of explants tested for target suitability. These included: pollen, embryogenic tissue or calli, embryogenic cell suspensions, zygotic embryos, somatic embryos, cotyledons, excised cotyledons, mature seeds, vegetative buds, fascicular buds, secondary xylem, seedlings, pollen tubes and

organogenic explants such as seedling hypocotyls, detached cotyledons and hypocotyls (Table 2.2).

Although embryogenic tissue has been mainly used in *Agrobacterium*-mediated investigations, this method has been most commonly applied to organogenic tissue. The effect of particle bombardment on shoot organogenesis has also been tested in *Pinus pinea* cotyledons (SUL and KORBAN 1998). Regenerated transgenic *Larix* plants were obtained after seedling hypocotyls were transformed with *A. rhizogenes* (HUANG *et al.* 1991; SHIN *et al.* 1994). *A. rhizogenes* was further used to transform *P. contorta* hypocotyls (YIBRAH *et al.* 1996). CHARITY *et al.* (2002) used *A. tumefaciens* to transform *P. radiata* detached cotyledons from zygotic embryos and apical meristematic domes. Detached cotyledons of *P. pinea* were co-cultivated with *A. tumefaciens* (HUMARA *et al.* 1999b). However, TANG *et al.* (2001) reported both stable transformation and regeneration of *P. taeda* plants derived from co-cultivated mature zygotic embryos.

Advances in conifer somatic embryogenesis have created opportunities for genetic improvement in various species that will have application in the forest industry (SUTTON 2002). Regeneration of stably transformed plants after *Agrobacterium*-mediated transformation has been demonstrated in *P. strobus* somatic embryos (LEVÉE *et al.* 1999), *P. radiata* (CHARITY *et al.* 2001; CHARITY *et al.* 2005), and *Larix kaempferi* x *L. decidua* embryogenic tissue (LEVÉE *et al.* 1997); embryogenic tissue of *Picea glauca*, *P. mariana* and *P. abies* (KLIMASZEWSKA *et al.* 2001), *P. pinaster* embryonal suspensor masses (ESM) (TRONTIN *et al.* 2002) and in *Betula platyphylla* var *japonica* (MOHRI *et al.* 1997). Other *Agrobacterium*-mediated investigations using a variety of tree species include those of: HOLLAND *et al.* 1997, CHARITY *et al.* 2002 and PAPPINEN *et al.* 2002.

Significant progress has been made in the transfer of foreign genes to conifer species through biolistic and *Agrobacterium*-mediated means in the last ten years (see reviews WALTER *et al.* 2002; TANG and NEWTON 2003). The freedom from limiting technology and improved regeneration regimes have expanded the application of gene transfer technology in conifers, concentrating on areas such as herbicide resistance (CONFALONIERI *et al.* 2000; BISHOP-HURLEY *et al.* 2001),

insect resistance (ELLIS *et al.* 1993; GRACE *et al.* 2005), wood modification (LI *et al.* 2003) and furthering the knowledge base of lignin biosynthesis. However, few studies to date have both incorporated traits of commercial interest and evaluated their gene expression (GRACE *et al.* 2005).

Table 2.2 Summary of current literature on gene transfer studies in conifers

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|--|---|---|---------------------------------------|
| <i>Abies procera</i> | <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | Callus | MORRIS <i>et al.</i> (1989) |
| <i>Abies nordmanniana</i> | <i>A. tumefaciens</i> strains | | CLAPHAM and EKBERG (1986) |
| <i>Betula papyrifera</i> (paper birch) | <i>A. tumefaciens</i> – crown gall tissues induced | | MACKAY <i>et al.</i> (1997) |
| <i>Betula pendula</i> (silver birch) | <i>A. tumefaciens</i> | 2-month-old seedlings | ARONEN (1997) |
| <i>B. pendula</i> | <i>A. tumefaciens</i> | Base of stem | ARONEN and HÄGGMAN (1995) |
| <i>B. pendula</i> | Biolistic transfer <i>Agrobacterium</i> -mediated transfer | Small leaves Nodal stem pieces | KEINONEN-METTÄLÄ <i>et al.</i> (1998) |
| <i>B. pendula</i> | <i>Agrobacterium</i> -mediated transfer | Nodal stem pieces | LEMMETYINEN <i>et al.</i> (1998) |
| <i>B. pendula</i> | <i>A. tumefaciens</i> carrying <i>htp</i> in the pPCV8125 vector | Stem segments | MAKOVEYCHUK and SANDBERG (1998) |
| <i>B. pendula</i> | <i>Agrobacterium</i> -mediated transfer | | PAPPINEN <i>et al.</i> (2002) |
| <i>B. pendula</i> | Biolistic transfer of <i>rbcS</i> and <i>nptII</i> | <i>In vitro</i> callus and shoot cultures | VALJAKKA <i>et al.</i> (2000) |
| <i>Betula platyphylla</i> var <i>japonica</i> (white birch) | <i>Agrobacterium</i> -mediated transfer | Regeneration | MOHRI <i>et al.</i> (1997) |
| <i>Larix spp</i> | Biolistic transfer | Haploid and diploid embryogenic cells | DUCHESNE <i>et al.</i> (1993) |
| <i>Larix decidua</i> (European larch) | <i>A. rhizogenes</i> | Plantlets | DINER and KARNOSKY (1988) |
| <i>L. decidua</i> | <i>A. rhizogenes</i> | | ELLIS <i>et al.</i> (1994) |
| <i>L. decidua</i> | <i>A. rhizogenes</i> | Organogenic - Seedling hypocotyls | HUANG <i>et al.</i> (1991) |
| <i>L. decidua</i> | Variety of <i>Agrobacterium</i> strains | Seedlings | HUANG <i>et al.</i> (1993) |
| <i>L. decidua</i> | <i>A. tumefaciens</i> and <i>A. rhizogenes</i> mediated transfer | Hypocotyls | KARNOSKY <i>et al.</i> (1988) |
| <i>L. decidua</i> | <i>A. rhizogenes</i> | Sterile seedlings | KARNOSKY <i>et al.</i> (1995) |
| <i>L. decidua</i> | <i>A. rhizogenes</i> transfer of insect (Bt toxin synthesis, regenerated transformant) and herbicide resistance | Organogenic - Hypocotyls | SHIN <i>et al.</i> (1994) |
| <i>Larix x eurolepis</i> (hybrid larch) | Electroporation with GUS, <i>nptII</i> and chloramphenicol acetyltransferase (CAT) | Protoplasts | CHAREST <i>et al.</i> (1991) |
| <i>Larix x eurolepis</i> | Effect of promoter sequence following biolistic transfer | Embryogenic calli | DUCHESNE and CHAREST (1992) |

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|--|---|--|------------------------------------|
| <i>L. kaempferi</i> x <i>L. decidua</i> | <i>A. tumefaciens</i> with <i>nptII</i> under two different promoters | Embryogenic tissue | LEVEE <i>et al.</i> (1997) |
| <i>Larix laricina</i> (tamarack) | Particle bombardment with pRT99GUS and pB1426 | Mature cotyledonary somatic embryos and suspensions from embryonal masses. | CHAREST <i>et al.</i> (1996) |
| <i>L. laricina</i> | Transfer of pBI426, pRT99GUS, pRT66GUS via bombardment | Embryonal masses and somatic embryo cells | KLIMASZEWSKA <i>et al.</i> (1997) |
| <i>L. laricina</i> | Root induction using <i>A. rhizogenes</i> | Mature embryos, shoots, de-rooted seedlings | McAFEE <i>et al.</i> (1993) |
| <i>Libocedrus decurrens</i> (incense cedar) | <i>A. tumefaciens</i> | 2-4 month old seedlings with 3-5 cm epicotyls | STOMP <i>et al.</i> (1990) |
| <i>Picea abies</i> (Norway spruce) | Biolistic transfer | Embryogenic tissue | BERCETCHE <i>et al.</i> (1994) |
| <i>P. abies</i> | Particle bombardment with <i>bar</i> and <i>nptII</i> | Embryogenic tissue | BISHOP-HURLEY <i>et al.</i> (2001) |
| <i>P. abies</i> | Particle bombardment | Embryogenic cultures | BRUKHIN <i>et al.</i> (2000) |
| <i>P. abies</i> | <i>A. tumefaciens</i> strains | | CLAPHAM and EKBERG (1986) |
| <i>P. abies</i> | <i>A. tumefaciens</i> A281 | 3-month old seedlings | CLAPHAM <i>et al.</i> (1990) |
| <i>P. abies</i> | Biolistic transfer | Embryogenic suspension | CLAPHAM <i>et al.</i> (1995) |
| <i>P. abies</i> | Regeneration following biolistic transfer | Embryogenic cultures | CLAPHAM <i>et al.</i> (2000) |
| <i>P. abies</i> | | Embryogenic cells | ELFSTRAND <i>et al.</i> (2001) |
| <i>P. abies</i> | <i>A. tumefaciens</i> A281 | 3-month-old seedlings | HOOD <i>et al.</i> (1990) |
| <i>P. abies</i> | Regeneration after <i>A. tumefaciens</i> cocultivation | Embryogenic tissue | KLIMASZEWSKA <i>et al.</i> (2001) |
| <i>P. abies</i> | <i>A. rhizogenes</i> | 2-3-week-old seedlings | MAGNUSSEN <i>et al.</i> (1994) |
| <i>P. abies</i> | Particle acceleration | Pollen grains | MARTINUSSEN <i>et al.</i> (1994) |
| <i>P. abies</i> | Microprojectile bombardment | Pollen | MARTINUSSEN <i>et al.</i> (1995) |
| <i>P. abies</i> | Particle acceleration | Zygotic embryos, embryogenic callus, embryogenic cell suspensions | NEWTON <i>et al.</i> (1992) |
| <i>P. abies</i> | Microprojectile bombardment | Somatic embryos | ROBERTSON <i>et al.</i> (1992) |
| <i>P. abies</i> | Biolistic transfer | Embryogenic tissue | WALTER <i>et al.</i> (1999) |
| <i>P. abies</i> | <i>A. tumefaciens</i> | Embryogenic suspension-cultured cells | WENCK <i>et al.</i> (1999) |

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|---|--|---|---|
| <i>P. abies</i> | Microprojectile bombardment | Embryogenic suspension | YIBRAH (1994) |
| <i>P. abies</i> | Microprojectile bombardment | Embryogenic suspension | YIBRAH <i>et al.</i> (1994) |
| <i>P. abies</i> and <i>P. sylvestris</i> | GUS transfer by particle bombardment Electrofusion of protoplasts | Pollen Protoplasts | HÄGGMAN <i>et al.</i> (1997) ARONEN <i>et al.</i> (1998) KIRSTEN <i>et al.</i> (1987) |
| <i>P. engelmannii</i> (Engelmann spruce) | Various strains of <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | 6-month-old seedlings | ELLIS <i>et al.</i> (1989) |
| <i>Picea glauca</i> (white spruce) | Electroporation | Protoplast isolated from embryogenic cultures | BEKKAOUI <i>et al.</i> (1988) |
| <i>P. glauca</i> | Electroporation with CAT or GUS | Protoplasts from suspension cultures of somatic embryos | BEKKAOUI <i>et al.</i> (1990) |
| <i>P. glauca</i> | Biolistic transfer of GUS: <i>npt</i> II | Cotyledonary somatic embryos | BOMMINENI <i>et al.</i> (1993) |
| <i>P. glauca</i> | Particle bombardment with pBI426 | Somatic embryos | BOMMINENI <i>et al.</i> (1997) |
| <i>P. glauca</i> | Microprojectile bombardment with ABA-inducible <i>uidA</i> construct | Somatic embryos | BOMMINENI <i>et al.</i> (1998) |
| <i>P. glauca</i> | Microprojectile transformation using GUS and <i>nptII</i> | Embryogenic tissue | CHAREST <i>et al.</i> (1993) |
| <i>P. glauca</i> | Various strains of <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | 6-month-old seedlings | ELLIS <i>et al.</i> (1989) |
| <i>P. glauca</i> | Particle acceleration | Zygotic embryos, seedlings, embryogenic callus | ELLIS <i>et al.</i> (1991) |
| <i>P. glauca</i> | Regeneration after particle bombardment, with Bt <i>cryIA</i> gene | Embryogenic callus, embryos and seedlings | ELLIS <i>et al.</i> (1993) |
| <i>P. glauca</i> | Expression patterns of CaMV35S promoter in transgenic <i>P. glauca</i> lines | Embryogenic callus, somatic embryos, somatic embryo-derived seedlings | ELLIS <i>et al.</i> (1994b) |
| <i>P. glauca</i> | Particle acceleration | | ELLIS <i>et al.</i> (1994a) |
| <i>P. glauca</i> | Particle transfer | Embryogenic callus | ELLIS (1997) |
| <i>P. glauca</i> | Electroporation of ADH reporter ¹ genes | Protoplasts | GOOD <i>et al.</i> (1990) |
| <i>P. glauca</i> | Regeneration after <i>A. tumefaciens</i> cocultivation | Embryogenic tissue | KLIMASZEWSKA <i>et al.</i> (2001) |
| <i>P. glauca</i> | Particle transfer | Pollen, embryogenic tissue | LI <i>et al.</i> (1994) |
| <i>P. glauca</i> | Regeneration after <i>A. tumefaciens</i> transformation | Embryogenic tissue | LE <i>et al.</i> (2001) |

¹ *Adh* genes transcribe Alcohol Dehydrogenase proteins

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|---|---|---|-----------------------------------|
| <i>P. glauca</i> | Microprojectile transfer of green fluorescent protein (GFP) | Pollen, embryogenic suspension mass, and somatic embryos | TIAN <i>et al.</i> (1997) |
| <i>P. glauca</i> | PEG-mediated expression of GUS & CAT genes | Protoplasts from embryogenic suspension cultures | WILSON <i>et al.</i> (1989) |
| <i>Picea mariana</i> (black spruce) | Biolistic bombardment | Cotyledonary somatic embryos, immature embryos | BOMMINENI <i>et al.</i> (1994) |
| <i>P. mariana</i> | Microprojectile bombardment | Embryogenic callus | CHAREST <i>et al.</i> (1993) |
| <i>P. mariana</i> | Particle bombardment with pRT99GUS and pB1426 | Mature cotyledonary somatic embryos and suspensions from embryonal masses | CHAREST <i>et al.</i> (1996) |
| <i>P. mariana</i> | Particle bombardment | Embryogenic callus | DUCHESNE and CHAREST (1991) |
| <i>P. mariana</i> | Biolistic transfer | Embryogenic calli | DUCHESNE and CHAREST (1992) |
| <i>P. mariana</i> | Particle transfer | Zygotic embryos, seedlings | GRAY <i>et al.</i> (1996) |
| <i>P. mariana</i> | Microparticle bombardment | Mature pollen | HAY <i>et al.</i> (1994) |
| <i>P. mariana</i> | Regeneration after <i>A. tumefaciens</i> cocultivation | Embryogenic tissue | KLIMASZEWSKA <i>et al.</i> (2001) |
| <i>P. mariana</i> | Electroporation | Protoplasts isolated from embryogenic suspension cultures | TAUTORUS <i>et al.</i> (1989) |
| <i>P. mariana</i> | Particle transfer of GFP | Pollen, ESM, suspension culture, somatic embryos | TIAN <i>et al.</i> (1997) |
| <i>P. mariana</i> | Particle transfer of GFP <i>Agrobacterium</i> transformation | 6 w somatic embryos Embryogenic tissues | TIAN <i>et al.</i> (1999) |
| <i>P. mariana</i> | Particle bombardment | Mature somatic embryos | TIAN <i>et al.</i> (2000) |
| <i>Picea sitchensis</i> (sitka spruce) | Various strains of <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | 6-month-old seedlings | ELLIS <i>et al.</i> (1989) |
| <i>P. sitchensis</i> | <i>A. tumefaciens</i> | ESM | PATTERSON <i>et al.</i> (1998) |
| <i>P. sitchensis</i> | <i>A. tumefaciens</i> | Embryogenic cell lines | DRAKE <i>et al.</i> (1997) |
| <i>Pinus ayacahuite</i> | <i>A. tumefaciens</i> | <i>In vitro</i> derived shoots | SABORIO <i>et al.</i> (1999) |
| <i>Pinus aristata</i> (bristlecone) | Particle delivery | Pollen tubes | FERNANDO <i>et al.</i> (2000) |
| <i>Pinus banksiana</i> (jack pine) | Microparticle bombardment | Mature pollen | HAY <i>et al.</i> (1994) |

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|---|--|---|--|
| <i>P. banksiana</i> | Root induction using <i>A. rhizogenes</i> | Mature embryos, shoots, de-rooted seedlings | McAFEE <i>et al.</i> (1993) |
| <i>P. banksiana</i> | Electroporation | Protoplasts | TAUTORUS <i>et al.</i> (1989) |
| <i>Pinus contorta</i> (lodgepole) | Microparticle bombardment | Mature pollen | HAY <i>et al.</i> (1994) |
| <i>P. contorta</i> | <i>A. rhizogenes</i> | | LINDROTH <i>et al.</i> (1999) |
| <i>P. contorta</i> | <i>A. rhizogenes</i> | 2-3-week-old seedlings | MAGNUSSEN <i>et al.</i> (1994) |
| <i>P. contorta</i> | <i>A. rhizogenes</i> | Organogenic - Hypocotyls | YIBRAH <i>et al.</i> (1996) |
| <i>Pinus echinata</i> (short leaf) | <i>A. tumefaciens</i> | Seedlings | HUANG and TAUER (1994) |
| <i>Pinus eldarica</i> (Afgan pine) | <i>A. tumefaciens</i> | 2-4 month old seedlings with 3-5 cm epicotyls | STOMP <i>et al.</i> (1990) |
| <i>Pinus elliottii</i> (slash pine) | Adventitious rooting stimulated by <i>Agrobacterium</i> | Hypocotyledonary explants | BURNS and SCHWARZ (1996) |
| <i>P. elliotti</i> | Particle bombardment | Embryogenic cell suspensions | NEWTON <i>et al.</i> (1994) |
| <i>P. elliotti</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>P. elliottii</i> | Particle bombardment | Suspension cells | WU <i>et al.</i> (1999) |
| <i>P. griffithii</i> (blue pine) | Particle delivery | Pollen tubes | FERNANDO <i>et al.</i> (2000) |
| <i>P. halepensis</i> (Aleppo pine) | <i>A. rhizogenes</i> | Shoot cultures | ALTMAN <i>et al.</i> (1995) |
| <i>P. halepensis</i> | <i>A. rhizogenes</i> -mediated DNA transfer | Embryos, seedlings and shoots | TZFIRA <i>et al.</i> (1996) |
| <i>Pinus jeffreyi</i> (Jeffrey pine) | <i>A. tumefaciens</i> | 2-4 month old seedlings with 3-5 cm epicotyls | STOMP <i>et al.</i> (1990) |
| <i>Pinus lambertiana</i> (sugar pine) | <i>A. tumefaciens</i> containing plasmid pTiBo542 | Shoots | LOOPSTRA <i>et al.</i> (1990) |
| <i>P. lambertiana</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>Pinus maximartinezii</i> | <i>A. rhizogenes</i> | Shoots | VILLALOBOS-AMADOR <i>et al.</i> (2002) |
| <i>Pinus monticola</i> (western white pine) | Particle delivery | Pollen tubes | FERNANDO <i>et al.</i> (2000) |
| <i>P. monticola</i> | Root induction using <i>A. rhizogenes</i> | Mature embryos, shoots, de-rooted seedlings | McAFEE <i>et al.</i> (1993) |
| <i>Pinus nigra</i> (black pine) | Transfer of β -lucuronidase by <i>A. tumefaciens</i> | Cotyledonous cells | ORDÁS <i>et al.</i> (1994) |
| <i>P. nigra</i> | Particle bombardment | 1-year old cotyledons | HUMARA <i>et al.</i> (1998) |
| <i>P. nigra</i> | <i>A. rhizogenes</i> | Primary explants | MIHALJEVIC <i>et al.</i> (1996) |

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|---|--|---|--|
| <i>P. nigra</i> | Particle bombardment | Excised cotyledons | REY <i>et al.</i> (1996) |
| <i>Pinus palustris</i> (longleaf pine) | <i>A. rhizogenes</i> | Germlings with roots (1-5 mm) | DINER and SOLIMAN (1993) |
| <i>P. palustris</i> | Particle bombardment | Embryogenic masses | NAGMANI <i>et al.</i> (1993) |
| <i>Pinus patula</i> | Particle bombardment | ESM | NIGRO <i>et al.</i> (2004) |
| <i>Pinus pinceana</i> | <i>A. rhizogenes</i> | Shoots | VILLALOBOS-AMADOR <i>et al.</i> (2002) |
| <i>Pinus pinea</i> (stone pine) | Induction of virulence response in <i>A. tumefaciens</i> by exudates | Embryonic exudates of cotyledons | HUMARA <i>et al.</i> (1999a) |
| <i>P. pinea</i> | Particle bombardment | 1-year old cotyledons | HUMARA <i>et al.</i> (1998) |
| <i>P. pinea</i> | <i>A. tumefaciens</i> | Organogenic - Excised cotyledons from embryos | HUMARA <i>et al.</i> (1999b) |
| <i>P. pinea</i> | Particle bombardment | Excised cotyledons | REY <i>et al.</i> (1996) |
| <i>P. pinea</i> | Particle bombardment | Cotyledons | SUL and KORBAN (1998) |
| <i>Pinus pinaster</i> (maritime pine) | Microprojectile bombardment | Pollen | MARTINUSSEN <i>et al.</i> (1995) |
| <i>P. pinaster</i> | Particle transfer | ESM | TRONTIN <i>et al.</i> (2001a) |
| <i>P. pinaster</i> | <i>A. tumefaciens</i> | ESM | TRONTIN <i>et al.</i> (2001b) |
| <i>P. pinaster</i> | Regeneration following particle bombardment or <i>A. tumefaciens</i> | ESM | TRONTIN <i>et al.</i> (2002) |
| <i>Pinus ponderosa</i> (ponderosa) | <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | Callus | MORRIS <i>et al.</i> (1989) |
| <i>P. ponderosa</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>Pinus radiata</i> (Monterey) | <i>A. tumefaciens</i> -mediated transformation | Shoots and rooted plantlets | BERGMANN and STOMP (1992) |
| <i>P. radiata</i> | Particle bombardment | Embryogenic tissue | BISHOP-HURLEY <i>et al.</i> (2001) |
| <i>P. radiata</i> | Electroporation and particle bombardment | Suspension-derived protoplasts | CAMPBELL <i>et al.</i> (1992) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> | Embryogenic tissue | CERDA <i>et al.</i> (2002) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> | Embryogenic tissue | CHARITY <i>et al.</i> (2001) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> -mediated transformation using vacuum infiltration | Organogenic tissue – detached cotyledons, apical meristematic domes | CHARITY <i>et al.</i> (2002) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> , Regeneration with stable expression of <i>nptII</i> , <i>uidA</i> and <i>bar</i> genes | Embryogenic cell cultures placed on nurse cultures | CHARITY <i>et al.</i> (2005) |
| <i>P. radiata</i> | Microprojectile bombardment | Embryogenic tissue | GRACE <i>et al.</i> (2001) |
| <i>P. radiata</i> | Regeneration after bombardment with insect resistant Bt toxin gene (<i>cryIAc</i>) | Embryogenic tissue | GRACE <i>et al.</i> (2005) |

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|---|--|---|----------------------------------|
| <i>P. radiata</i> | <i>Agrobacterium</i> | Excised cotyledons | GRANT <i>et al.</i> (1998) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> | Cotyledons | GRANT <i>et al.</i> (2004) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> | Cotyledons | HOLLAND <i>et al.</i> (1997) |
| <i>P. radiata</i> | | Embryogenic and xylogenic tissue | MOYLE <i>et al.</i> (2002) |
| <i>P. radiata</i> | Particle acceleration with pBIN121 plasmid | Mature seeds | REY <i>et al.</i> (1994) |
| <i>P. radiata</i> | Particle bombardment | Cotyledons | REY <i>et al.</i> (1995) |
| <i>P. radiata</i> | Particle bombardment | Excised cotyledons | REY <i>et al.</i> (1996) |
| <i>P. radiata</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>P. radiata</i> | Particle bombardment | Embryogenic cultures | WALTER <i>et al.</i> (1994) |
| <i>P. radiata</i> | Regeneration following biolistic transfer with <i>nptII</i> and <i>uidA</i> | Embryogenic tissue | WALTER <i>et al.</i> (1998b) |
| <i>Pinus roxburghii</i> (chir pine) | Particle bombardment with pAHC25 vector | Mature zygotic embryos; regeneration from epicotyl and adventitious buds | PARASHARAMI <i>et al.</i> (2006) |
| <i>P. radiata</i> | Particle delivery | Embryogenic tissue | WAGNER <i>et al.</i> (1997) |
| <i>Pinus Salzmanii</i> | Particle bombardment | 1-year old cotyledons | HUMARA <i>et al.</i> (1998) |
| <i>Pinus strobus</i> (white pine) | Regeneration following <i>A. tumefaciens</i> cocultivation | Embryogenic tissue | LEVÉE <i>et al.</i> (1999) |
| <i>P. strobus</i> | Microprojectile transfer of GFP | Pollen, ESM, and somatic embryos | TIAN <i>et al.</i> (1997) |
| <i>P. strobus</i> | Particle transfer of GFP <i>Agrobacterium</i> transformation | 6 w somatic embryos Embryogenic tissues | TIAN <i>et al.</i> (1999) |
| <i>P. strobus</i> | Particle transfer of GFP or GUS | Embryogenic cultures | ZIPF <i>et al.</i> (2001) |
| <i>Pinus sylvestris</i> (Scots pine) | Particle bombardment | Vegetative buds, bud-derived calli and suspension cells | ARONEN <i>et al.</i> (1994) |
| <i>P. sylvestris</i> | Particle bombardment | Buds | ARONEN <i>et al.</i> (1995) |
| <i>P. sylvestris</i> | <i>A. rhizogenes</i> | Fascicular buds | ARONEN <i>et al.</i> (1996) |
| <i>P. sylvestris</i> | <i>A. tumefaciens</i> | Base of stem | ARONEN and HÄGGMAN (1995) |
| <i>P. sylvestris</i> | Particle bombardment | Regenerating cotyledons and embryogenic cultures | HÄGGMAN and ARONEN (1998) |
| <i>P. sylvestris</i> | Particle bombardment prior to pollination in controlled crossings | Pollen lots | ARONEN <i>et al.</i> (2003) |
| <i>P. sylvestris</i> | Induction of hairy and normal roots by <i>A. rhizogenes</i> strains | 2-3 week-old seedlings | MAGNUSSEN <i>et al.</i> (1994) |
| <i>P. sylvestris</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |

| Species | Method of Gene Transfer | Explant/ Tissue | Reference |
|--|---|---|--------------------------------|
| <i>Pinus taeda</i> (loblolly pine) | <i>A. tumefaciens</i> EHA101 | Shoot apex | GOULD <i>et al.</i> (2002) |
| <i>P. taeda</i> | Electroporation | Protoplasts isolated from embryogenic cell cultures | GUPTA <i>et al.</i> (1988) |
| <i>P. taeda</i> | <i>A. tumefaciens</i> | Seedlings | HUANG and TAUER (1994) |
| <i>P. taeda</i> | Microprojectile bombardment | Secondary xylem | LOOPSTRA <i>et al.</i> (1992) |
| <i>P. taeda</i> | Particle bombardment <i>A. tumefaciens</i> | Embryogenic cell suspensions Shoots | NEWTON <i>et al.</i> (1994) |
| <i>P. taeda</i> | <i>Agrobacterium</i> transformation | Callus | SEDEROFF <i>et al.</i> (1986) |
| <i>P. taeda</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>P. taeda</i> | Microprojectile bombardment | Cotyledon cells | STOMP <i>et al.</i> (1991) |
| <i>P. taeda</i> | <i>A. tumefaciens</i> | | TANG (2001) |
| <i>P. taeda</i> | Regeneration after <i>A. tumefaciens</i> | Mature zygotic embryos | TANG <i>et al.</i> (2001) |
| <i>P. taeda</i> | | | TANG and TIAN (2003) |
| <i>P. taeda</i> | <i>A. tumefaciens</i> | Embryogenic suspension-cultured cells | WENCK <i>et al.</i> (1999) |
| <i>Pseudotsuga menziesii</i> (Douglas-fir) | <i>A. tumefaciens</i> transformation, containing derivatives of pTiA6 | Tumors on micropropagated shoots and seedlings | DANDEKAR <i>et al.</i> (1987) |
| <i>P. menziesii</i> | Various strains of <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | 6-month-old seedlings | ELLIS <i>et al.</i> (1989) |
| <i>P. menziesii</i> | Transient expression following biolistic transfer | Cotyledons | GOLDFARB <i>et al.</i> (1991) |
| <i>P. menziesii</i> | Two strains of <i>A. tumefaciens</i> | Micropropagated shoots and seedlings | GUPTA <i>et al.</i> (1989) |
| <i>P. menziesii</i> | <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | Callus | MORRIS <i>et al.</i> (1989) |
| <i>P. menziesii</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>Pinus virginiana</i> | Particle bombardment | Embryogenic cell suspensions | NEWTON <i>et al.</i> (1994) |
| <i>P. virginiana</i> | <i>A. tumefaciens</i> | 2-4 month old seedlings | STOMP <i>et al.</i> (1990) |
| <i>Sequoia sempervirens</i> (redwood) | <i>A. rhizogenes</i> | Microcloned shoot-tips | MIHALJVIC <i>et al.</i> (1999) |
| <i>Tsuga heterophylla</i> (western hemlock) | <i>A. tumefaciens</i> and <i>A. rhizogenes</i> | Callus | MORRIS <i>et al.</i> (1989) |

2.9 Perception of Transgenic Trees: Science divided

The technology of genetically modifying trees has now joined the heated public debate concerning other fields of biotechnology. The scientific community is divided in opinion concerning the extent of potential risks, while more vehement opinions are expressed by activist groups, such as the Forest Stewardship Council (MATHEWS and CAMPBELL 2000) and Greenpeace, determined to prohibit the environmental release of transgenic trees and crops. The activist groups view transgenic trees as a precedent for the uncontrolled release of genetically modified plants in forests (reviewed in TANG and NEWTON 2003). It would be at the research community's own peril to ignore the public debate (MERKLE and DEAN 2000) and considered wise to obtain environmental acceptance of transgenic conifers before field release (TANG and NEWTON 2003).

Forest trees have lagged behind in biotechnological progress in comparison to the agricultural sector as they have traditionally been less valuable to mankind. WRIGHT (1976) stated this to be due to their abundance in natural forests and their long life cycles. Further challenges (briefly mentioned in Section 2.3) that have hampered the progress of molecular biology for forest trees, including genetic engineering, relate to the long rotation time of trees and difficulties with tissue culture and genetic transformation protocols (TZFIRA *et al.* 1998; WALTER *et al.* 1998a). Towards the end of the nineteenth century forest trees were only traditionally bred using artificial selection. As a result the gene pool has changed very little genetically and phenotypically. Rapid global deforestation in response to increased demand for timber products, however, has brought scientific research and commercial attention on the forest gene pool for improved productivity and quality.

MATHEWS and CAMPBELL (2000) outline the three main ethical concerns regarding genetic engineering (GE) of forest trees. The greatest fear is the potentially deleterious impact of genetically modified (GM) crops or trees upon human health, natural and anthropogenic ecosystems that could become apparent only after a build-up effect. A second concern is the monopoly of large profit-

driven companies being the sole providers of forest genetic material. Thirdly, the moral issue surrounding GM, colloquially termed “playing God”.

Potential environmental hazards caused by the introduction of transgenic trees need to be investigated. The risks have been identified as the spread of transgenic trees or their transgenes into surrounding ecosystems, evolution of pest biotypes resistant to transgenes conferring pest toxins and the possibility of new GM by- or end- products of trees exerting a negative impact on the environment (MATHEWS and CAMPBELL 2000). The likelihood of transgene spread and transgenic species reaching weed status depends on the chance of horizontal gene transfer or outcrossing which is further dependent on sexual compatibility and physical proximity of the transgenic stand with other species. As many forest trees are dioecious and wind-pollinated a rigorous management strategy would be required. AHUJA (1997) suggested that ecological niches in natural ecosystems are occupied and not open for transgene invasion in contrast to artificially-vacant ones populated by man. However, this is not the rule as many sensitive natural ecosystems such as the South African Fynbos are not always able to resist invasion from exotics (EWEL *et al.* 1999).

Field assessment of GM impact is not as advanced as the technique itself - most trees are planted on small plots (not representative of potential transgene-environment interactions) that are seldom more than 12 years old. Further concern is that the hybridization of an environmentally safe transgenic with a wild type could produce hazardous traits due to altered gene expression or epigenetic changes (PHILLIPS *et al.* 1994). Engineered sterility or engineering for delayed sexual maturity (harvesting trees before sexual reproduction) could be a solution to this hazard (MATHEWS and CAMPBELL 2000).

Pests developing resistance to toxins inherent in GM trees is a common concern. However, tree-feeding insects unlike agricultural pests are subject to variable exposure patterns, infrequent treatments and incomplete spray deposition, making immunity to pesticides rare. The final concern is the negative impact that gene products could have on the recipient environment through enhanced competitiveness or accumulation of transgenically produced toxins. Some of the

traits (modified lignin content, reproductive sterility and herbicide resistance where no herbicides are used) conferred in GM programmes are unlikely to enhance competitive ability of pests. The least understood aspect of risk-assessment is the adverse effects of novel toxic products on soil-organisms (MATHEWS and CAMPBELL 2000).

Traditionally, high-yielding exotic tree species were introduced to silvicultural systems rather than indigenous species that retained long life cycles and expressed the phenomenon of linkage (where both desirable and undesirable traits were frequently expressed together) (MATHEWS and CAMPBELL 2000). In contrast, modern transformation techniques aim to introduce transgenes to indigenous species, thereby avoiding the possibility of weed status (TZFIRA *et al.* 1998).

Among the advantages of genetic transformation, are the overcoming of interspecific barriers by hybridizing transgenes into the genomes of different species and as well as providing recombination opportunities with synthetic genes designed by humans (NEWTON *et al.* 2001). The speed and accuracy with which better genotypes may be secured, without disturbing existing gene arrangements, are distinct advantages over classical methods of plant breeding. The genetic engineering of conifers benefit the scientific community by providing useful protocols that could provide functions to genes used in the emergence of functional genomics (CHARITY *et al.* 2002). *Arabidopsis* is a widely used plant system but whose qualities (small plant size, rapid generation time, small genome) cannot be practically exploited in conifer transformation systems (BIRCH 1997). For this reason the successful (regenerated) reports of conifer transformations (Table 2.2) could be a start to developing conifer model systems (TANG and NEWTON 2003), and perhaps one day *P. patula* would also be featured in this group.

2.10 Research Objectives

2.10.1 Aims of Study

Pinus patula, one of the South African forestry's most important softwood species for pulp production, has been improved through biotechnological research with the establishment of protocols for somatic embryogenesis and cryopreservation. The focus of this study centred on the inherent ability of this commercially significant species to undergo genetic transformation. Prolific literature exists on transformation studies in the *Pinus* genus. To our knowledge, there are no published reports for gene transfer in *P. patula* in the literature. Thus the aims of this study were to develop a genetic transformation regime for *P. patula*, through the introduction of the pAHC25 plasmid, containing the selectable herbicide resistance *bar* gene and the *uidA* reporter gene.

This project aimed to develop two transformation protocols: using direct particle bombardment of isolated plasmid DNA from *E. coli* (pAHC25), by means of a Genebooster™, and indirect transfer through *Agrobacterium*-mediated infection and co-cultivation with a LBA4404 *Agrobacterium* strain containing the pAHC25 vector.

2.10.2 Transformation approach

Genetically different lines of embryogenic tissue (ESM and mature somatic embryos) and organogenic tissue (mature zygotic embryos) of *Pinus patula* were tested for their ability to undergo gene transfer. *In vitro* propagation of *P. patula* via somatic embryogenesis involves initiation, maintenance, maturation and germination (discussed in Chapter 3). The histological GUS assay detected transient expression in newly transformed tissue. Stable transformants were selected for, by culture on the herbicide BASTA®-supplemented medium before undergoing PCR and Southern hybridization analyses.

CHAPTER 3

IN VITRO PROTOCOLS FOR *PINUS PATULA*

3.1 Introduction

The cloning of herbaceous and woody plants originates from Neolithic Britain, with the coppicing of alders, and more than 1000 years as with *Cryptomeria japonica* (BONGA and ADERKAS 1992). *In vitro* culture of tree species began in 1934 with cambial tissue of *Pinus pinaster* and *Abies alba* amongst the woody species (GAUTHERET 1934). The first gymnosperm callus was established with continuous culture of *Sequoia sempervirens* in 1950 (BALL 1950, cited in BONGA and ADERKAS 1992). A complete *in vitro* cloned conifer was reported in 1975 with the longleaf pine (*Pinus palustris*) (SOMMER *et al.* 1975). Adventitious shoots were produced, excised and subsequently rooted. Coniferous, embryo-like structures were reported soon after in jack pine (*Pinus banksiana*) by DURZAN and CHALUPA (1976).

Protocols in woody micropropagation include: shoot initiation (embryonic and juvenile tissues as explants); shoot multiplication and elongation (careful excision of shoot apices and apical tufts of primary needles); *in vitro* rooting (a limiting factor to whole plant regeneration of woody species, especially conifers); enhancement of axillary bud break (clonal propagation of herbaceous ornamentals, fruit trees and some hardwoods); adventitious budding (production of shoot primordia on tissue such as cotyledons, leaves, stems etc.); protoplast culture (GUPTA *et al.* 1988), with the most commonly used methods being axillary shoot elongation, organogenesis and somatic embryogenesis (BONGA and ADERKAS 1992).

Although *in vitro* regeneration has been achieved in many conifers, propagation from juvenile tissue was initially limited to few species: *Pinus radiata* (AITKEN-CHRISTIE 1984), *Pinus taeda* (MOTT and AMERSON 1984),

Pseudotsuga menziesii (RITCHIE and LONG 1986; BOULAY 1977). Higher production costs of tissue culture, compared to conventional propagation such as cuttings, due to labour intensive work hampered progress. Improved robotic automation of some of the steps can significantly reduce labour costs, which can amount to 60% of the total production cost. This was recently demonstrated in mass propagated liquid Douglas-fir cultures (GUPTA and TIMMIS 2005). Somatic embryogenesis was first achieved in *Pinus patula* in 1992 (JONES *et al.* 1993) with subsequent improvements to the maturation process and establishment of somatic embryos (JONES and VAN STADEN 1995; JONES and VAN STADEN 2001). Other economically important coniferous species from *Pinus* and *Picea* have been produced using somatic embryogenesis or organogenesis (TANG and NEWTON 2005) such as *P. strobus* and *P. virginiana* (KLIMASZEWSKA *et al.* 2000; TANG *et al.* 2004) and *P. abies* (JALONEN and VON ARNOLD 1991).

Organogenesis has been widely used for genotype preservation in clone banks and for clonal seed establishment. Somatic embryogenesis, however, is favoured for its potential for high multiplication rates, delivery via bioreactor and synthetic and seed technologies and is an excellent source of target tissue for gene transfer (MERKLE and DEAN 2000). This system is also able to capture elite individuals and has an amenity to cryopreservation. The terms “embryonal suspensor masses (ESM)”, “filamentous embryos” and “embryogenic tissue” have become the accepted terminology to describe the highly organized structures present in the translucent mass of immature embryos (TAUTORUS *et al.* 1991; ATTREE and FOWKE 1993; STASOLLA and YEUNG 2003).

The work discussed in this Chapter aimed to provide suitable tissue for subsequent transformation experiments (see Chapter 5 and 6) through the somatic embryogenesis and as well as the organogenesis pathways. The specific focus of the ensuing sections was to attempt to induce adventitious budding, not previously reported in *P. patula*, in genetically transformed mature zygotic embryos. The *in vitro* study also aimed to reproduce ESM and mature somatic embryos using the somatic embryogenesis protocol previously described by JONES and VAN STADEN (1995; 2001).

3.2 Materials and Methods

3.2.1 Formulation of MSG-3 Medium and Derivatives

The basal MSG (BECWAR *et al.* 1990) medium is a modification of the medium proposed by MURASHIGE and SKOOG (1962), where the NH_4NO_3 was replaced with L-glutamine, KCl was added and the KNO_3 level reduced. BECWAR *et al.* (1990) formulated a range of variations in the MSG medium supplements resulting in the development of three different media. MSG3 medium is composed of MSG basal medium (Table 3.1) supplemented with 0.1 g l^{-1} myo-inositol; 1.5 g l^{-1} L- glutamine; 2.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA. Selective MSG3 medium was supplemented with filter sterilized BASTA® with the active ingredient at stepwise concentrations of 1 mg l^{-1} and 3 mg l^{-1} glufosinate ammonium. BASTA® is a registered product of AgrEvo South Africa (Pty) Ltd. that is water soluble and contains an active ingredient of glufosinate ammonium at 200 g l^{-1} .

MSG3 medium was prepared from concentrated stock solutions of the relevant inorganic compounds (Table 3.1). The pH of the medium was adjusted to 5.8 using KOH and HCl, prior to the addition of 3 g l^{-1} Gelrite® (Labretoria, Pretoria, South Africa). The carbohydrate component was provided in the form of maltose. A 30% solution of maltose [w/v] was prepared and the pH adjusted to 5.8. The solution was autoclaved separately from the medium to prevent the sugar from caramelizing. An aqueous stock solution of L-glutamine (1.5 g l^{-1}) was filter-sterilized twice (with $0.22 \mu\text{m}$ Millipore aqueous filters) using Sartorius® (Sartorius AG, Germany) filter units, after correcting the pH to 5.8. The sterile L-glutamine was then added to the cooled medium together with the maltose. Approximately 10 ml of medium was poured into 65 mm plastic sterile Petri dishes.

3.2.2 Collection of Plant Material

Green female cones from selected *Pinus patula* families were provided by SAPPI Forests Research in the Natal Midlands ($29^\circ 29' \text{ S } 30^\circ 11' \text{ E}$) for induction of embryogenic tissue. Cones were collected from selected elite families based on

their growth performance in the field. The collection period spanned eight weeks from December 1999 to 21 January 2000. The cones (Figure 3.1 A) were harvested from a 10-year-old clonal seed orchard and were kept in brown paper bags at 4 °C until seeds were placed in culture within four days of collection.

Investigations involving organogenic tissue used open pollinated seeds derived from the Tweedie clonal seed orchards, also provided by SAPPI Forests Research. Seed from two families, AP133 and P42 (coded OP1 and OP2 respectively), which were harvested in 2002, were used in these studies.

Table 3.1: The formulation of MSG3 basal medium for induction and maintenance of *Pinus patula* embryogenic tissue (adapted from BECWAR *et al.* 1990; JONES and VAN STADEN 1995)

| Inorganic Compounds (mg l⁻¹) | |
|--|------------|
| KNO ₃ | 100.0 |
| MgSO ₄ .7H ₂ O | 370.0 |
| KH ₂ PO ₄ | 170.0 |
| CaCl ₂ .2H ₂ O | 440.0 |
| KCl | 745.0 |
| KI | 0.83 |
| H ₃ BO ₃ | 6.2 |
| MnSO ₄ .H ₂ O | 16.9 |
| ZnSO ₄ .7H ₂ O | 8.6 |
| Na ₂ MoO ₄ .2H ₂ O | 0.25 |
| CuSO ₄ .5H ₂ O | 0.025 |
| CoCl ₂ .6H ₂ O | 0.025 |
| FeSO ₄ .7H ₂ O | 27.8 |
| Na ₂ EDTA | 37.3 |
| Vitamins (mg l⁻¹) | |
| Nicotinic acid | 0.5 |
| Pyridoxine-HCl | 0.1 |
| Thiamine-HCl | 0.1 |
| Supplements (g l⁻¹) | |
| Myo-inositol | 0.1 |
| L-glutamine | 1.5 |
| Plant Growth Regulators (mg l⁻¹)¹ | |
| 2,4-D | 2.0 |
| BA | 1.0 |
| Carbohydrate and Gelling Agent (g l⁻¹) | |
| Maltose | 30.0 |
| Gelrite® | 3.0 |
| pH | 5.8 |

¹Concentrations of plant growth regulators were later lowered to a 10% strength

3.2.3 Induction of Embryogenic Tissue

After a brief wash under running water, the green cones were surface decontaminated by immersion into 75% ethanol [v/v] for 4-5 min. They were then transferred to a solution of 1.3% NaOCl [w/v] for 20 min, before being rinsed several times with sterile distilled water. Both solutions contained a few drops of Tween®20 (Merck, Germany), with an active ingredient of polyethylene sorbitan mononaurate, as a surfactant. The seeds were excised aseptically and the seed coats removed. The female gametophytes, containing immature embryos, were placed on MSG3 initiation media (BECWAR *et al.* 1990), a modified MURASHIGE and SKOOG (1962) medium, supplemented with 30 g l⁻¹ sucrose which encouraged induction of semi-translucent ESM (Figure 3.1 B) (JONES *et al.* 1993). Other medium supplements included: 1.5 g l⁻¹ L-glutamine (filter-sterilized twice with 0.22 µm Millipore aqueous filters using Sartorius® filter units prior to addition to the medium); 0.1 g l⁻¹ myo-inositol, 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg l⁻¹ benzyladenine (BA) (Table 3.1). Media were solidified with 3 g l⁻¹ Gelrite® (Labretoria, South Africa). The pH was adjusted to 5.8 with 1 N HCl or 1 N KOH prior to autoclaving at 110 °C, 1.21 kPa for 20 min.

Previous studies have shown that immature seeds are the most responsive explants for induction of embryogenic cultures for *Pinus* species (KRIEBEL and FINER 1990; BECWAR *et al.* 1990; SALAJOVÁ and SALAJ 1992; JONES and VAN STADEN 2001). JONES and VAN STADEN (2001) optimized the protocol for initiation of embryogenic tissue of *P. patula* from excised megagametophyte explants containing immature zygotic embryos (Figure 3.1 B). Induction was achieved using MSG (BECWAR *et al.* 1990) and Douglas-fir Cotyledon Revised, DCR (GUPTA and DURZAN 1985) media with present induction frequencies ranging from 2.6 to 8.5% (FORD *et al.* 2005). After reaching a few millimetres in diameter, the embryogenic tissue was transferred to a solid maintenance medium. Towards the later half of the study maintenance medium contained lower amounts (10% strength) of growth regulators (STASOLLA and YEUNG 2003). Irrespective of the medium (MSG or DCR) on which the embryogenic cultures were initiated, all embryogenic tissue used in this project was maintained on MSG medium, where it

was also tested for its ability to undergo transformation. Tissue was kept in the dark at 25 °C and subcultured every 2 weeks to maintain the ESM in a proliferative state.

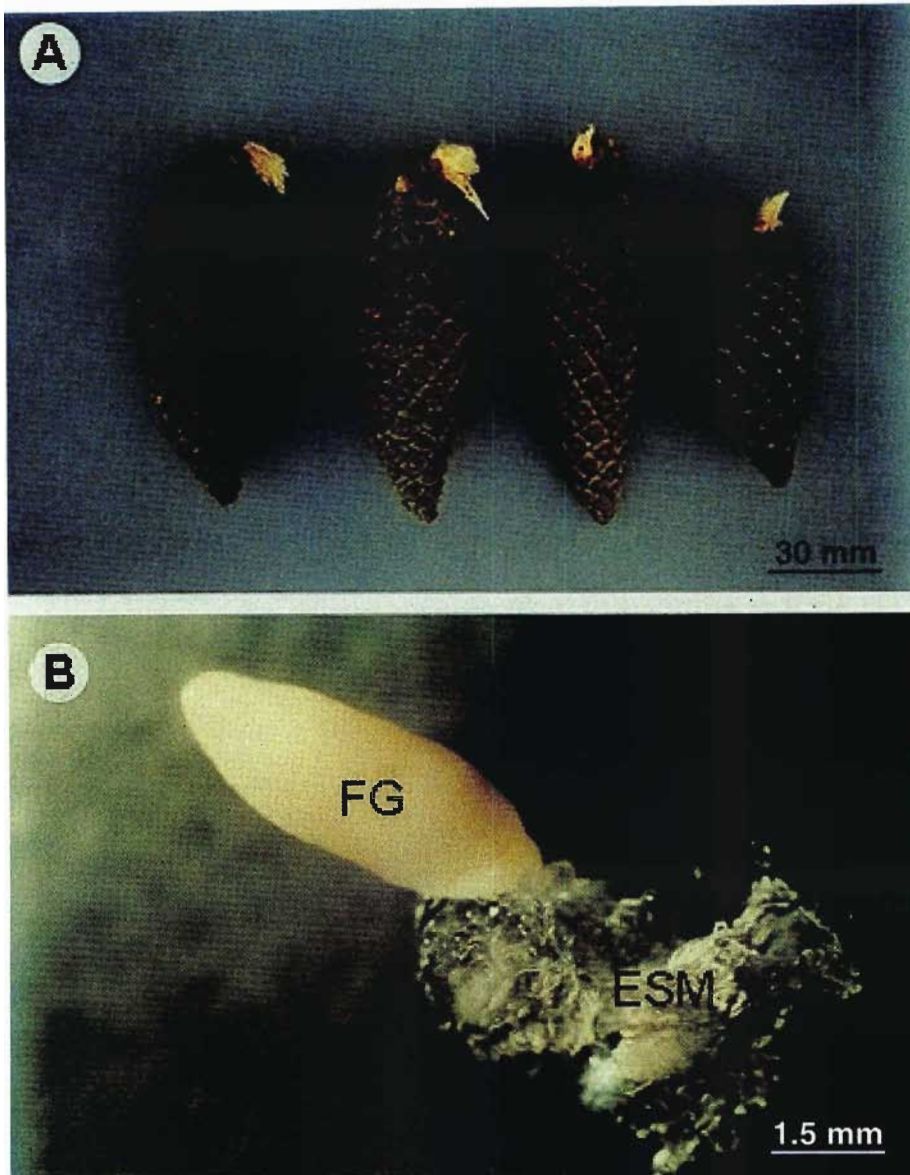


Figure 3.1 Immature green cones and seed of *P. patula*. (A) Phenotypic variation in harvested cones of different families. (B) The female gametophyte (FG), extracted from immature seed, had the seed coat removed and was placed on initiation medium for the proliferation of translucent embryonal suspensor masses (ESM)

Genetic lines in the biolistic study were designated codes (1), (2), (3) and (4). Three lines (1, 2 and 3) were selected for final biolistic transformation experiments on the basis of the relative ease with which they proliferated in culture. Lines 1

and 2 were cryopreserved (FORD *et al.* 2000a; 2000b) and later revived by Sappi Forests Research Laboratory for continued use in the present author's *Agrobacterium*-mediated transformation study (see Chapter 6), with other prolific lines which were also code encrypted: (5), (6) and (7).

All of the remaining lines were initiated into suspension culture, which was also used for bulking up of tissue before transformation experiments. Embryogenic suspension cultures were initiated by inoculating approximately 200 mg of friable tissue into 35 ml liquid MSG3 medium in 100 ml Erlenmeyer flasks. The flasks were stoppered with a sterile cotton wool bung and covered with aluminium foil. The cultures were placed on a rotary shaker at 120 rpm in the dark at 25 °C for 10 d, or until polar embryos were observed.

3.2.4 Formulation of 240 Maturation Medium

The 240 maturation medium (PULLMAN and WEBB 1994) was made up to either contain 5% [w/v], 7.5% [w/v] or no polyethelene glycol (PEG) 8000, using concentrated basal stock solutions (Table 3.2). No PEG was included in maturation media in the last half of the study. The medium was supplemented with 0.1 g l⁻¹ myo-inositol and 0.5 g l⁻¹ casein hydrolysate. The medium pH, prior to autoclaving, was corrected to 5.7 and then solidified with 3 g l⁻¹ Gelrite®. Both the PEG component and the 60% maltose [w/v] solution were prepared separately and the pH adjusted to 5.7 prior to autoclaving.

The L-glutamine (4.5 g l⁻¹) and ABA (10 mg l⁻¹) components of the medium also required separate preparation and pH adjustment. Both these components are thermolabile and therefore required filter sterilization using 0.22 µm Millipore aqueous filters in Sartorius® filter units. After autoclaving, the medium, PEG and maltose solutions were combined. The medium was allowed to cool to 55 °C or below before the addition of the ABA and L-glutamine solutions and then poured into 65 mm sterile plastic Petri dishes.

Table 3.2: The formulation of 240 maturation medium (PULLMAN and WEBB 1994)

| Inorganic Compounds (mg l⁻¹) | |
|--|---------------------|
| NH ₄ NO ₃ | 200 |
| KNO ₃ | 909.9 |
| MgSO ₄ .7H ₂ O | 246.5 |
| KH ₂ PO ₄ | 136.1 |
| Ca(NO ₃) ₂ .4H ₂ O | 236.2 |
| Mg(NO ₃) ₂ .6H ₂ O | 256.5 |
| MgCl ₂ .6H ₂ O | 101.7 |
| KI | 4.15 |
| H ₃ BO ₃ | 15.5 |
| MnSO ₄ .H ₂ O | 10.5 |
| ZnSO ₄ .7H ₂ O | 14.4 |
| Na ₂ MoO ₄ .2H ₂ O | 0.125 |
| CuSO ₄ .5H ₂ O | 0.125 |
| CoCl ₂ .6H ₂ O | 0.125 |
| FeSO ₄ .7H ₂ O | 13.9 |
| Na ₂ EDTA | 18.65 |
| Vitamins, Amino Acids and Growth Regulators (mg l⁻¹) | |
| Thiamine.HCl | 1.0 |
| Pyridoxine.HCl | 0.5 |
| Nicotinic acid | 0.5 |
| Glycine | 2.0 |
| Abscisic Acid | 10 ¹ |
| Supplements (mg l⁻¹) | |
| Casein hydrolysate | 500 |
| Myo-inositol | 100 |
| L-glutamine | 450 |
| Polyethylene glycol MW 8,000 | 50,000 ¹ |
| Carbohydrate and Gelling Agents (g l⁻¹) | |
| Maltose | 60.0 |
| Gelrite® | 3.0 |
| pH | 5.7 |

¹Concentrations of ABA and PEG varied during study

3.2.5 Partial Drying of Mature Somatic Embryos

Mature somatic embryos were harvested once full cotyledons and hypocotyls had developed. The excised embryos were placed into sterile Nunclon® (Nalge Nunc International, USA) 4 well dishes, which were lined with small sterile discs of Whatman No. 1 filter paper. The space around the wells was filled with 3 ml sterile distilled water to allow harvested embryos to dry under high relative humidity. The embryo-containing multiwells were sealed with clingwrap and placed in the dark for 21 d to simulate the process that occurs naturally in seed development.

3.2.6 Germination of Mature Somatic Embryos

A hormone-free medium containing half the amount of maltose (30% [w/v]) was prepared for the desiccated embryos. The 240 medium was supplemented with 0.5% activated charcoal [w/v] and solidified with 2.75 g l⁻¹ Gelrite®. Activated charcoal helps to simulate a darkened environment to encourage germination. It also adsorbs residual plant growth regulators (DODDS and ROBERTS 1985). The mature somatic embryos were placed on the medium surface and incubated in the dark for up to 2 weeks. The appearance of characteristic “embling” (somatic plantlet) morphology with slender hypocotyls and green cotyledons prompted transfer to a hormone-free 240 medium without activated charcoal (Figure 3.6 A). Germination with observed root development was achieved in Magenta vessels (Sigma-Aldrich, Germany) placed in the light (16 h light: 8 h dark photoperiod), using cool white fluorescent light at a PAR of 67.7 μ mol m⁻² s⁻¹ at 25 °C.

3.2.7 Maturation of Bombarded Cultures

For purposes of more efficient bombardment and better maturation of somatic embryos, a process of embryo singulation was included through the use of suspension cultures. Newly-grown ESM from peripheral layers were harvested and placed into liquid MSG3 medium at a concentration of 1 g regenerating tissue/ 10 ml of liquid MSG3 medium or 0.5 g/ 5 ml, which was then filtered onto 10 or 5 plates respectively. This suspension was shaken vigorously. Using a Sartorius® filter, 1 ml was then filtered onto Whatman No. 1 filter paper using cut tips to avoid shearing embryos and placed together with the filter paper onto solid 240 maturation medium (Figure 3.2). Results of regenerated mature somatic embryos are discussed in Section 5.5.3.

A modified maturation medium was employed for the tissue used in the final bombarded experiments due to poor response on the maturation medium employed in earlier maturation protocols. These changes included a double volume of ABA (20 mg l⁻¹) and an increased gelling agent content of 9 g l⁻¹.

3.2.8 Anatomical Studies

In order to determine tissue viability, the stage at which embryos should be transferred to maturation medium and also to monitor morphological differences between the lines studied, anatomical studies were conducted (Figure 3.4 A and B). Samples of suspension and solid cultures were placed onto glass slides, and stained with acetocarmine (0.5%) and Evans blue (0.05%) (GUPTA and DURZAN 1987) before gently covering them with a cover slip. Microscopic observations were recorded photographically using an Olympus BH-2 light microscope (Germany). Tissue integrity was routinely confirmed in this way before bombardment experiments were undertaken.

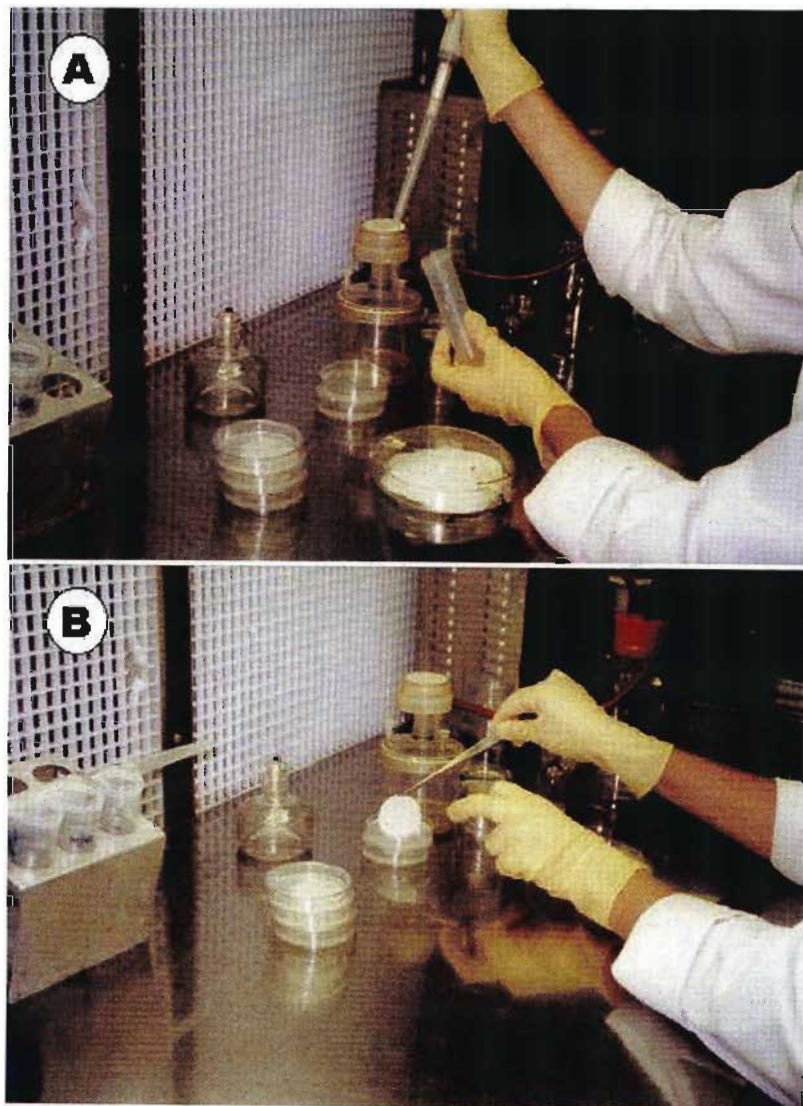


Figure 3.2 Plating out liquid cultures (A) Method of filtering off excess liquid MSG, and **(B)** subsequent transfer of filter paper supporting ESM onto solid medium

3.2.9 Culture Growth Assessments of ESM

Culture growth of four ESM lines, used in biolistic transformations, was determined using the settled cell volume (SCV) technique. One gram of tissue/ 100 ml liquid MSG3 medium was placed in a 500 ml side-arm flask. The flasks were placed on a rotary shaker set at 120 rpm throughout the experiment. Growth data was collected almost daily by suspending flasks so that the arm angle of each flask was perpendicular for a period of 10 min to allow the final settling of cells at the base of the arm. Each experiment consisted of five replicates (which were meaned) per line and the experiments were repeated twice. The data collected were analyzed using the Microsoft Excel 95 computer software to determine the mean and least significant difference at a 95% confidence interval (mean+CI), and represented graphically using Sigma Plot (Figure 3.7). The experiment showing the most representative growth trends for the four lines were presented.

3.2.10 Plant Material and Adventitious Bud Induction

Open pollinated seeds were surface sterilized by immersion into 75% ethanol [v/v] for 4-5 min. They were then transferred to a solution of 1.3% NaOCl [w/v] for 20 min, before being rinsed several times with sterile distilled water. The mature zygotic embryos were dissected aseptically and placed on half-strength Douglas fir cotyledon revised medium ($\frac{1}{2}$ DCR) supplemented with 2% sucrose [w/v], 2.5 μ M N⁶-benzyladenine (BA), 0.025 μ M thidiazuron (TDZ) (MATHUR and NADGAUDA 1999) and 0.5 g l⁻¹ myo-inositol (Table 3.3).

Induction of adventitious budding (organogenesis) using mature zygotic embryos (MZE's) was attempted immediately after excision with selected families: OP2 and OP1, just before the transformation event (Section 6.2.4.5). Newly excised embryos were placed on $\frac{1}{2}$ DCR medium supplemented with 2.5 μ M BA and 0.025 μ M TDZ (Figure 3.3). After a 24h - 36h exposure to BA and TDZ, the MZE's were removed from the hormones and placed on $\frac{1}{2}$ DCR without hormones for co-cultivation immediately after *Agrobacterium* infection.

Table 3.3 Formulation of modified full strength DCR basal media (GUPTA and DURZAN 1985)

| Inorganic Compounds (final concentration mg l⁻¹) | |
|--|------------|
| NH ₄ NO ₃ | 400 |
| KNO ₃ | 340 |
| CaCl ₂ .2H ₂ O | 85 |
| MgSO ₄ .7H ₂ O | 370 |
| KH ₂ PO ₄ | 170 |
| Ca(NO ₃) ₂ .4H ₂ O | 556 |
| KI | 0.83 |
| H ₃ BO ₃ | 6.2 |
| MnSO ₄ .H ₂ O | 22.3 |
| ZnSO ₄ .7H ₂ O | 8.6 |
| Na ₂ MoO ₄ .2H ₂ O | 0.25 |
| CuSO ₄ .5H ₂ O | 0.25 |
| CoCl ₂ .6H ₂ O | 0.025 |
| FeSO ₄ .7H ₂ O | 27.8 |
| Na ₂ EDTA | 37.3 |
| NiCl ₂ | 0.025 |
| Vitamins, Amino Acids and Growth Regulators (mg l⁻¹) | |
| Thiamine.HCl | 1.0 |
| Pyridoxine.HCl | 0.5 |
| Nicotinic acid | 0.5 |
| Glycine | 2.0 |
| Supplements (mg l⁻¹) | |
| Casein hydrolysate | 1000 |
| Myo-inositol | 200 |
| L-glutamine | 450 |
| PVP | 0.2 |
| Carbohydrate and Gelling Agents (g l⁻¹) | |
| Sucrose | 30 |
| Gelrite® | 3.0 |
| pH | 5.8 |

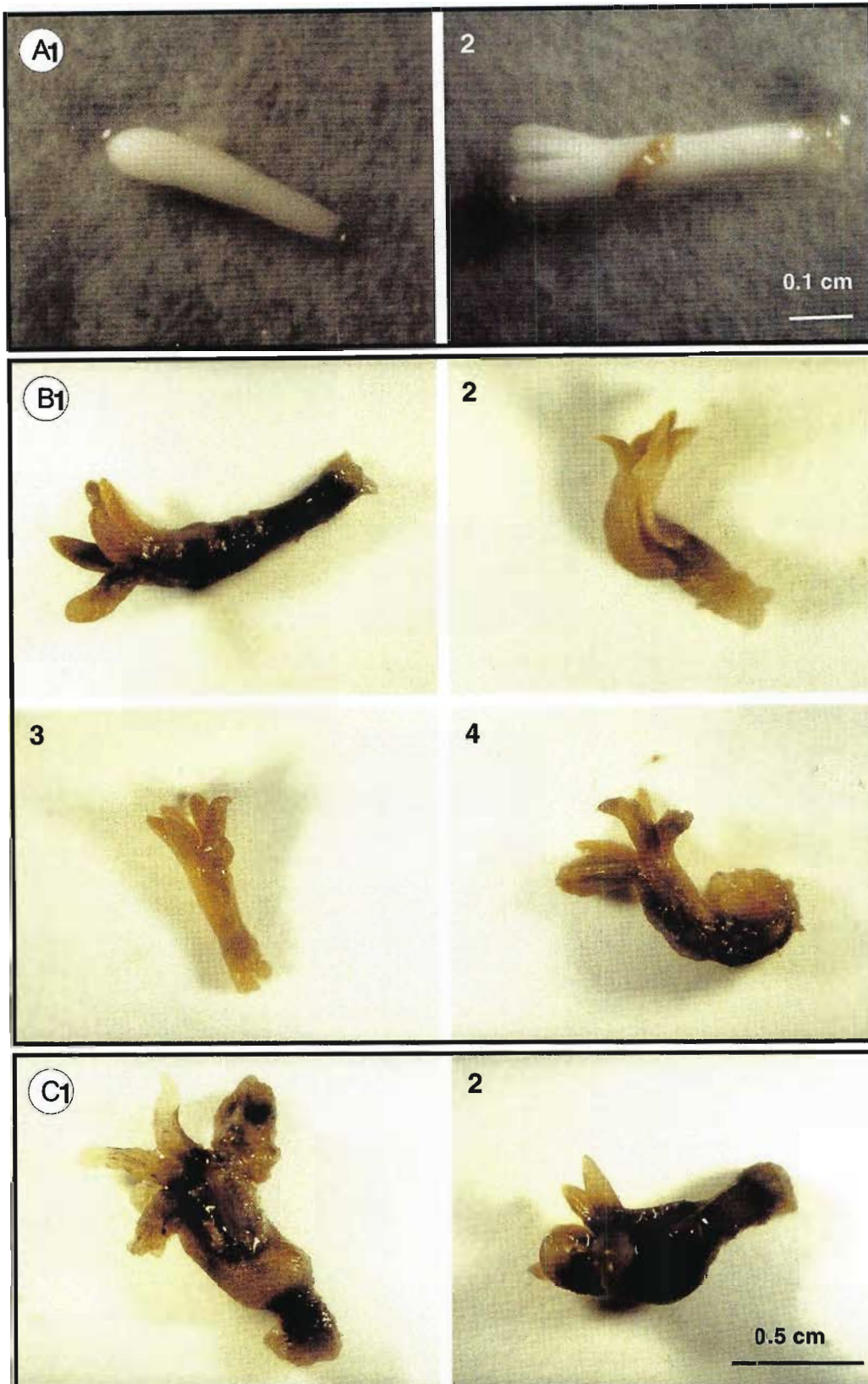


Figure 3.3 Response of mature zygotic embryos (MZE) after placement on hormone-supplemented $\frac{1}{2}$ DCR organogenic medium. **A1-2** Newly excised MZE's **B1-4** organogenic-type responses in MZE. **C1-2** Abnormal development of MZE's showing swollen hypocotyl and splayed cotyledons

3.3 Results and Discussion

3.3.1 Anatomical Studies

Embryogenic tissue staining was used to determine the appearance of the developing embryonal heads and to confirm that cultures contained embryos and not single suspensor cells. The embryonal heads which were densely packed with isodimetric DNA-containing cells, stained red with acetocarmine (SHARMA and SHARMA 1980). The viability of the embryos was screened for by the application of Evans blue stain as the less viable cells permit dye to enter. The histological features of all stages of PEMs (I, II, III) were observed in these routine screenings (not included in results). Morphological analysis indicated that each line exhibited a specific morphology characteristic for that line. RAMAROSANDRATANA *et al.* (2001b) observed the performance of lines with “spiky” and “smooth” tissue morphology on maturation medium. The “spiky” (embryos at the periphery) ESM proliferation was characteristic to Line 1 whereas the prolific lines 2 and 3 demonstrated a cottonwool-like appearance and the slow growing Line 4 had small embryogenic apices (Figure 3.4 A).

3.3.2 Maturation of *Pinus patula*

All lines underwent somatic embryo maturation culminating in the development of cotyledons and defined hypocotyls, which was termed Stage IV. Embryos characteristic of stages described by HAKMAN and VON ARNOLD (1988) developed across all lines.

Dense ESM displayed semi-translucent projections of long, highly vacuolated suspensor cells (Stage I). These structures soon developed into Stage II embryos with a prominent embryonal apex that became opaque with a smooth glossy appearance. Stage III embryos were portrayed by “teeth-like” serrations which developed into cotyledons. In the final stage of maturation (Stage IV), just before harvest, embryos consisted of an elongated hypocotyl with full cotyledonary development. The time taken to reach Stage I was genotype dependent and took

between 2-4 months from the beginning of Stage I through to final Stage IV (Figure 3.5).

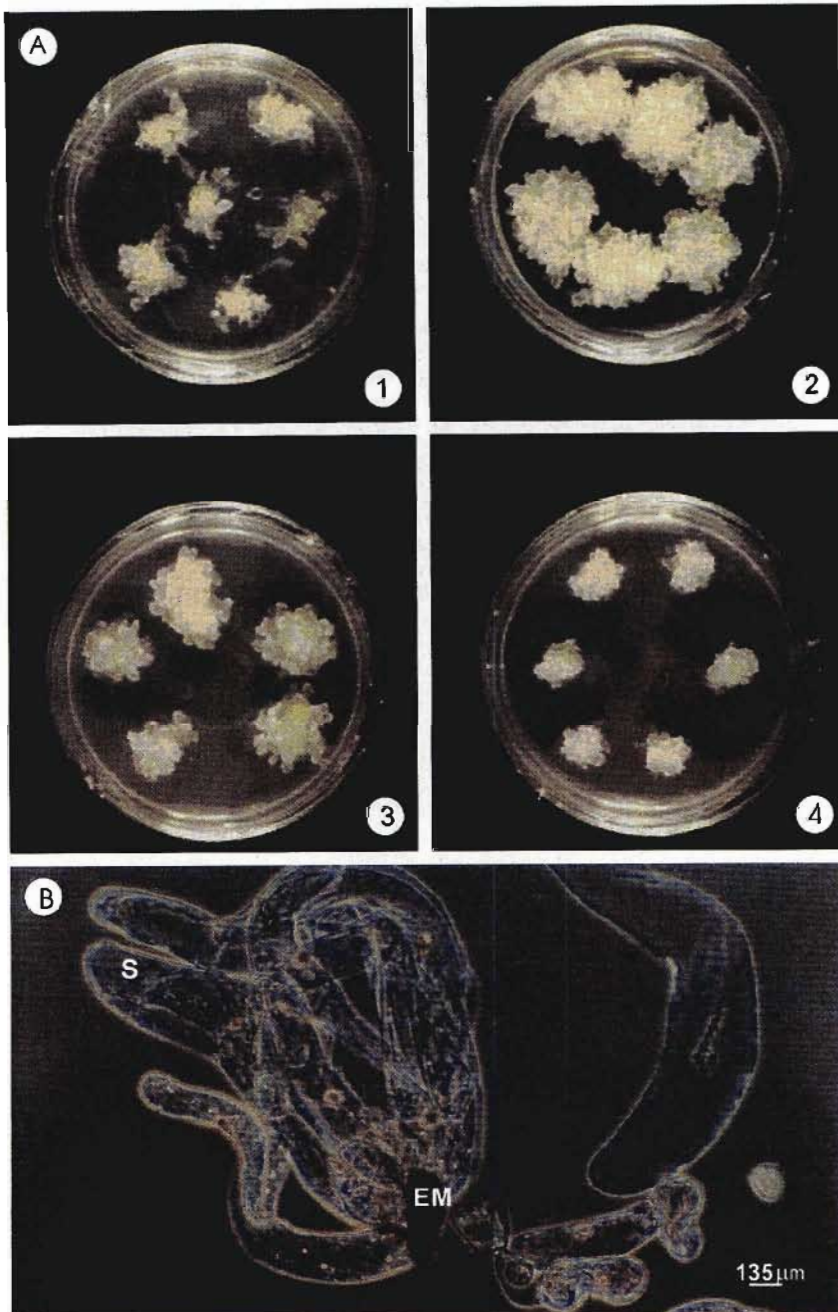


Figure 3.4 Differences observed between lines through morphological (A) and microscopic (B) analysis. (A) Clumps of ESM ten days after subculture: (1)“spiky” Line 1, (2) prolific Line 2, (3) prolific Line 3 and (4) slow growing Line 4. (B) Double staining (with acetocarmine and Evan’s blue) of single embryo possessing embryonal mass (EM) and suspensor (S). Loose suspensor cells on right side of embryonal head

The elongated embryos depicted in Figure 3.6 are from lines 2 and 3. Line 2 continued prolific production of mature somatic embryos after the first harvest. Pronounced differences of somatic embryo maturation yield between different lines and sometimes between Petri dishes (replicates) of the same line, were observed, as is the case in many micropropagation protocols (RAMAROSANDRATANA *et al.* 2001b).

Although all lines produced embryos, the prolific lines (2 and 3) had the highest embryo production and as a consequence had better survival rates as they were the only lines to result in embryo germination (Figure 3.6 A). The germinating embryos did not reach a hardening-off stage, moreover, root development was indicated by the emergence of a radical in some plantlets. Only one geminating embryo from Line 1 survived for a short time. Abnormal embryos were produced by all lines and as a result were not isolated or recorded as these emblings were incapable of normal development during germination, as seen in Figure 3.6 B. Embryo hyperhydricity was a problem encountered with all lines.

PEG was excluded from the maturation protocol due to the better responses (larger sized embryos) to germination, observed in another study (JONES *et al.* 2000). In the same study a greater production of embryos on PEG-containing medium was reported, although germination potential was compromised.

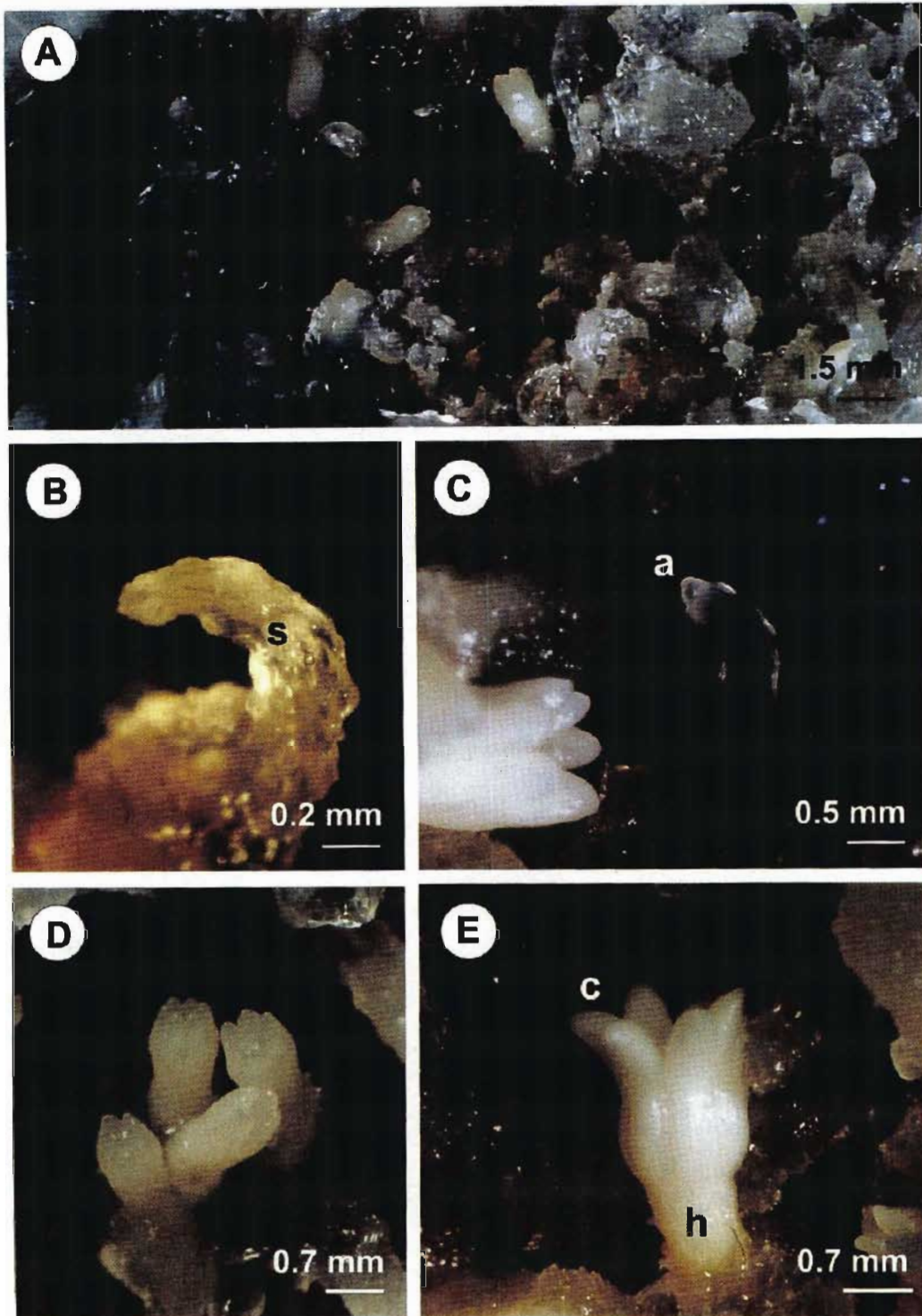


Figure 3.5 (A) Various stages of maturing somatic embryos. (B) Stage I showing the semi-translucent suspensor (s) region. (C) The opaque embryonal apex (a) subtending the semi-translucent suspensor defines Stage II, a late Stage III is visible on left. (D) Early Stage III embryos, showing cotyledonary initials or teethlike projections beginning to develop at the apex. (E) Stage IV: full development and enlargement of cotyledons (c) and hypocotyl elongation (h)

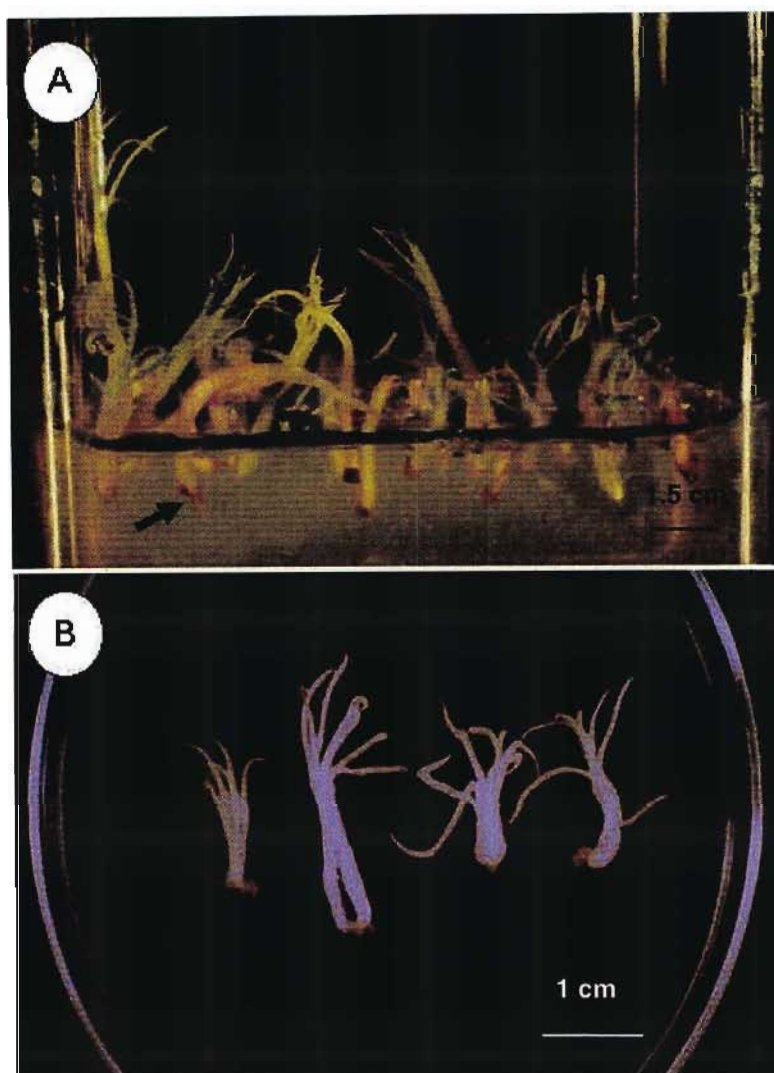


Figure 3.6 (A) Elongated embryos in hormone-free 240 medium to encourage root development. Emergence of radical is indicated by arrow. **(B)** Typical hyperhydric (abnormal) embryos on 240 activated charcoal medium characterized by split and/or short thickened hypocotyls

3.3.3 Organogenesis

Mature zygotic embryos did not exhibit characteristic budding as described by PULIDO *et al.* (1992), but the MZE's did display organogenic-type responses, which resembled budding (Figure 3.3). The budding response ranged from 10–20% in experiments performed with the OP2 and OP1 families (Table 3.4). Other observations of the responses from the MZE's included: (1) no development or (2) swollen hypocotyledons with splayed cotyledons. Detailed results are shown

concomitantly with *Agrobacterium*-mediated transformation experiments in Section 6.3.

Table 3.4 A summary of percentage (%) viability and organogenic-type responses (% budding) observed in mature zygotic embryos (MZE's) after placement on hormone-supplemented ½ DCR medium

| Experiment | Viability (%) | Budding (%) |
|--------------------------|---------------|--------------|
| 1: Family OP2 | 43.64 | 12.73 |
| 2: Family OP1 | 27.27 | 16.36 |
| 3a: Family OP2 | 14 | 10 |
| 3b: Family OP2 (control) | 48 | 22 |
| Mean % | 33.23 | 15.27 |

Trial experiments undertaken with OP1 using 23 (experiment) and 22 (control) MZE's yielded 0% "budding" overall with 0% and 68.18% viability respectively (results not shown). Experiment 3b control refers to the *Agrobacterium* strain used that did not contain the pAHC25 vector (Section 6.3).

This method of explant production was discontinued, as the holistic focus of the study was not to formulate and implement an organogenesis protocol via adventitious budding in *P. patula*, but rather to investigate an optimum method of gene transfer with suitable explants.

3.3.4 Determination of Growth Trends Between Different Lines

The growth of ESM in suspension culture provided an indication of the rate of cleavage polyembryogenesis exhibited by each line (Figure 3.7). None of the four lines showed typical sigmoidal growth, characterized by a lag phase and plateau effect. Despite this, each line displayed a characteristic "end point" for reaching the maximum settled cell volume (SCV) percentage. A lag phase was commonly observed in all experiments, although less pronounced in the more vigorous and prolific lines, 2 and 3. These lines also reached their maximum potential SCV volume in the shortest time suspended in MSG liquid medium.

The exponential burst of growth demonstrated by the liquid cultures has already been mentioned and needs to be emphasized as a point of reference for selection of tissue or lines as target tissue in transformation studies. This phase of active growth due to cleavage polyembryogenesis would be an ideal stage to conduct gene transfer. The prolific lines 2 and 3 reached a point of exponential growth within a week of culture: between 4-8 d for both experiments of line 2 and 5-7 d for line 3. Line, 4, showed consistently poor or slow tissue growth throughout its use in the study as could be seen by an exponential increase in growth between day 16 and 19 in suspension, more than double the time required for the prolific lines. The liquid medium was not replenished during the experiment so perhaps nutrients were limited for this line. It proved unfeasible to obtain sufficient tissue for a repeat of the preliminary growth study: an obstacle that deterred its continued use in further bombardment experiments.

Growth of ESM in liquid cultures responded well and this system was also used to increase ESM material for further transformation experiments (described in Chapters 5 and 6). Fast-growing cell lines with good embryogenic potential are a noteworthy consideration in transformation experiments, as these lines tend to multiply quicker and therefore expedite the transformation procedure. No correlation between embryogenic potential and important breeding traits such as growth and phenology was found in *P. abies* (EKBERG *et al.* 1993; HÖGBERG *et al.* 1998), implying that no unfavourable selection would take place. Significant differences in transient expression have been observed between genotypes and did not correlate with the growth rate or embryogenic potential (DUCHESNE and CHAREST 1991; DUCHESNE *et al.* 1993), but these findings contrasted with tobacco studies by IIDA *et al.* (1991). YIBRAH *et al.* (1994) found that variation in transient expression with time of bombardment after subculture was characteristic for promoter and cell-line. However, pooled results from this study suggested that highest GUS expression was obtained 6 d after subculture. This corresponds to the trend of exponential growth observed in the prolific lines 1, 2 and 3, until 10 d of growth.

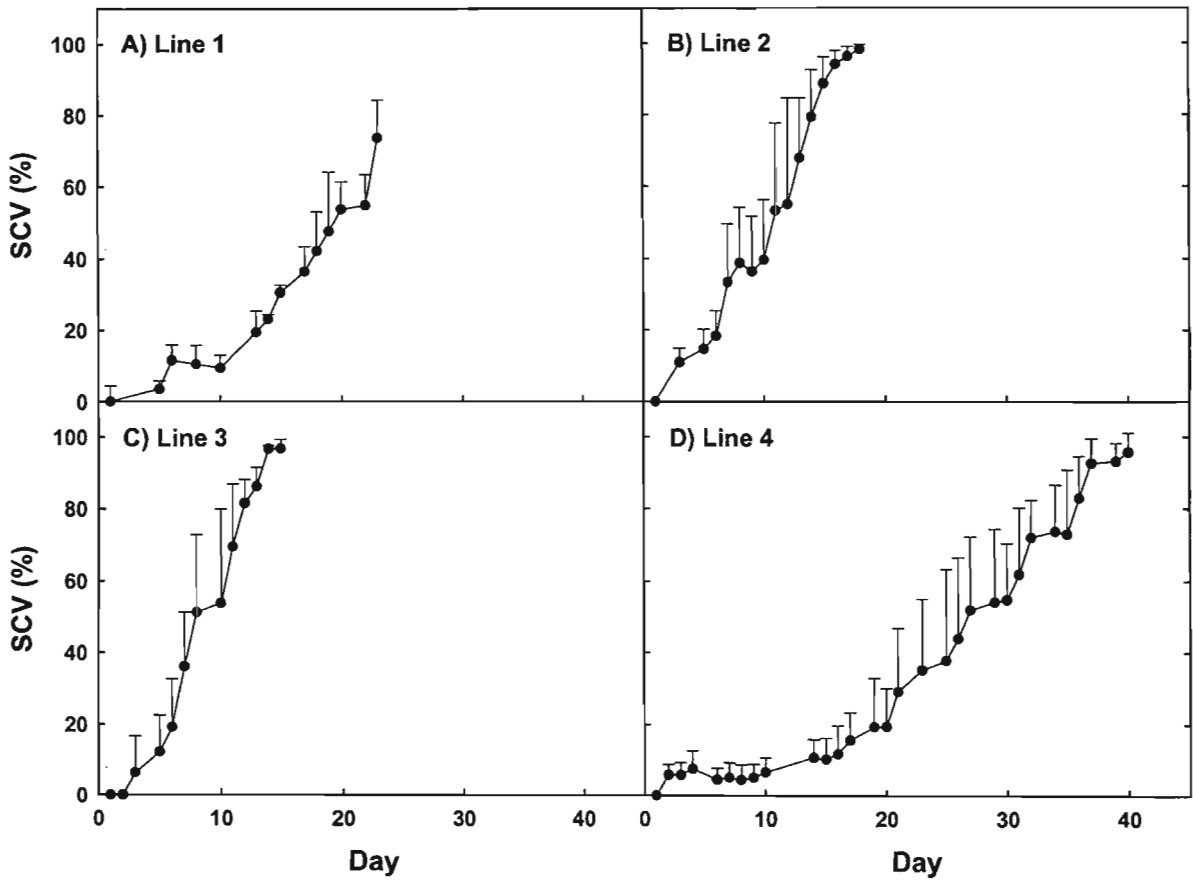


Figure 3.7 A-D Growth of embryogenic suspensor masses shown by four different lines. Bar indicates the least significant difference at the 95% confidence interval (mean+CI)

CHAPTER 4

MOLECULAR BIOLOGICAL TECHNIQUES RELATING TO THE TRANSFORMATION VECTOR

4.1 Introduction

The pAHC25 plasmid construct was one of several plasmids generated by CHRISTENSEN and QUAIL (1996) with the aim of creating high-level gene expression of selectable markers. Efficient monocotyledon transformation would thereby be achieved due to the high activity of the ubiquitin (Ubi-I) promoter in monocotyledons. Ubiquitin is one of the most highly conserved proteins known, with functions in numerous vital cellular processes in organisms such as man, yeast and maize (CHRISTENSEN *et al.* 1992). The pAHC25 construct has been successfully transferred to barley (KOPREK *et al.* 1996; YAO *et al.* 1997), Norway spruce (CLAPHAM *et al.* 2000), wheat (RASCO-GAUNT *et al.* 2001), Brazilian elite Indica-type rice (MUNIZ de PÁDUA *et al.* 2001), chir pine (PARASHARAMI *et al.* 2006), and turmeric (SHIRGURKAR *et al.* 2006), while pAHC25-derived constructs (with the *bar* gene) have been used for other transformations such as switchgrass (RICHARDS *et al.* 2001), *Pinus radiata* and *Picea abies* (BISHOP-HURLEY *et al.* 2001). The maize ubiquitin promoter (CORNEJO *et al.* 1993), strongly expressed in monocotyledonous plants (but not in dicotyledons), showed high histochemical expression in *P. radiata*, *Pinus taeda*, *Pinus elliottii* and *Pseudotsuga menziesii* (WALTER and SMITH 1999). Surprisingly, the constitutive CaMV35 promoter (GUILLEY *et al.* 1982), normally strongly expressed in angiosperms, showed the lowest levels of expression compared with the maize ubiquitin promoter in the same study (WALTER and SMITH 1999).

The pAHC25 vector consists of both the selectable marker *bar* gene (THOMPSON *et al.* 1987), which encodes for phosphinothricin acetyl transferase (DE BLOCK *et al.* 1987) and renders transformants resistant to the herbicide BASTA® within a pUC8 vector (VIERA and MESSING 1982) backbone, and the *uidA* reporter gene

encoding β -glucuronidase (JEFFERSON *et al.* 1987). Each is under the control of a maize Ubi-1 promoter and first intron (CHRISTENSEN *et al.* 1992) terminating in a nopaline synthase gene (*nos*) sequence from *Agrobacterium tumefaciens* (BEVAN *et al.* 1983) at the 3' end. The Ubi-BAR and Ubi-GUS chimaeric genes were assembled separately in pAHC20 and pAHC27 respectively. These genes were then spliced together creating a double construct in the resultant pAHC25 vector, with both genes in the same orientation. The Ubi-BAR chimaeric gene was assembled by ligating a 570 bp *Bam*HI-*Bcl* I fragment containing the *bar* gene into the *Bam*HI site of pAHC17 (CHRISTENSEN and QUAIL 1996). The unique *Hind*III site at the 5' end of the Ubi-I sequence made this construct adaptable for the insertion of a second chimaeric gene such as GUS in pAHC25.

The bialaphos resistance (*bar*) gene of *Streptomyces hygroscopicus* ATCC 21705 (THOMPSON *et al.* 1987) encodes phosphinothricin acetyl transferase (PAT). PAT acetylates the free NH₂ group of phosphinothricin (PPT), preventing autotoxicity in the producing plant (MURAKAMI *et al.* 1986), and renders it inactive against glutamine synthetase (GS) (refer to Section 5.1). As a result, the *bar* gene provides tolerance to PPT-based herbicides such as BASTA[®] and bialaphos (DE BLOCK *et al.* 1987; DENNEHEY *et al.* 1994). The *bar* gene has a 68.3% content of cytosine (C) and guanine (G) bases (VICKERS *et al.* 1996), a percentage higher than most plant genes where the average GC content is 50% (DONN 1991).

The aims of this Chapter were to confirm the integrity of the pAHC25 vector using fundamental molecular biological protocols such as DNA isolation, restriction digestion and subsequent analysis by gel electrophoresis. These techniques were routinely and proficiently executed in transformation experiments performed throughout the study. Characterization of the genes used in plasmid DNA manipulations provides integrative knowledge necessary to reach the experimental aims.

4.2 Reagents

All chemicals used were of the highest quality available. Inorganic salts and HPLC-grade water (High-performance liquid chromatography), HiPerSolv[®], were

obtained from British Drug Houses (BDH, England). Agar, tryptone and yeast extract were purchased from Oxoid (England) while casein acid hydrolysate was obtained from Saarchem (South Africa). Sigma (Germany) was the supplier of the phenol [pH 8]. Roche (Germany) was the supplier for Tris-HCl (2-Amino-2-[hydroxymethyl]1,3-propanediol) and BDH for EDTA (ethylenediamine tetra-acetic acid). The agarose was purchased from Whitehead Scientific (South Africa), or Promega (United States of America). Unless otherwise specified, all enzymes and antibiotics were purchased from Roche Products (Germany).

4.3 Materials and Methods

4.3.1 Transformation of Bacterial Strains with Plasmid DNA

A strain of *Escherichia coli* HB101, which had been cryopreserved at $-70\text{ }^{\circ}\text{C}$, was revived by dilution streaking on Luria Bertani (LB) plates (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 10 g l^{-1} sodium chloride, 10 g l^{-1} agar). The plates were incubated at $37\text{ }^{\circ}\text{C}$ for one day, until the appearance of single colonies. A single colony was used to inoculate LB broth in a sterile Erlenmeyer flask and transferred to a $37\text{ }^{\circ}\text{C}$ rotary shaker. Dr Jénés from the Agricultural Biotech Centre in Gödöllő, Hungary donated a variety of plasmid vectors (pAHC25, pff19GUS, DH5 VIII, pRT103GUS, pD554, pACTIF, pG310), which were used to transform *E. coli* cells. The protocol of AUSUBEL *et al.* (1988) was followed using sterile conditions. Cells were grown overnight or to log phase. The following day, or at log phase, 1.5 ml cells were centrifuged at $10\ 000\text{ rpm}$ for 2 min in a SIGMA 113 desktop microfuge. Pelleted cells were made competent for transformation through the addition of $400\ \mu\text{l}$ 0.1 M MgCl_2 , and centrifuged at $10\ 000\text{ rpm}$ for 2 min and the supernatant decanted. The cells were resuspended in $200\ \mu\text{l}$ 0.1 M CaCl_2 and incubated on ice for 30 min . Aliquots of $0.2\ \mu\text{l}$ and $0.5\ \mu\text{l}$ of each plasmid DNA ($1\ \mu\text{g}\ \mu\text{l}^{-1}$), were added to the competent cells.

After the addition of the plasmid DNA, the cells were heat-shocked for 2 min at $42\text{ }^{\circ}\text{C}$. One ml of pre-warmed LB broth was added to the heat-shocked cells, which were then incubated at $37\text{ }^{\circ}\text{C}$ for 30 min . Transformed cells were plated on LB

agar plates supplemented with $100 \mu\text{g ml}^{-1}$ ampicillin (amp^+), in order to select for *E. coli* plasmids containing ampicillin resistance, and grown overnight. Freezer stocks of the transformed plasmid-containing cells were prepared by adding an equal volume of glycerol solution (65% glycerol [v/v], 0.1 M magnesium sulphate, 0.025 M Tris-chloride [pH 8]) to bacterial solutions in a 1.8 ml cryovial and quick freezing in liquid nitrogen prior to storage at -70°C .

The *Agrobacterium tumefaciens* LBA4404 strain was also transformed in the same manner with the pAHC25 plasmid, as described above, with amendments (Section 6.2.1), in preparation for the transformation of *P. patula* tissue (Chapter 6).

4.3.2 Isolation of pAHC25 Plasmid DNA

A modification of the LI *et al.* (1995) alkaline lysis method was employed. An overnight culture of *E. coli*, containing the pAHC25 vector, was grown at 37°C in LB medium supplemented with 100 mg ml^{-1} filter-sterilized ampicillin. Cultures were agitated on a rotary shaker at approximately 60 rpm for 16-24 h. Aliquots of inoculated cells were transferred to 15 ml Sterilin centrifuge tubes and centrifuged for 5 min at 8287 rpm in a Hettich Universal KT2S centrifuge. Each bacterial pellet was resuspended in 1 ml Buffer I (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA). Bacteria were lysed with two volumes lysate solution (0.2 M NaOH, 1% SDS [w/v]), and kept on ice for 15 min. The addition of 1.5 volumes 5 M potassium acetate, pre-chilled at -20°C , neutralized the bacterial suspension. Tubes were mixed by gentle inversion and placed on ice for 30 min. Cell debris and proteins were removed by centrifugation at 8287 rpm for 10 min; this was repeated using fresh tubes, until all the cellular debris was removed. The DNA suspension was transferred to sterile 15 ml tubes where it was treated with $1 \mu\text{g } \mu\text{l}^{-1}$ DNase-free RNase and incubated for 15 min at 37°C . The supernatant was extracted with an equal volume of buffered phenol [pH 8.0] and briefly vortexed, before a further volume of chloroform: iso-amyl alcohol (24:1 [v/v]) was added in the fumehood to purify the DNA. The aqueous phase was collected after separation by centrifugation at $10\,000 \times g$ for 10 min and placed in a fresh tube. The phenol-chloroform extraction was repeated to remove any visible residual

protein material in the DNA extract at the interface. Plasmid DNA was precipitated by addition of an equal volume of ice-cold iso-propanol and incubated for at least an hour at $-20\text{ }^{\circ}\text{C}$. The DNA was collected by centrifugation at 8287 rpm for 15 min. The DNA pellet was washed with 200 μl 70% ethanol [v/v], to remove remaining salts, and then washed with 100% ethanol [v/v] for precipitation, before open tubes were vacuum- or air-dried on the laminar flow bench. The isolated DNA pellet was resuspended in 50 μl TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA).

4.3.3 Quantification of DNA

DNA was quantified by ultraviolet (UV) absorbance spectrophotometry (VARIAN CARY 50 Conc UV-Visible Spectrophotometer) on the principle that the amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA present in solution. Two μl of plasmid DNA solution was placed in a quartz cuvette containing 0.998 ml of TE buffer [pH 8.0]. The zero value was established by a cuvette containing 1 ml TE buffer.

The following formula was used to establish the DNA concentration (AUSUBEL *et al.* 1988):

$$\frac{\text{Corrected } A_{260} \times \text{dilution factor} \times \Delta E}{1000} = \text{DNA concentration } (\mu\text{g } \mu\text{l}^{-1})$$

Where corrected $A_{260} = A_{260} - A_{320}$ and $\Delta E = 50\text{ } \mu\text{g ml}^{-1}$ double stranded DNA when A_{260} is 1.0.

The sample purity was estimated by calculating the A_{260}/A_{280} ratio with 1.8 reflecting 100% purity. A ratio of greater than 1.8 indicated RNA contamination and less than 1.8 indicated protein and phenol contamination (BROWN 1995).

4.3.4 Electrophoresis

The integrity of isolated plasmids was analyzed on a 1.5% agarose gel [w/v] (0.6 g agarose in 40 ml of 1X TAE Buffer: 0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]). Five μl of dyed loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% glycerol [v/v] and 0.05% bromophenol blue [w/v]) were added to 5 μg of plasmid DNA before loading of the gel. The plasmids were electrophoresed at 80 V at room temperature and stained with 0.5 $\mu\text{g ml}^{-1}$ ethidium bromide (EtBr) stock. Gels were stained by including 2-4 μl EtBr stock to the 40 ml agarose solution or stained in 100 ml 1XTAE with 40 μl EtBr stock for 10 min before destaining in 1XTAE for 10 min. The DNA was visualized by UV illumination of the gel at 312 nm (SPECTROLINE MODEL TC-312A Transilluminator 312 nm Ultraviolet). For size determination of isolated plasmid samples, and/or restriction digests, Molecular Weight Marker III (Roche, Germany), MIII, or Molecular Weight Marker MXIV (Roche), MXIV respectively, was run alongside the plasmid DNA. MIII is a mixture of 13 fragments resulting from cleavage of λ -DNA with *EcoRI* and *HindIII*. MXIV is a 15-fragment mixture prepared through cleavage of a specially constructed Roche plasmid with specific restriction enzymes. Isolated results were initially recorded photographically using a Pentax ME Super with an orange filter and later with the UVItec Gel-Documentation system DOC-008.TFT (England).

4.3.5 Restriction Analysis

Two attempts were made to isolate the *bar* gene from the pAHC25 plasmid. Trial enzyme combinations (Table 4.1), which included an uncut control, were performed according to standard procedures (SAMBROOK *et al.* 1989) in an attempt to restrict the GUS and *bar* gene individually in a 25 μl reaction volume: 5 μg of plasmid DNA was digested with 2.5 units of restriction enzyme, 2.5 μl compatible 10X buffer, 0.1 M spermidine, 2.5 μl bovine serum albumin (BSA) (100 $\mu\text{g ml}^{-1}$), 5 μl RNase (10 $\mu\text{g ml}^{-1}$) and distilled water and incubated for 1.5 h in a 37 °C water bath.

Double (*Bam*HI and *Sac*I) and single digests (*Sac*I) were performed to isolate the GUS gene (Table 4.1). Restriction Buffer L (10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithioerythritol, [pH 7.5 at 37 °C]), as supplied by the manufacturer, was used for both the single and double digests.

Table 4.1: Combinations of restriction reactions performed to characterize single genes

| GENE | Reaction 1 | Reaction 2 | Reaction 3 | Reaction 4 |
|--|--|--|--|---|
| <i>Bar</i> gene (single cut) | <i>Bam</i> HI <i>Kpn</i> I BSA Buffer L | <i>Bam</i> HI <i>Kpn</i> I BSA Buffer L | <i>Bam</i> HI <i>Kpn</i> I BSA Buffer L | <i>Bam</i> HI <i>Kpn</i> I BSA Buffer L Spermidine RNase |
| GUS gene (double cut) | <i>Bam</i> HI <i>Sac</i> I Buffer L | <i>Bam</i> HI <i>Sac</i> I Buffer L | <i>Bam</i> HI <i>Sac</i> I Buffer L | <i>Bam</i> HI <i>Sac</i> I Buffer L Spermidine RNase |
| GUS gene (single cut) | <i>Sac</i> I Buffer L | <i>Sac</i> I Buffer L | <i>Sac</i> I Buffer L | <i>Sac</i> I Buffer L Spermidine RNase |

The *bar* gene was restricted with *Bam*HI and *Kpn*I (Table 4.1), which requires supplementation with 100 µg ml⁻¹ bovine serum albumin (BSA), in the presence of Buffer L. The schematic diagram of the pAHC25 expression vector illustrates the restriction sites available (Figure 4.1).

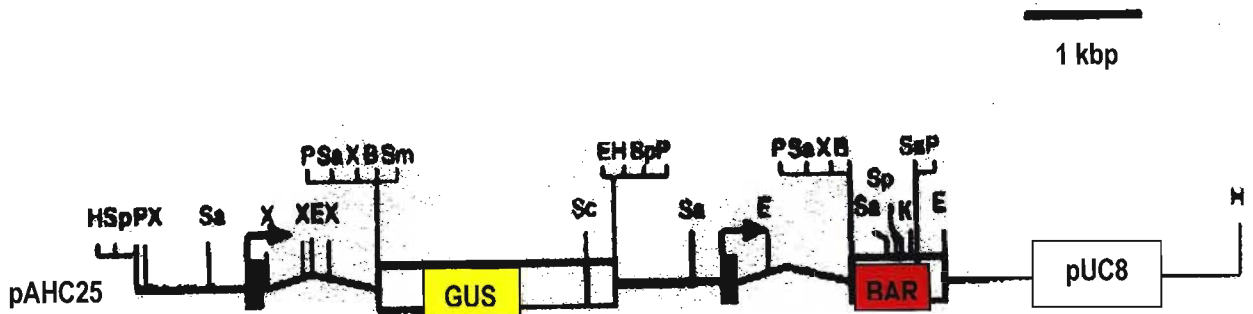


Figure 4.1 Schematic diagram of the pAHC25 expression vector showing restriction sites used in construction of the chimaeric genes and in adjacent polylinker sequences. Bold straight line, *Ubi-1* promoter sequence; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; labelled open boxes, reporter gene sequences; blank open boxes, nopaline syntases 3' untranslated sequence; thin straight line, pUC8 sequence; B, *Bam*HI; E, *Eco*RI (which cuts very inefficiently); H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. Arrow at the *Ubi-1* exon signifies transcription start site and direction (CHRISTENSEN and QUAIL 1996)

The second attempt to isolate the *bar* gene employed use of information provided by the restriction map in Figure 4.2. The restriction of the *bar* gene was performed in a two-step digestion in a 25 μ l reaction volume: 5 or 10 μ g of plasmid DNA was digested with 2.5 units of restriction enzyme, using the relevant restriction buffer, and incubated for 3 - 4 h in a 37 $^{\circ}$ C water bath. Two restriction enzymes were used separately to generate fragments containing the *bar* gene: *Bam*HI incubated with Buffer B (10 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, 1 mM 2-mercaptoethanol [pH 8.0 at 37 $^{\circ}$ C]) and *Xba*I incubated with Buffer H (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithioerythritol [pH 7.5 at 37 $^{\circ}$ C]).

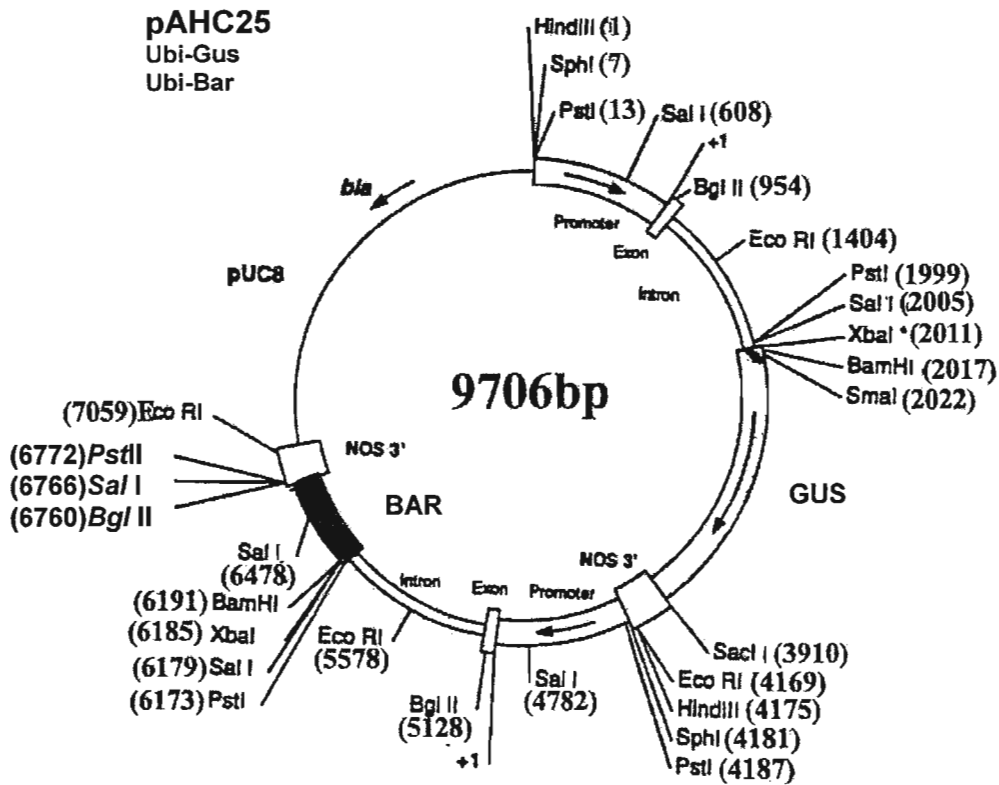


Figure 4.2 A restriction map of pAHC25 detailing restriction sites and position. Additional *XbaI* sites exist in plasmid at 45, 1035, 1390, 1618, 4219, 5209, 5564, 5792 bp (CHRISTENSEN *et al.* 1992)

The reaction products were separated on a 1% agarose gel [w/v] and the 5532 bp fragment (from the *Bam*HI restriction) and the 3566 bp fragment (from the *Xba*I restriction) containing *bar* were extracted from the gel using the GeneClean II® system (Bio101, USA) according to the manufacturer's instructions. To further restrict the *bar* fragment, the purified DNA was restricted with *Bgl*II incubated with Buffer M (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithioerythritol [pH 7.5 at 37 °C]). Following electrophoretic separation, the resultant *bar* fragment of 569 bp was excised from a 2% agarose gel [w/v] and purified as described above. No band was observed from the *Xba*I after the *Bgl*II restriction, although a 575 bp fragment would have been expected.

4.4 Results and Discussion

4.4.1 Transformation of Bacterial Strains with Plasmid DNA

Successful transformation was observed by the growth of bacterial colonies on LB agar plates supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. Many plasmid DNA isolations were needed for bombardment experiments in particular. In this respect the host for the pAHC25 vector needed to be efficient and relatively easy to isolate plasmid DNA from. *Escherichia coli* was therefore chosen as the harbouring host due to the inherent difficulties associated with plasmid preparations from *A. tumefaciens* (LI *et al.* 1995). The vector plasmids and the large (>200kb) Ti plasmids are both maintained at a low copy number, and mini-preparations can yield both plasmid types making characterization analysis through restriction digestion difficult (LI *et al.* 1995).

4.4.2 Isolation and Analysis of DNA

Intact plasmid DNA was successfully isolated using a modified method of LI *et al.* (1995). Plasmid sizes were determined after separation on a 1.5% agarose [w/v] gel. All plasmids co-migrated with the 21.2 kb marker band (Figure 4.4 A). The exact size of pAHC25 is 9706 bp but was not distinguishable from the largest Mill fragment as a 1.5% agarose gel is only able to effectively separate linear DNA molecules in the range 0.2-3 kb (AUSUBEL *et al.* 1988). As this plasmid contained the *bar* gene it was used as the exogenous DNA in microprojectile bombardment experiments. High quality, intact DNA is essential for a successful and high transformation efficiency. The inherent ability of DNA to be coated onto microparticles is influenced by the purity of the DNA. Protein contamination in particular limits the efficient use of DNA and is the principal cause of microparticle agglomeration, therefore additional phenol extraction steps are recommended for DNA used in biolistic experiments (SANFORD *et al.* 1993).

4.4.3 Confirmation of pAHC25 Integrity

Structure of the pAHC25 plasmid was confirmed by double restriction analysis. DNA samples contaminated by RNA were treated with RNase (intact RNA inhibits restriction endonucleases), though digested RNA generally does not interfere with restriction analysis and need not be removed (DAVIS *et al.* 1980). Uncut plasmid DNA yielded a band co-migrating with 21.2 kb marker band (lane 2 of Figure 4.3 A). Bands were produced when digested with *Bam*HI and *Xba*I digestion enzymes. The *Bam*HI restriction yielded bands of 5532 bp and 4174 bp seen in Figure 4.3 A (lane 3), the larger fragment containing *bar*. Eight bands were visualized after *Xba*I cleavage (lane 4), the *bar* gene was within the 3566 bp restriction product. However, some *Xba*I restriction sites are *dam* methylated and therefore the true fragment size would not be clear (parveez@mpob.gov.my).

The second restriction resolved the cleavage of the *bar* fragment (Figure 4.3 B) that was yielded from the *Bam*HI digestion product excised with *Bgl*II. No *bar*-containing product was seen from the *Xba*I digestion products cleaved with *Bgl*II, possibly as a smaller quantity (perhaps less than 2 µg template DNA) was loaded for the reaction.

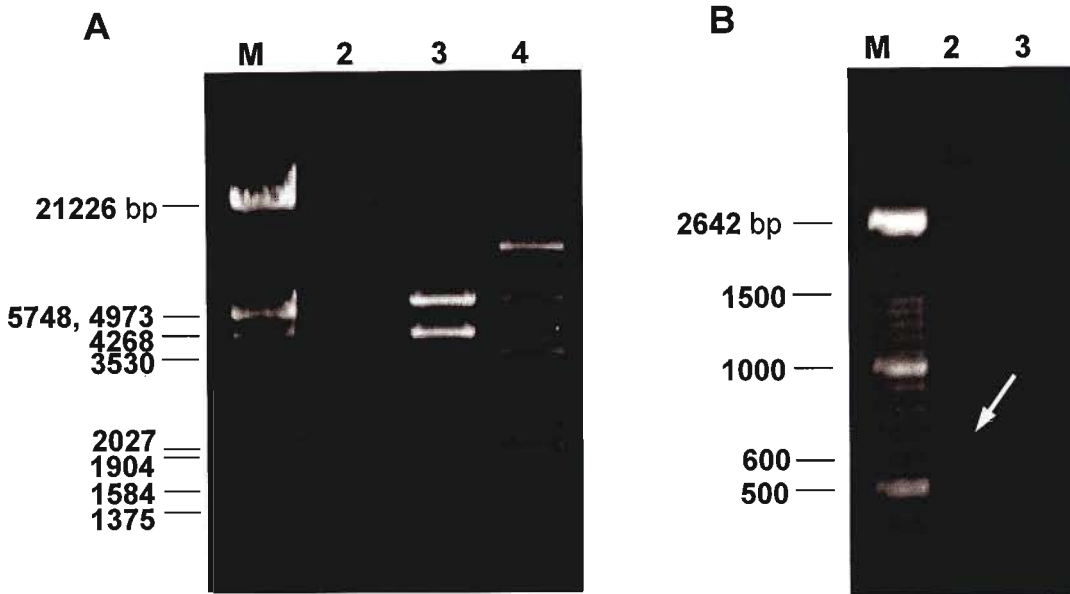


Figure 4.3 Confirmation of the pAHC25 structure by restriction analysis. (A) Restriction of uncut pAHC25 with *Bam*HI and *Xba*I digestion enzymes respectively and viewed on a 1% gel [w/v]. The DNA contents of the lanes are: lane M – Molecular weight marker MIII (Roche); lane 2 - 5 μ g uncut plasmid DNA; lane 3 - 5 μ g *Bam*HI-digested pAHC25; lane 4 - 5 μ g *Xba*I-digested pAHC25. (B) Appearance of restricted *bar* at 569 bp (arrow) on a 2% gel [w/v], after excision and purification of *bar*-containing fragments as seen in (A). The lane contents are: lane M – MXIV (Roche); lane 2 - 14 μ l purified DNA cleaved with *Bgl*II from *Bam*HI digestion; lane 3 - 14 μ l purified DNA cleaved with *Bgl*II from *Xba*I digestion

Characterization of the pAHC25 structure yielded the expected gene fragments according to the restriction map followed (Figure 4.2). Further sequence analysis would demonstrate these conclusions with greater accuracy. It is pertinent to note that the GUS and *bar* genes are not linked, a factor that could affect the percentage regeneration if target material only received the GUS gene, and could be selected against using BASTA[®]-containing selective media.

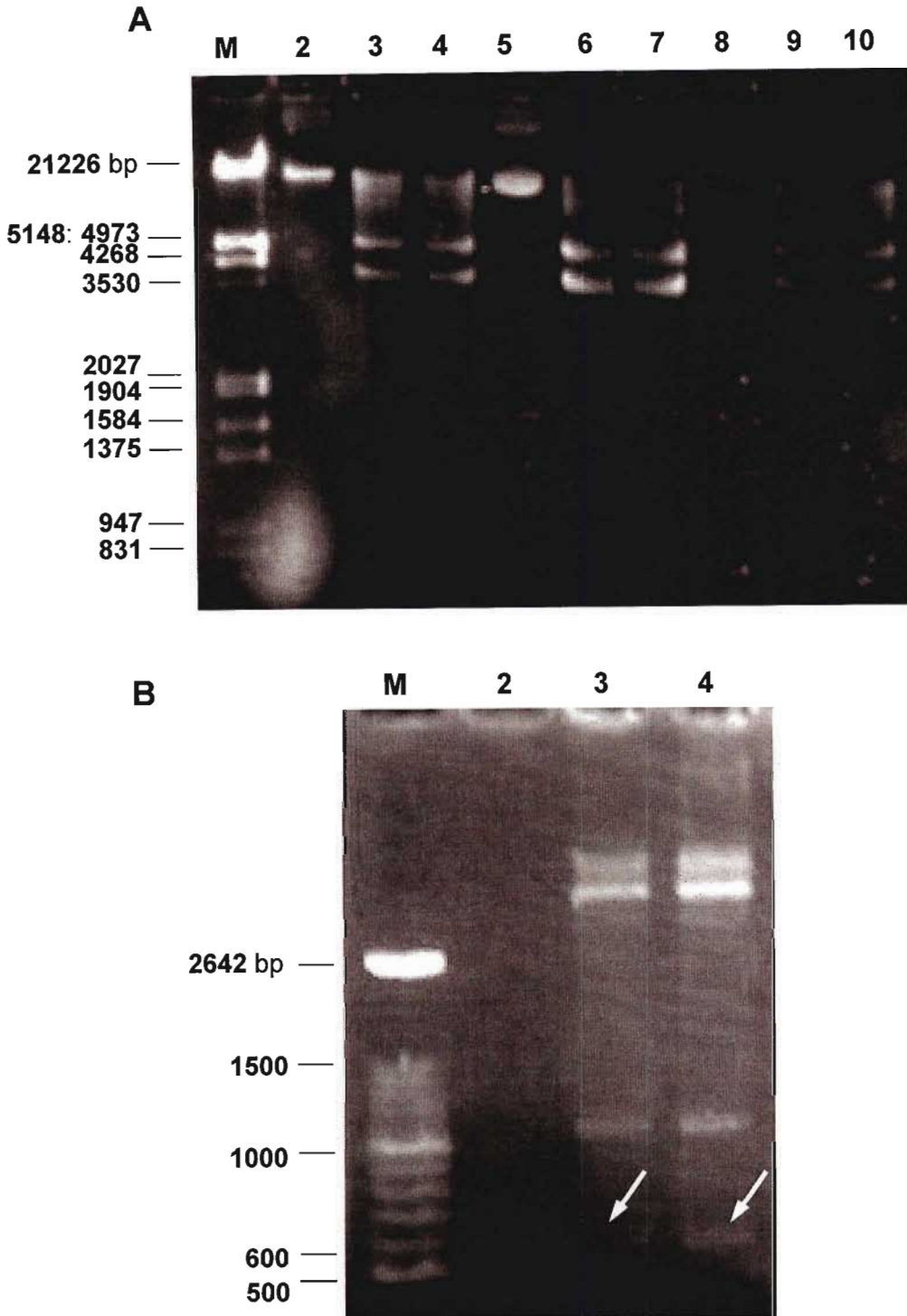


Figure 4.4 Optimized double restriction digestion of pAHC25 to obtain *bar* probe. (A) Contents of lanes are: lane M - MIII (Roche); lanes 2, 5, 8 - three uncut DNA samples; rest of lanes (3, 4, 6, 7, 9, 10) - samples cut by the *Bam*HI restriction enzyme. (B) Contents of lanes are lane M - MXIV (Roche); lanes 3 and 4 - restriction of purified *bar*-containing fragments by *Bgl*II yielded the *bar* gene of approximately 569 bp (indicated by arrows)

The generation of the *bar* probe (Section 4.3.5) was successfully achieved through an optimized double restriction digestion before each Southern transfer. Digestion of plasmid pAHC25 with *Bam*HI yielded two bands at 4973 bp and 3530 bp (Figure 4.4 A). Purified *bar*-containing fragments yielded the *bar* gene at 569 bp when cleaved by *Bgl*II (Figure 4.4 B).

It was important that the basic molecular biology principles were mastered before their regular conjunct use with the transformation protocols and subsequent analyses. Biolistic transformations, described in the following Chapter 5, require numerous isolations of pure plasmid DNA.

CHAPTER 5

BIOLISTIC TRANSFORMATION OF *PINUS PATULA*¹

5.1 Introduction

Two main systems of selection are used during transformation: positive and negative. As the names suggest, negative selection kills cells that do not have the introduced gene of interest and normally include use of antibiotic- and herbicide-based selection. Positive selection provides an environment that gives transformed cells the capacity to grow using specific nitrogen, carbon or growth regulators as the selective agents. Public concern over the presence of antibiotic resistance genes in transgenic plants has prompted the European Union to ban the use of antibiotic resistance genes for selection of plant cells (LAFAYETTE *et al.* 2005). Reports relating to antibiotic toxicity and genome-wide alterations in DNA methylation due to the presence of antibiotics, are incentive to research alternative selection strategies (LAFAYETTE and PARROTT 2001; LAFAYETTE *et al.* 2005).

Herbicides have acquired an established niche in modern agriculture, though the development of effective yet environmentally safe herbicides is continually revised. Most herbicides do not discriminate between crops and weeds and therefore it is necessary to modify plants to become herbicide-resistant for selective use of herbicides for crop protection (DE BLOCK *et al.* 1987), particularly as there are no selective herbicides available (BISHOP-HURLEY *et al.* 2001). Herbicide resistance in forestry has the potential to improve cost efficiency and management

¹ The work described in this chapter led to the publication of: NIGRO SA, MAKUNGA NP, JONES NB, VAN STADEN J (2004) A biolistic approach towards *Pinus patula* Scheide et Deppe embryonal suspensor masses (ESM). *Plant Growth Regulation* 44: 187-197; and FORD CS, FISHER LJ, JONES NB, NIGRO SA, MAKUNGA NP, VAN STADEN J (2005) Somatic embryogenesis in *Pinus patula*. In: SM JAIN and P GUPTA (Eds) Protocol for Somatic Embryogenesis in- Woody Plants. Springer, Dordrecht, The Netherlands. Pp 121-139. ISBN - 10 1-4020-2984-5.

skills especially in intensively managed forests. The use of post-emergence herbicides can also be implemented in a simpler and more cost efficient fashion with herbicide tolerant planting stock or to distinguish between re-growth of old stock and newly planted, genetically engineered, superior stock (BISHOP-HURLEY *et al.* 2001).

BASTA® is one of the most commonly used non-selective herbicides for the control of weeds, especially during forage grass establishment when weeds compete for space, light, water and nutrients (SHU *et al.* 2005). It is reported to have a short half-life in soil as it is quickly degraded by microbial activity (GÖTZ *et al.* 1983). Phosphinothricin (PPT), the active ingredient of BASTA®, is a potent inhibitor of glutamine synthetase (GS) (LEASON *et al.* 1982). This enzyme assimilates ammonia and regulates nitrogen metabolism in plants, rendering it essential for the metabolism of ammonia: a natural byproduct of nitrate reduction, photorespiration and amino acid catabolism (MIFLIN and LEA 1977). Glutamine synthetase is the only plant enzyme that can detoxify released ammonia from nitrate reduction, amino acid degradation and photorespiration (DE BLOCK *et al.* 1987). Inhibited action of GS disrupts photosynthesis and leads to a build-up of ammonia, ultimately causing the plant to die (TACHIBANA *et al.* 1986). PPT is chemically synthesized as BASTA® (Hoechst AG, Germany), while bialaphos is obtained through *Streptomyces hygroscopicus* fermentation (DE BLOCK *et al.* 1987). BASTA® is a commercial formulation of glufosinate, which is the ammonium salt of PPT (FROMM *et al.* 1990).

Poplar was the first softwood to demonstrate conferred resistance to glyphosate (FILLATTI *et al.* 1987), while BISHOP-HURLEY *et al.* (2001) were the first to transfer an herbicide resistance gene, *bar*, into conifers. The BASTA® resistant gene (*bar*) is widely used in transformation research ranging from sorghum (ZHAO *et al.* 2000), maize (DENNEHEY *et al.* 1994), Brazilian sugarcane (FALCO *et al.* 2000), Chinese leymus grass (SHU *et al.* 2005) and the conifers Norway spruce (BISHOP-HURLEY *et al.* 2001), radiata pine (BISHOP-HURLEY *et al.* 2001; CHARITY *et al.* 2005) and chir pine (PARASHARAMI *et al.* 2006). This array of species used makes this gene more readily available than the patented 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene resistant to

Roundup® (STEINRUCKEN and AMRHEIN 1980), a commonly used herbicide in commercial forestry.

The efficiency of transformation can be estimated by the survival rate observed through selection. This can be improved by noting the growth phase of cells and, the concentration of osmoticum amongst other factors (previously detailed in Section 2.4).

5.2 Influence of Osmoticum

Optimal bombardment conditions vary according to cell preparation methods to make the target tissue more receptive to gene transfer. This includes the use of cells at the proper phase of growth (ARMALEO *et al.* 1990) or at the proper density (FINER *et al.* 1992), as well as the type and quality of target cells and tissues, and media requirements for somatic embryogenesis (VAIN *et al.* 1993). Optimization of physical bombardment parameters (KLEIN *et al.* 1988; ARAGAO *et al.* 1993) such as distance from stopping plate, retaining screen and modification to the actual bombardment device (WILLIAMS *et al.* 1991; SAUTTER *et al.* 1991; FINER *et al.* 1992) ensured earlier successful transformation reports.

Osmotic treatment was first shown to have a positive effect on transient expression and recovery of stable transformants after particle bombardment of micro-organisms (ARMALEO *et al.* 1990; SHARK *et al.* 1991; SANFORD *et al.* 1993) and non-embryogenic plant cells (RUSSEL *et al.* 1992a) using mannitol- and sorbitol-supplemented media. Further studies have included exposure of cell suspensions to osmoticum treatment in tobacco (YE *et al.* 1990; RUSSEL *et al.* 1992a), scutellar wheat calli (PERL *et al.* 1992), *Zea mays* (VAIN *et al.* 1993) and *Picea glauca* (LI *et al.* 1994). IGLESIAS *et al.* (1994) used immature somatic embryos from *Triticum aestivum*. PERL *et al.* (1992) observed a higher survival rate in transformed maize cells treated with mannitol, possibly preventing leakage after bombardment.

VAIN *et al.* (1993) placed maize suspension cells 4 h prior to and 16 h after bombardment on medium supplemented with 0.2 M sorbitol and 0.2 M mannitol.

This resulted in a 2.7-fold increase in transient GUS expression, and a 6.8-fold recovery of stably transformed maize clones. Osmotic enhancement was suggested to have resulted from plasmolysis of the cells that may have reduced cell damage by preventing or making protoplasm extrusion less likely from bombarded cells (VAIN *et al.* 1993; LI *et al.* 1994) and may have improved particle penetration itself (SANFORD *et al.* 1993). RUSSEL *et al.* (1992a) postulated that cells (suspension-cultured tobacco cells) exposed to certain sugars are hardened by mechanisms resembling those of cold acclimation (CLAPHAM *et al.* 1995). According to ATTREE and FOWKE (1993) an osmotic stress can be applied by either permeating or non-permeating solutes. Low molecular weight compounds such as sucrose and sorbitol are permeating solutes, which cross the cell wall causing plasmolysis as water is withdrawn from the protoplast. However, prolonged incubation results in absorption of the osmoticum into the plant cell vacuole and subsequent osmotic recovery or deplasmolysis through adjustment of the tissue osmotic potential. The consequences of this are: water content is not decreased and direct or indirect metabolic effects on specific plant metabolites occur due to solute toxic effects or its utilization (ATTREE and FOWKE 1993).

Many transformation studies have since been executed and several authors have used the previously described work as a platform. CLAPHAM *et al.* (1995) maintained embryogenic suspension cultures (1-3 h pre-treatment and 24 h post-bombardment) of *Picea abies* on 0.25 M myo-inositol which is reported to favour growth from conifer protoplasts (ATTREE *et al.* 1989; EGERTSDOTTER and VON ARNOLD 1993). WALTER *et al.* (1994) plated *Pinus radiata* embryogenic tissue onto medium supplemented with 0.25 M sorbitol or mannitol and left the culture overnight in an unsealed Petri dish in the laminar flow hood. In stable transformation experiments, WALTER *et al.* (1998b) used 0.25 M sorbitol and left Petri dishes closed overnight in the laminar airflow hood, before bombardments.

5.3 Biolistic Transfer and its Use in Conifers

Particle bombardment offers a universal technique of gene transfer for use in all species, particularly those that exhibited recalcitrance to earlier methods of transformation (BATTY and EVANS 1992), increasing the potential range of host

species to undergo transformation. In early experiments biolistic transfer was the only method that had the capacity to target intact explants for transformation experiments (CHRISTOU 1996). Basic plant development could be monitored using chromogenic markers in regenerated plants. GRAY and FINER (1993) described several advantages over *Agrobacterium*-mediated transformation. Transformation protocols were simplified due to the elimination of complex, specific bacteria: plant relationships. Monocotyledons which were initially recalcitrant to *Agrobacterium* were more easily transformed and escapes (false positives) resulting from growth of *Agrobacterium* in host tissue were eliminated. Co-transformation (transfer of several plasmids) could also be conducted instead of using large plasmid sizes thereby excluding DNA sequences essential for T-DNA replication and *Agrobacterium* transfer in plasmid construction. This has been shown by HADI *et al.* (1996) who transferred 12 plasmids into embryogenic tissue of soybean and BISHOP-HURLEY *et al.* (2001) who co-transformed embryogenic tissue of *P. abies* and *P. radiata* with two plasmids. Complications of using fragile protoplasts and host-range limitations associated with *Agrobacterium*-mediated transfer can also be avoided with biolistic transfer (HADI *et al.* 1996).

Although particle transfer has been the most commonly used method of transfer in conifers (comprehensively listed in Table 2.2), a low transformation frequency abounds. Conifers that have regenerated plants after transformation by microparticle bombardment are *Picea glauca* (ELLIS *et al.* 1993), *Picea mariana* (CHAREST *et al.* 1996), *Pinus radiata* (WALTER *et al.* 1998b) and more recently *Pinus roxburghii* (PARASHARAMI *et al.* 2006). Biolistic transformations have, in contrast to *Agrobacterium*-mediated transformations, resulted in fragmented or multicopy integration events of the transgene at single loci (FINNEGAN and McELROY 1994; MEYER 1995; MATZKE and MATZKE 1995; WALTER *et al.* 1998b), which may lead to gene silencing (KUMPATLA *et al.* 1997).

The objective of the ensuing work was to introduce new genetic material to *Pinus patula* using a direct biolistic method. The intended transfer vector was the pAHC25 plasmid that contains the herbicide resistance *bar* gene and the *uidA* (GUS) gene for use with solid- and liquid-derived ESM, mature somatic embryos and germinating embryos.

5.4 Materials and Methods

5.4.1 Determination of BASTA® Concentration for Selection

Aliquots of 1.5 ml of non-transformed *P. patula* (Lines 2 and 3) embryogenic suspension culture were pipetted onto MSG3 medium supplemented with a range of concentrations of BASTA® herbicide AgrEvo South Africa (Pty) Ltd to determine the minimum concentration capable of inhibiting growth after two subcultures. BASTA® is water-soluble and contains an active ingredient of glufosinate ammonium at 200 g l⁻¹. The range tested included: 0, 1, 2, 3, 4, 5, 10, 50 and 100 mg l⁻¹ glufosinate ammonium (Figure 5.1). Ten replicates (Petri dishes) per selection concentration were employed for both genotypes. Later, once the growth inhibition concentration of BASTA® was established, Line 5 (also used for *Agrobacterium*-mediated transformations, see Chapter 6) was tested for its tolerance to BASTA® by subculturing ESM tissue onto 0, 1 and 3 mg l⁻¹ glufosinate ammonium (3 replicates per selection). A similar “kill-curve” experiment was conducted for two subcultures of Line 2 on 240 maturation medium containing various levels of polyethelene glycol (PEG) at 0, 5 and 7.5% [w/v], to test the effect of selection, if any, on maturing somatic embryos (Table 5.2). Five replicates per media type were used. Initial experiments employed selective 240 maturation medium, charcoal-supplemented 240 medium and hormone-free germination medium. Assessment of tissue development was conducted and the final Stage (I-IV) of maturation reached was recorded.

5.4.2 Pre-bombardment Preparation

The laminar flow bench, as well as the interior and exterior of the Genebooster™ (ELAK Ltd, Hungary), was sprayed and wiped with 70% alcohol [v/v] at least 15 min prior to bombardment. Macroparticles were stored in 100% ethanol [v/v] overnight, placed onto an autoclaved Petri dish and left to air dry.

5.4.3 Treatment of Target Tissue

Preliminary bombardment experiments tested various pre-treatment conditions for ESM for Lines 1-4 (Table 5.1), in an effort to enhance transformation efficiency and recover bombarded tissue.

Table 5.1: Summary of treatments applied to preliminary bombardment experiments using solid and liquid-derived cultures.

| Day | Treatment | All lines | |
|-----|--------------------|-----------|--------|
| | | Solid | Liquid |
| 0 | Unsealed overnight | - | √ |
| | Untreated | √ | √ |
| | Sorbitol | - | √ |
| | Sorbitol-Mannitol | - | √ |
| | Myo-inositol | - | - |
| 5 | Unsealed overnight | - | - |
| | Untreated | √ | √ |
| | Sorbitol | - | √ |
| | Sorbitol-Mannitol | - | √ |
| | Myo-inositol | - | √ |
| 10 | Unsealed overnight | - | - |
| | Untreated | √ | √ |
| | Sorbitol | - | √ |
| | Sorbitol-Mannitol | √ | √ |
| | Myo-inositol | - | - |
| 14 | Unsealed overnight | √ | √ |
| | Untreated | √ | √ |
| | Sorbitol | √ | √ |
| | Sorbitol-Mannitol | √ | √ |
| | Myo-inositol | - | √ |

Untreated – negative control for pre-treatment where ESM is bombarded without treatment, (-) not tested

Target tissue was placed onto Whatman No. 2 filter paper supports and placed onto MSG3 solid medium (refer to Figure 3.2 B) supplemented with either of the following osmoticums or treatments: 0.25 M sorbitol (WALTER *et al.* 1999), 0.25 M equimolar sorbitol-mannitol (YAO *et al.* 1997), 0.25 M myo-inositol (CLAPHAM *et al.* 1995), untreated (no osmoticum) on plain MSG3 medium, or Petri dishes were left unsealed and kept on the laminar flow bench overnight before the bombardment event. The “untreated” treatment served as a control for pre-treatment. Tissue derived from suspension culture, was filtered in 1.5 ml aliquots

onto the filter paper supports. Target tissue from Lines 1-4 ranged from solid- and liquid-derived cultures (Table 5.3 and Table 5.4) to various developmental stages of somatic embryo maturation and germinating somatic embryos (Table 5.5). Liquid and solid-derived cultures were bombarded after 0, 5, 10 and 14 d growth on solid medium and subcultured onto maintenance medium the following day. Maturing embryos and germinating embryos did not undergo any pre-treatment before bombardment. Mixed stages of Line 2 embryos were bombarded using ten Petri dishes at each PEG concentration (5% and 7.5%). Additional bombardments were performed (using sorbitol pre-treatment) with Line 5 ESM tissue after 10 d growth on solid medium (percentage survival results not shown), for purposes of gene analysis and stable integration tests (refer to Section 7.3.2. and 7.3.3).

5.4.4 DNA Coating of Microparticles

One hundred mg of 1.5 μm tungsten microparticles (ELAK Ltd, Hungary) were sterilized by overnight incubation in 2 ml 70% ethanol [v/v]. The following day the particles were briefly spun down at 2400 g . The ethanol was removed and the microparticles were washed twice with 2 ml sterile dH_2O . The sterile particles were stored in sterile 50% glycerol [v/v] solution at $-20\text{ }^\circ\text{C}$ for the duration of the transformation event. Macroparticles were stored in 100% ethanol overnight, placed onto an autoclaved Petri dish and left to air dry. Plasmid DNA was isolated as described by LI *et al.* (1995) (refer to Section 4.3.2) and then coated onto the tungsten particles using the PERL *et al.* (1992) method, described below, to obtain a concentration of 4 μg DNA mg^{-1} tungsten particles.

DNA-coated particles were prepared by adding 25 μg isolated plasmid DNA to 125 μl sterile tungsten particle suspension and mixed well, prior to the addition of 125 μl ice-cold 1 M CaCl_2 . The mixture was vigorously shaken to prevent DNA tungsten aggregation. Incubation on ice for 10-12 min was sufficient to allow precipitation and sedimentation. Two hundred μl of the clear supernatant were discarded and the particles were resuspended in the remaining liquid. This was sufficient for 20 discharges of the particle gun.

5.4.5 Biolistic Protocol

All experiments were performed using a gene gun (Genebooster™) with a nitrogen-driven biolistic delivery system. The settings of the sterile Genebooster™ remained constant at -0.4 bar vacuum and at 40 bar for shooting pressure of the nitrogen gas. Initial experiments were constant at -0.20 bar vacuum and 34 bar shooting pressure. The microcarrier travel distance was 70 mm from the stopping plate to the target tissue. A sterile macroparticle, which contained 5 µl (later amended to 10 µl) of DNA-coated particles was inserted into the acceleration barrel. The closing plug was used to stab the opposite end of the macroparticle, inverting it when inserted into the barrel, and shot. Microparticles were released from the macroparticle and dispersed evenly through a sterile mesh into the prepared target material placed in the Genebooster™ chamber. The mesh benefits transformation efficiency by reducing shock-generated trauma to cells (SANFORD *et al.* 1993) and zones of cell death. The target tissue, which was bombarded twice (later amended to only once with 10 µl DNA coated suspension), was removed and incubated in the dark at 25 °C. The macroprojectile was removed from the stopping plate and discarded.

5.4.6 Selection of Transformed Tissue

Allowing 2-3 d of recovery after the bombardment event, filter paper disks supporting bombarded ESM were transferred onto selection MSG3 medium supplemented with BASTA® bioactive ingredient (glufosinate ammonium). Selection was initially used at 3 mg l⁻¹ BASTA® before implementing a stepwise selection regime in the biolistic protocol. This consisted of 1 mg l⁻¹ followed by 3 mg l⁻¹ BASTA® inclusion in the MSG3 medium at each subculture.

5.4.7 Optimization of Bombardment Protocol

ESM derived from solid cultures (Gelrite®-containing medium) were discontinued as target tissue for final bombardment experiments. Attempts were made to improve bombardment efficiency. The revised biolistic protocol included one shot of 10 µl of DNA-coated particles at -0.4 bar vacuum and 40 bar shooting pressure

for the nitrogen gas. Target material (liquid medium-derived cultures after 0, 5, 10 and 14 d growth on solid medium shown in Figure 5.5) was treated with 0.25 M sorbitol or untreated and left in unsealed Petri dishes on the laminar flow bench overnight. Non-bombarded ESM constituted the control. The survival (%) of viable Petri dishes after the optimised biolistic protocol was monitored (Table 5.6).

The bombarded ESM further underwent an improved regeneration regime (refer to Section 3.2.7) by suspending the resultant potentially transformed viable tissue, as seen in Table 5.6, in liquid medium before being transferred back to the solid medium (Table 5.7). Regenerated, bombarded tissue is represented in Figure 5.6. Harvested mature somatic embryos were partially dried, initially for 18 d, before being placed onto charcoal-supplemented germination medium (240 hormone-free). Further embryo harvests were desiccated for 10 d.

5.5 Results and Discussion

5.5.1 Determination of BASTA® Concentration for Selection

After 10 d of subculture, embryogenic tissue growth was completely inhibited at 4 mg l⁻¹ and higher concentrations of glufosinate ammonium (Figure 5.1), the lowest concentration capable of inhibiting the growth of ESM on MSG3 maintenance medium. Decreasing amounts of new embryogenic tissue growth was observed at 1 mg l⁻¹, 2 mg l⁻¹ and 3 mg l⁻¹ BASTA® bioactive ingredient, respectively. These observations were also reflected in Line 5 tissue, which after 12 d exhibited prolific growth at 0 mg l⁻¹; started to senesce and discolour to a brown colour at 1 mg l⁻¹; and show retarded growth with senescence and brown spots at 3 mg l⁻¹ glufosinate ammonium (results not shown). Subculture of bombarded Line 5 ESM onto 3 mg l⁻¹ BASTA® resulted in an initial yellowing response, presenting further evidence to implement a stepwise selection regimen. However, in order to reduce toxicity to regenerating or recovering bombarded ESM all selection media contained a maximum of 3 mg l⁻¹ BASTA® active ingredient. Other methods of assessment include use of fresh weight measurements together with qualitative analysis to evaluate effects of BASTA® bioactive ingredient as a

selection agent. The inclusion of PPT powder instead of the commercial herbicide could also be an alternative to avoid unaccounted effects from other ingredients, present in the herbicide on the cultures (SNYMAN *pers comm.* 2001).

Several authors have developed selection systems using phosphinothricin. ABRIE (1998) tested a phosphinothricin gradient of 0, 1, 5, 10, 50 and 100 mg l⁻¹ using cotyledonary explants of the melon cultivar Hales Best 36 and observed no shoot regeneration on phosphinothricin levels of 5 mg l⁻¹ or more, but employed 10 mg l⁻¹ to make selection more stringent. WALTER and SMITH (1999) observed significant resistance of *P. radiata* embryogenic tissue to relatively high concentrations (25 mg l⁻¹) of phosphinothricin. It was concluded that the inclusion of glutamine in the medium and the lack of photosynthetic activity by the embryogenic cells would explain this result. WEHRMANN *et al.* (1996) concluded that phosphinothricin is an effective selective agent at 0.005 - 0.025 µM.

Two successive subcultures on selective 240 maturation medium (containing 0, 5 and 7.5% PEG [w/v]) resulted in various stages of mature somatic embryo development (as defined by HAKMAN and VON ARNOLD 1988) (Table 5.2). Mature somatic embryo development was sustained on 0% and 5% PEG-containing [w/v] medium supplemented with 2 mg l⁻¹ or less BASTA® bioactive ingredient. Some mature somatic embryo development was noted in tissue grown on 5% PEG-containing [w/v] medium supplemented with 3 mg l⁻¹ glufosinate ammonium. The effects of 0% and 5% PEG-containing [w/v] 240 maturation medium supplemented with BASTA® concurred with results obtained previously with the maintenance medium selection kill-curve: no growth was supported on medium containing 4 mg l⁻¹ or more of glufosinate ammonium. The addition of BASTA® herbicide to 240 medium supplemented with 7.5% PEG [w/v] seemed to adversely affect mature somatic embryo development at all concentrations applied.

It was observed that non-bombarded, partially dried embryos elongated on 240 media supplemented with activated charcoal and 3 mg l⁻¹ or 4 mg l⁻¹ BASTA® bioactive ingredient, without any apparent deleterious effect to the germinating

embryos. It was concluded that the presence of activated charcoal masked the effect of the selection agent and therefore selection would not be accurate at the germination stage.

The survival of bombarded germinating embryos and the senescence of un-bombarded control embryos was observed after one week on selection medium using 3 mg l⁻¹ BASTA® bioactive ingredient (Figure 5.2).

Table 5.2: Overall effect of BASTA®-supplemented 240 maturation media on mature somatic embryo development from non-bombarded tissue

| Glufosinate ammonium (mg l ⁻¹) | PEG concentration [w/v] | | |
|--|---------------------------------------|------------|----------|
| | 0% | 5% | 7.5% |
| | Final stages of mature SE development | | |
| 0 | Stage III | Stages III | Stage II |
| 0.5 | Stages II | Stages III | ++ |
| 1 | Stages III | Stage II | ++ |
| 2 | Stages II | Stage II | + |
| 3 | ++ | Stage I | + |
| 4 | + | ++ | + |
| 5 | + | + | + |
| 10 | + | - | - |
| 50 | - | - | - |
| 100 | - | - | - |

SE - somatic embryo; (-) no growth; (+) very little growth; (++) some growth

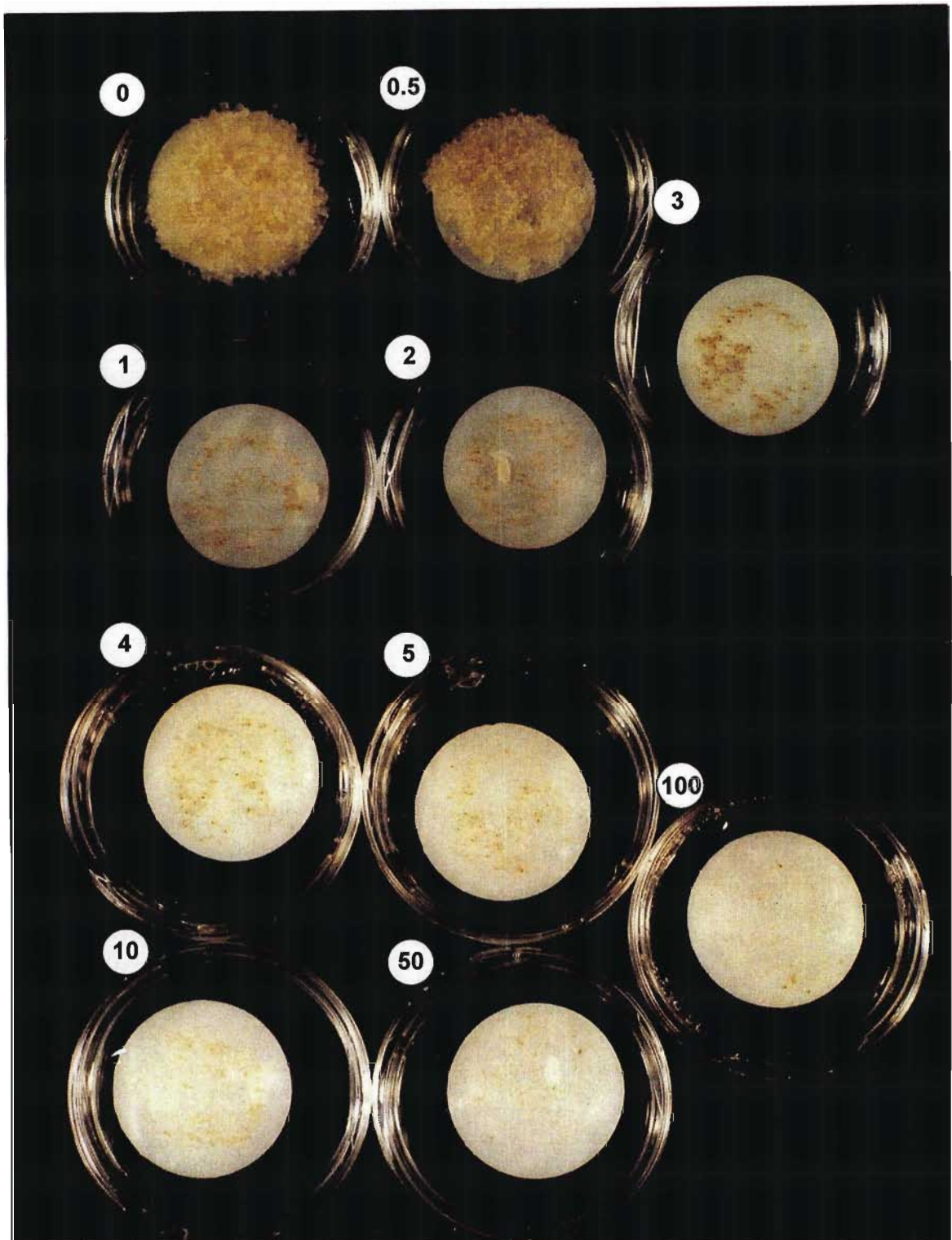


Figure 5.1 Determination of the lowest BASTA® concentration capable of inhibiting ESM growth on MSG3 medium. Concentrations (mg l^{-1}) of BASTA® active ingredient indicated in circles. Embryogenic tissue grew prolifically at 0 and 0.5 mg l^{-1} glufosinate ammonium. ESM growth was completely inhibited at 4 mg l^{-1} after 10 d of subculture

Further maintenance on selection medium resulted in senescence of the surviving “emblings”. These results indicated firstly, that selection would have to be carried out through a stepwise regime of subculturing putative transformants from 1 mg l⁻¹ to 3 mg l⁻¹ BASTA® selection media. Secondly, it was concluded that selection on 240 germination medium (in the absence of activated charcoal) constituted conditions that were too harsh for sensitive developing radicles and did not allow for optimum regeneration and development of bombarded somatic plantlets. Selection on 240 germination was therefore discontinued. However, the fact that the germinating bombarded embryos survived on selection medium, albeit for a short time, indicated promising results for putative transformation of *Pinus patula*.

5.5.2 Pre-treatment of Target Tissue

Cells must be exposed to high osmoticum both before and after bombardment to induce a significant increase (at least two fold) in GUS expression (CLAPHAM *et al.* 1995). Although cells exhibited a 15% decrease in GUS transient expression when the duration of the myo-inositol-supplemented pre-treatment was extended up to 5 h, the GUS expression increased when maintained on osmoticum up to 10 d post bombardment (CLAPHAM *et al.* 1995). Microscopic analysis after particle transfer showed cell-burst in cultures without osmotic treatment (Figure 5.2 A). A marked positive effect on the integrity of culture cells was observed after inclusion of an osmoticum in the medium (Figure 5.2 B).

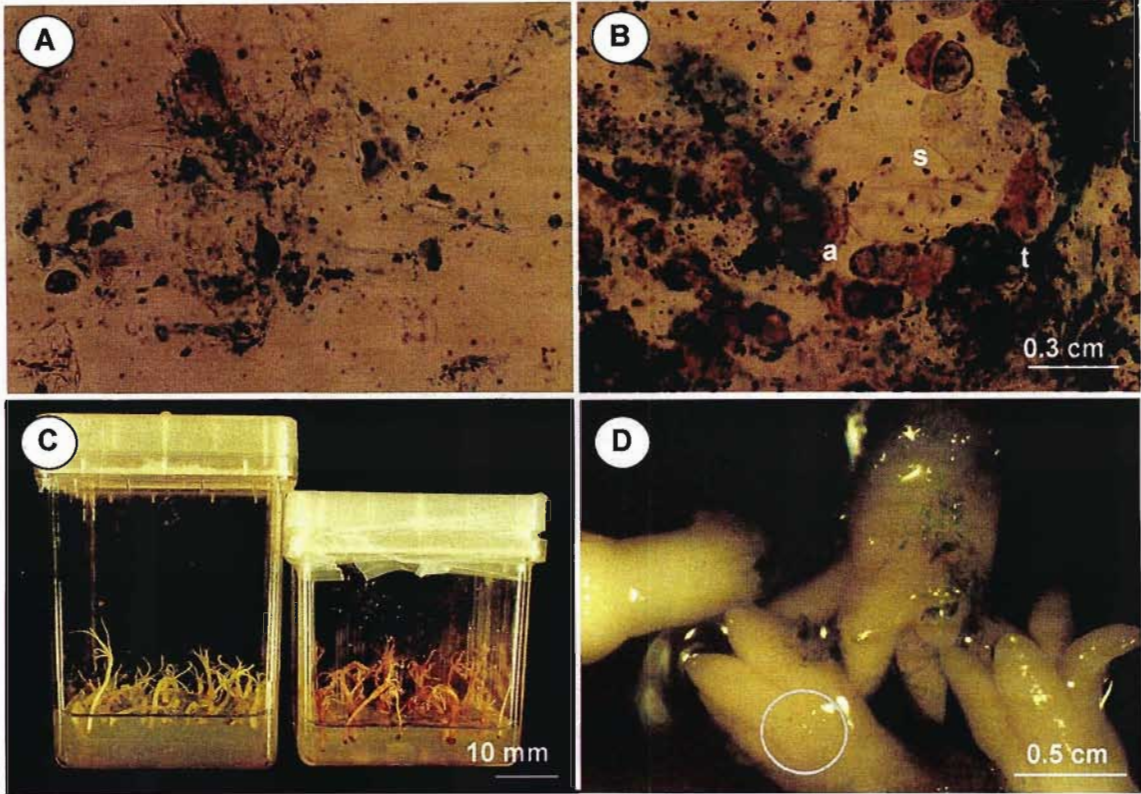


Figure 5.2 Effects of particle bombardment on tissue indicated by GUS expression and selective BASTA® pressure. Effects of particle bombardment on GUS stained target tissue before (A) and after (B) inclusion of the pre-treatment step. (A) Plasmolysis of embryonal cells after microparticle blast. (B) Intact blue embryonal heads indicate putatively transformed tissue: a – apex, s – suspensor cell, t – aggregates of tungsten particles. (C) Effects of selective medium on surviving bombarded germinating embryos (left) and senescing non-bombarded control embryos (right) supplemented with 3 mg l⁻¹ of BASTA® bioactive ingredient after one week. (D) Transient GUS expression observed in suspensor tissue surrounding harvested mature somatic embryos after particle transfer. GUS uptake by embryo tissue seen as blue spots in circle

5.5.3 Transformation Efficiency and Regeneration of Bombarded Tissue

Preliminary experiments were conducted to optimize the bombardment protocol to ascertain the performance of different genotypes and response to different treatments. Results were recorded after the second phase of selection on 3 mg l⁻¹ BASTA® bioactive ingredient. Target tissue preparation of solid medium-derived tissue relative to liquid medium-derived tissue was laborious and it was possible that the clumps of well-established tissue growth could compromise genetic transformation and regeneration of bombarded cultures. With solid medium-derived tissue there was a greater possibility of only transforming the outer-lying embryogenic tissue and not the entire clumps of tissue. The use of filtered tissue (liquid medium-derived cultures), one cell layer thick, would be more accessible and in better contact with the medium, which would promote better growth and a better response to the osmoticum. A uniform thin lawn of cells is optimal as it provides the greatest number of targets (SANFORD *et al.* 1993).

Table 5.3 Summary of preliminary results from bombardment of solid-derived cultures showing survival (%) of Petri dishes with live cultures

| Day | Treatment | Line 1 | Line 2 | Line 3 | Line 4 |
|-----|--------------------|--------|--------|--------|--------|
| 0 | Unsealed overnight | - | - | - | - |
| | Untreated | 70 | 100 | - | 70 |
| | Sorbitol | - | - | - | - |
| | Sorbitol-Mannitol | - | - | - | - |
| 5 | Unsealed overnight | - | - | - | - |
| | Untreated | 100 | 100 | - | 66 |
| | Sorbitol | - | - | - | - |
| | Sorbitol-Mannitol | - | - | - | - |
| 10 | Unsealed overnight | - | - | - | - |
| | Untreated | - | 100 | - | 50 |
| | Sorbitol | - | - | - | - |
| | Sorbitol-Mannitol | - | - | - | 0 |
| 14 | Unsealed overnight | - | - | 80 | - |
| | Untreated | - | - | 40 | - |
| | Sorbitol | - | - | 100 | - |
| | Sorbitol-Mannitol | - | - | 100 | - |

(-) not tested; 0 – no survival

Solid-derived cultures, specifically from the genotypes that were being tested, were an impractical source for providing large quantities of target tissue.

Consequently, insufficient tissue was generated to test all the different treatments. Untreated solid-derived tissue had high survival rates when bombarded at 0 (70 %) and 5 d (100%) for Line 1; and at 0, 5 and 10 d (100 % survival for all) for Line 2 (Table 5.3). Tissue bombarded after 14 d growth on solid medium, was tested for 4 different treatments in Line 3. Overall tissue survival (%) treatments were good, but the effect of pre-treatment was most noticeable in Line 3 (Table 5.3) with 100% survival of tissue where osmoticum-supplemented treatments were used (0.25 M sorbitol and 0.25 M sorbitol-mannitol). However, the effect of osmoticum was not apparent in Line 4 where no survival was observed when 0.25 M sorbitol-mannitol treatment was applied. In this case, survival could have been dependant on other factors such as physiological status of the tissue. A gradual decrease in survival was observed across the bombardment days (0, 5 and 10 d) in the slow-growing Line 4 despite the consistent treatment (untreated), where solid-derived cultures were bombarded but were not treated with an osmoticum (Table 5.3). All lines of the untreated solid-derived bombarded cultures (Table 5.3) survived selection pressure irrespective of bombardment day.

Table 5.4 Summary (% survival) of preliminary results from bombardment of liquid-derived cultures using various treatments across all lines

| Day | Treatment | Line | | | |
|-----|--------------------|------|-----|-----|-----|
| | | 1 | 2 | 3 | 4 |
| 0 | Unsealed overnight | - | - | 80 | - |
| | Untreated | 40 | 0 | 85 | 100 |
| | Sorbitol | 0 | 0 | 90 | - |
| | Sorbitol-Mannitol | 0 | 0 | 100 | 0 |
| 5 | Unsealed overnight | - | - | - | - |
| | Untreated | - | - | 80 | 0 |
| | Sorbitol | 80 | 60 | 60 | - |
| | Sorbitol-Mannitol | - | - | - | 0 |
| | Myo-inositol | 40 | 40 | 75 | - |
| 10 | Unsealed overnight | - | - | - | - |
| | Untreated | 100 | 0 | 60 | - |
| | Sorbitol | 100 | 0 | 100 | - |
| | Sorbitol-Mannitol | 0 | 40 | 75 | - |
| 14 | Unsealed overnight | 0 | - | 20 | - |
| | Untreated | 65 | 100 | 0 | 100 |
| | Sorbitol | 0 | 20 | 60 | - |
| | Sorbitol-Mannitol | 40 | 50 | - | - |
| | Myo-inositol | - | - | 0 | 0 |

(-) not tested; 0 – no survival

The myo-inositol pre-treatment was only tested in liquid-derived cultures and had variable response. The best survival percentage for this treatment was observed in Line 3 bombarded on day 5. This osmoticum is too expensive for use in many replicated bombardment experiments and was discontinued as a pre-treatment. Preliminary experiments using liquid-derived cultures, in general, responded well to sorbitol or sorbitol-mannitol treatments (Table 5.4). The unsealed response was tested in Line 3 only (liquid and solid-derived tissue) and elicited a good survival response (liquid-derived tissue at 14 d was the exception).

In the bombardments performed on the liquid medium-derived cultures (Table 5.4), a high selection pressure was observed particularly at bombardment days 5, 10 and 14 d after filtering onto solid medium. Selection pressure at 3 mg l⁻¹ was exerted irrespective of treatment used as shown by bombardment day 10. As expected there was a lower rate of ESM survival after bombardment on day 0. Most liquid-derived cultures of Lines 1, 2 and 4 bombarded on day 0 did not survive through to the first selection of 1 mg l⁻¹, indicating that liquid-derived cultures need time to establish (recovery period) on solid media before being able to buffer outside stresses such as bombardment. A high incidence of bombarded untreated liquid-derived Line 3 cultures survived selection pressure irrespective of bombardment day or treatment as indicated in Table 5.4, as was previously observed in solid-derived Line 3 cultures.

There was very little success in embryo maturation from bombarded tissue (Table 5.7), even though many cultures survived the second phase of selection on 3 mg l⁻¹ BASTA® bioactive ingredient (Table 5.3, Table 5.4, Figure 5.3, Table 5.6). Line 2 was a prolific line that was most consistent in embryo production (Table 5.5) and was the only line that produced embryos after using an improved regeneration regime (Table 5.7, Figure 5.4). This could be due to an inherently high embryogenic and maturation potential. Despite its consistent survival rate across all bombardments (Table 5.3, Table 5.4 and Figure 5.3 C), Line 3 seemed to exhibit a genotypic response of becoming mucilagenous after some time in subculture and did not exhibit the usual good maturation potential characteristic to that line, irrespective of particle transfer to target tissue.

Regeneration with quality somatic embryo production potential would be a desired outcome of a transformation event.

A trend was noted across all three lines subjected to bombardments using the optimized protocol (Section 5.4.7), where the greatest tissue survival was shown on bombardment day 14 by all treatments (Figure 5.3), particularly Line 1 (Figure 5.3 A) and Line 2 (Figure 5.3 B). Particle transfer to the mixed stages of maturation (Line 2) and germinating mature somatic embryos (Line 2 and 3) (Table 5.5) did not adversely affect further developmental changes in the maturation process as shown by the number of embryos harvested, which matured through to the germination phase of development. Tests for transient expression in harvested embryos showed GUS expression occurring in tissue surrounding the cotyledons and the superficial outer-lying cells of the embryo (Figure 5.2 D). This was an indication that higher vacuum and gas pressures need to be employed. Selection at the maturation (partial drying regime) and germination stages in the light is problematic as discussed in Section 5.5.1. Another problem that could arise when using differentiated tissue is the production of chimaeras. The worker cannot identify whether the whole target explant or only part thereof has been transformed.

Table 5.5: Number of maturing somatic embryos bombarded (without pre-treatment) at mixed stages of development and at germination

| Line | Stages of development | Pre-germination medium (PEG) | | Germination medium | |
|--|---------------------------|------------------------------|------|--------------------|------|
| | | 5% | 7.5% | 5% | 7.5% |
| No. of embryos and surviving germinating embryos | | | | | |
| 2 | Mixed stages ^o | 64 | 94 | 26 | 15 |
| | Mixed stages [*] | 54 | 21 | 1 | 0 |
| | Germinating ^o | 200 | 17 | 119 | 0 |
| 3 | Germinating ^o | 50 | 60 | 47 | 1 |

^{*}Bombarded twice on different bombardment date; ^o same bombardment date

Operator skill can produce drastic fluctuations in biolistic efficiency with seemingly routine procedures such as particle transfer. The procedure of microprojectile coating is considered one of the most important sources of variation that affects gene transfer efficiency as a uniform reaction is almost impossible to obtain.

Precipitation occurs quickly and tungsten is difficult to keep in suspension, so each precipitation event has a non-reproducible and unique pattern, which can produce variations in transformation rates from one microcentrifuge tube to the next (SANFORD *et al.* 1993). Fluctuations between day to day and month to month preparations have also been observed. Once tungsten particles have been coated it is essential that they are used immediately to avoid DNA degradation by these microparticles. Tungsten surface oxidation alters DNA binding and catalytically degrades DNA over time. Equal division of the DNA-tungsten mixture into respective aliquots and care to load in the centre of a macroparticle for each bombardment are further technical considerations to be kept constant (SANFORD *et al.* 1993). Causes of variation in biolistic efficiency can even be extended to the type of microcentrifuge tube used in DNA coating, and the type of Parafilm or wrapping used on plates which ultimately affect the microclimate in the plate. Tungsten and DNA are believed to stick to the surface of some brands of Eppendorf tubes (SANFORD *et al.* 1993). Parafilm is thought to create poor gaseous exchange, trapping excess ethylene, released after bombardment (KLEIN 1995), reducing the number of cells recovered (SANFORD *et al.* 1993).

Although the effect of tungsten toxicity was not specifically measured in this study, it is a factor that would have affected regeneration ability and general recovery of bombarded cultures. Gold particles are less toxic to plant cells than tungsten particles whose toxicity would also contribute to cell injury (RUSSEL *et al.* 1992b). SANFORD *et al.* (1993) reported drastic culture medium acidification from high tungsten concentrations resulting in cell death of pH-sensitive cells.

The physiological state of target tissue, and osmoticum concentration are according to SANFORD *et al.* (1993), the most important biological parameters that need to be optimized in a biolistic experiment. Actively dividing cells of tobacco suspensions at log-phase were optimum (SANFORD *et al.* 1993). *Pinus patula* ESM were in general best able to withstand the stresses of the bombardment process 14 d after being subcultured onto solid medium. Line 3 proliferated consistently regardless of the treatment or bombardment day. The effect of bombardment on cells after various days in suspension was not a parameter in this study, but should be investigated to optimize preparation of

target tissue before biolistic experiments. In this study it was expected that the treated bombarded cultures would outperform untreated and control target tissue. In general the opposite was observed in terms of percentage survival. Microscopic analysis, however, revealed an improvement in tissue integrity and transient expression (Figure 5.2 B) due to osmoticum-supplemented bombardment media.

5.5.4 Selection and Regeneration

Regeneration of bombarded cultures proved, not surprisingly, to be the most difficult process. The stepwise nature of ESM selection, before maturation, infringes on optimal conditions required for regeneration of putatively transformed embryos (Table 5.6). Tissue viability on maturation medium decreased as cultures were transferred from selection medium to maturation medium, the least viable shown in Line 2 across the bombardment days (Table 5.6). However, all lines showed resilience to the two-step selection in day 14 with 100% survival, presumably the tissue masses had grown too dense for selection pressure to be efficient. Ideal maturation conditions require a low ESM density to volume of media nourishing the mass (sustainable nutrients). However, during selection there is sufficient time for tissue proliferation, as tissue is transferred a few days after bombardment onto 1 mg l⁻¹ BASTA® bioactive ingredient and then subcultured onto 3 mg l⁻¹ BASTA® bioactive ingredient for a further week before being transferred to maturation medium. When ESM have proliferated to such a degree, there are limited nutrients available for emerging, and perhaps stressed, mature somatic embryos. This degree of proliferation can lead to selection problems. *Pinus radiata* had significant resistance to high levels of phosphinothricin where the selection effect could have been masked by the presence of glutamine, and embryogenic cells are not photosynthetically active (WALTER and SMITH 1999). Ubi-1, present on pAHC25 vector, is a stress inducible promoter where it could be speculated that higher expression levels of the selectable marker fusion genes are expected in initial stages of transformation when exposed to stress such as particle bombardment, growth in toxic conditions (selection) or high osmotic pressures (CHRISTENSEN and QUAIL 1996). Similarly a decrease in expression would be expected upon removal of these

selective conditions, such as removal of bombarded tissue from selection medium. In this study there was a high incidence of control replicates that survived selection at 3 mg l⁻¹ BASTA® bioactive ingredient. This concern was noted by PAPANIKOU *et al.* (2004) who showed that mechanisms for glyphosate (the active compound in Roundup®, a non-selective herbicide) resistance arose in plant tissue cultures that changed with length of time in culture.

Methods of transgenic plantlet recovery in conifers are far from optimal (CLAPHAM *et al.* 1995) as many transiently transformed cells are not receptive to stable integration or regeneration, or if the appropriate cells are stably transformed they do not continue to divide after transformation (McCABE and CHRISTOU 1993). The loss of embryogenic potential due to selective agents (ROBERTSON *et al.* 1992) or transgene silencing through methylation (HOLLIDAY 1984) are further factors that complicate the transformation progress.

KUAI and MORRIS (1995) have noted that the effect of cell growth cycle on GUS expression was closely correlated with growth rate and the rate of protein synthesis in protoplast cell cultures. However, prolonged subculturing of cells resulted in a gradual decline in transient transformation (apparently related to the developmental rather than the physiological stage). Cultures showed their highest GUS expression 10 weeks after initiation, which subsequently declined to barely perceptible levels one year after their initiation (KUAI and MORRIS 1995). Osmolarity of the medium (0.4 M sucrose) was also found to significantly increase GUS specific activity.

The mode of sampling ESM for suspension in order to re-establish regenerating material (Section 3.2.7) on maturation could have given great impetus to the regeneration process. This correlates with observations that embryogenic tissue collected from the periphery part of the ESM colony was more amenable to maturation than tissue from the inner part or the entire colony when plated onto maturation media (RAMAROSANDRATANA *et al.* 2001b). Maturation performance was greater in *Pinus pinaster* when plated onto medium containing 6% sucrose [w/v], 0% PEG [w/v] and 0.9% gellan gum [w/v] suggesting a positive correlation between the limitation of ESM proliferation and maturation yield.

Although both processes can occur simultaneously, the level of proliferation could affect developing embryo quality. Many of the small meristematic cells in the embryonic region have prominent vacuoles and the embryonic region is subtended by large vacuolar suspensor cells. These would be affected by the drying effect from the osmoticum and later the maturation media with the high gel strength. Turgor reduction is also affected by drying as it was observed that cells seemed to recover turgor once transferred to maintenance medium.

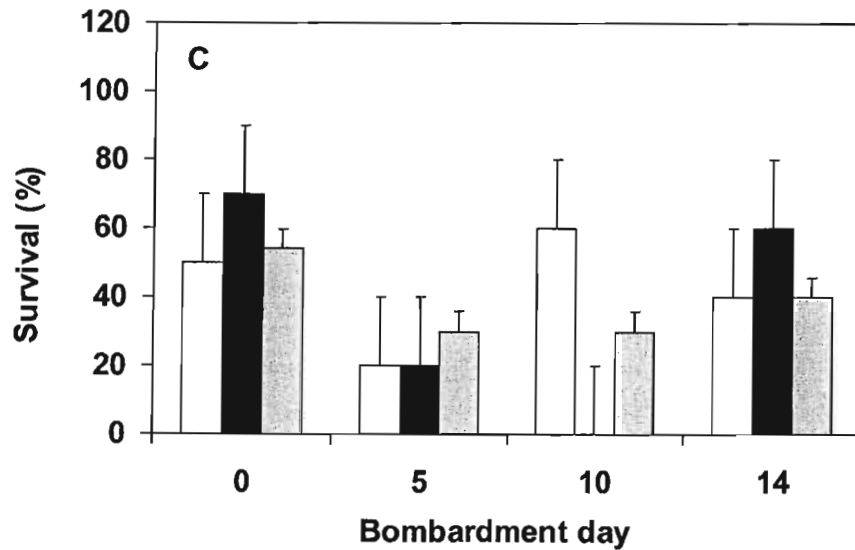
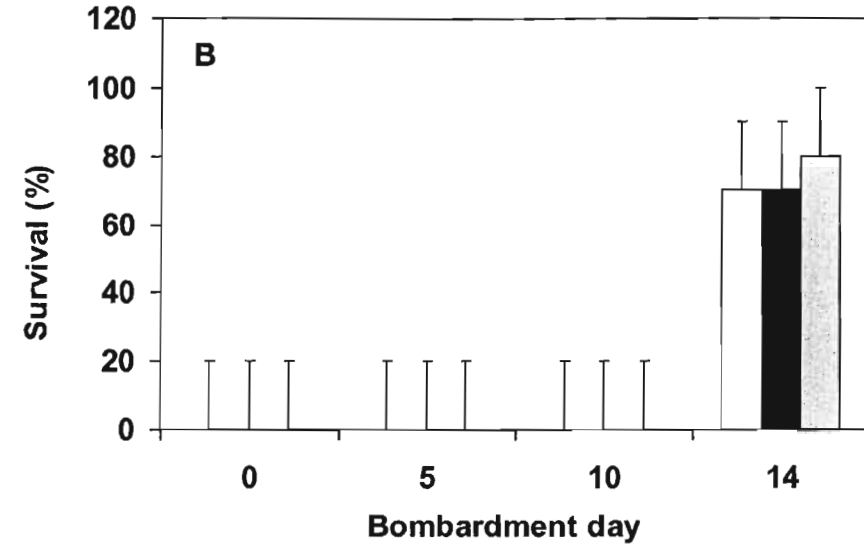
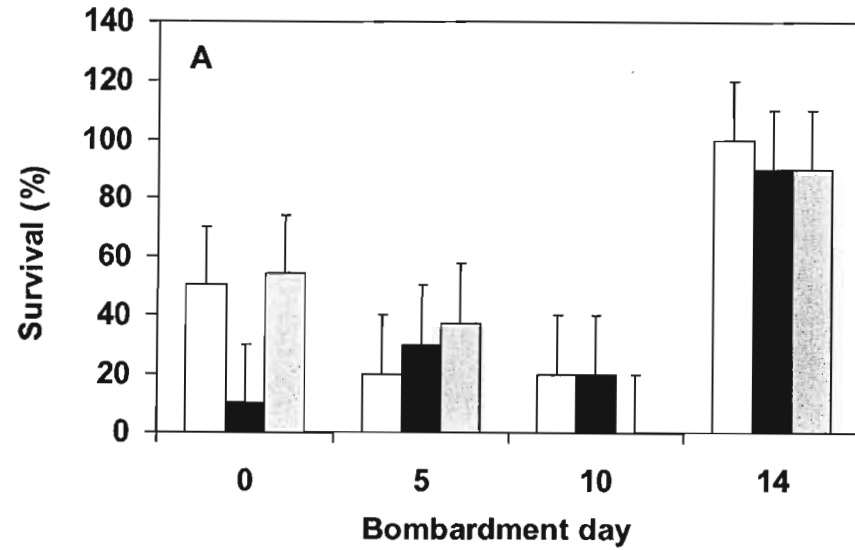


Figure 5.5: Survival (%) after using the optimized bombardment protocol for liquid medium-derived cultures of Line 1 (A); Line 2 (B) and Line 3 (C) bombarded on day 0, 5, 10 and 14 after establishment on solid medium. (Treatments: □Control - not bombarded; ■Untreated - bombarded tissue without pre-treatment; ▨Sorbitol - tissue bombarded after pre-treatment with sorbitol)

Table 5.6: Survival (%) of liquid-derived cultures on selection and maturation media following bombardment with an optimized biolistic protocol

| Bombardment Day | | | 0 | | | 5 | | | 10 | | | 14 | | |
|-----------------|---|-----|----------------------|-------------------|-------------------|-------------------|--------------------|--------------------|-------------------|------------------|------------------|-----------------|------------------|-----------------|
| Line | Procedure followed, culture medium used and BASTA selection concentration | | Control* | Untreated† | Sorbitol‡ | Control | Untreated | Sorbitol | Control | Untreated | Sorbitol | Control | Untreated | Sorbitol |
| 1 | Selection medium | MSG | 1 mg l ⁻¹ | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | Selection medium | MSG | 3 mg l ⁻¹ | 70 ^a | 50 | 90.9 | 90 | 70 | 100 | 100 | 100 | 100 | 100 | 100 |
| | Maturation medium | 240 | | 70 | 50 | 72.7 | 60 ^{a, c} | 70 ^{a, c} | 87.5 ^c | 50 | 60 | 12.5 | 100 | 100 |
| | Subculture ^x | 240 | | 50 ^e | 10 ^e | 54.5 ^e | 20 ^e | 30 ^e | 37.5 ^e | 20 ^e | 20 ^e | 0 | 100 ^e | 90 ^e |
| 2 | Selection medium | MSG | 1 mg l ⁻¹ | 100 | 100 | 100 | 88.9 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | Selection medium | MSG | 3 mg l ⁻¹ | 90 | 50 | 80 ¹ | 88.9 | 100 | 100 | 100 ^b | 100 ^b | 100 | 100 | 90 |
| | Maturation medium | 240 | | 20 | 20 | 20 | 11.1 | 25 | 11.1 | 0 | 0 | 0 | 100 | 90 |
| | Subculture ^x | 240 | | 0 ^{b, c} | 0 ^{b, c} | 0 ^{b, c} | 0 ^{b, c} | 0 ^{b, c} | 0 ^{b, c} | - | - | - | 70 ^e | 70 ^e |
| 3 | Selection medium | MSG | 1 mg l ⁻¹ | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | Selection medium | MSG | 3 mg l ⁻¹ | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 80 | 90 |
| | Maturation medium | 240 | | 100 | 100 | 90.9 | 40 | 60 | 66.7 | 100 | 40 | 50 | 100 | 80 |
| | Subculture ^x | 240 | | 50 ^d | 70 ^d | 54.5 | 20 | 20 | 33.3 ^e | 60 | 0 | 30 ^e | 40 ^f | 60 |

(*) – non-bombarded control; (†) – bombarded tissue with no pre-treatment; (‡) – bombarded tissue with a sorbitol pre-treatment; (x) – second subculture on 240 medium; (a) – poor growth; (b) – no growth; (c) – brown; (d) – yellow, very little new growth; (e) – small clumps of tissue; (f) – glassy appearance

Table 5.7: Regenerated bombarded liquid-derived ESM after improved regenerating regime (% surviving plates)

| Line | Bombardment Day | Treatment | | | | | | | | | | | |
|------|--|-----------|------------------------|-----------------------|---------|-----------|----------|---------|-----------|----------|---------|-----------|----------|
| | | 0 | | | 5 | | | 10 | | | 14 | | |
| | Maturation Protocol and Number of embryos harvested per treatment tested | Control* | Untreated [†] | Sorbitol [‡] | Control | Untreated | Sorbitol | Control | Untreated | Sorbitol | Control | Untreated | Sorbitol |
| 1 | Initial survival on 240 0% | 100 | 10 | 50 | 40 | 30 | 40 | 20 | 20 | - | 100 | 100 | 100 |
| | 240 0% (subculture 2) | 100 | 10 | 50 | 40 | 30 | 40 | 20 | 20 | - | 100 | 100 | 90 |
| | 240 0% (subculture 3) | 0 | 0 | 10 | 30 | 20 | 10 | 0 | 0 | - | 80 | 80 | 60 |
| | No. embryos harvested per treatment | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 |
| 2 | Initial survival on 240 0% | 100 | 100 | 100 | 90 | 80 | 90 | 30 | 50 | 90 | 100 | 100 | 100 |
| | 240 0% (subculture 2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 |
| | 240 0% (subculture 3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 |
| | No. embryos harvested per treatment | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 54 |
| 3 | Initial survival on 240 0% | 50 | 50 | 100 | 50 | - | 50 | 50 | - | 50 | 50 | 100 | 50 |
| | 240 0% (subculture 2) | 0 | 0 | 0 | 0 | - | 0 | 0 | - | 0 | 0 | 0 | 0 |
| | No. embryos harvested per treatment | 0 | 0 | 0 | 0 | - | 0 | 0 | - | 0 | 0 | 0 | 0 |

Control* – non-bombarded control; Untreated[†] - bombarded tissue with no pre-treatment; Sorbitol[‡] – bombarded tissue with a sorbitol pre-treatment; (-) not tested; 0 - death

Decreasing the water availability could be more pertinent than increasing the osmotic potential of the medium for the development of mature somatic embryos (RAMAROSANDRATANA *et al.* 2001a). PEG-supplementation did not improve maturation rates in *P. pinaster* cultures. Similar results of gradually decreasing ESM water potential were obtained by increasing the gelling agent to a higher concentration (9 g l⁻¹ or 0.9% [w/v]) in this study as compared to results procured by RAMAROSANDRATANA *et al.* (2001a). Superior suspensor elongation has been associated with perception of signals related to water stress and was certainly highlighted as a trait of regenerating maturation hardiness seen in the “spiky” Line 1, instead of the normally prolific Line 3 which had merely proliferated in mass but had exhibited a “smooth” appearance with a poor display of Stage I embryos. A subculture effect could have decreased the maturation potential of all lines (which were all chosen based on their superior maturation potential) used in the study over 2 years.

This effect had perhaps been manifested more acutely in Line 3, which did not manage to regenerate at all. An overall decrease in embryo production was observed with embryos obtained after the first year of study (results not shown). This was attributed to a subculture effect. It would be recommended that each transformation study use material that has been initiated within a year and that cryopreservation protocols are implemented to maintain tissue juvenility. The potential of stable integration (Chapter 7) could also be affected by prolonged subculture. Harvested embryos from regeneration material did not survive 18 d of desiccation (normally a 21 d regime) suggesting partial desiccation had already taken place on the maturation medium containing higher gel strength suggesting the partial desiccation regime needed to be shortened.

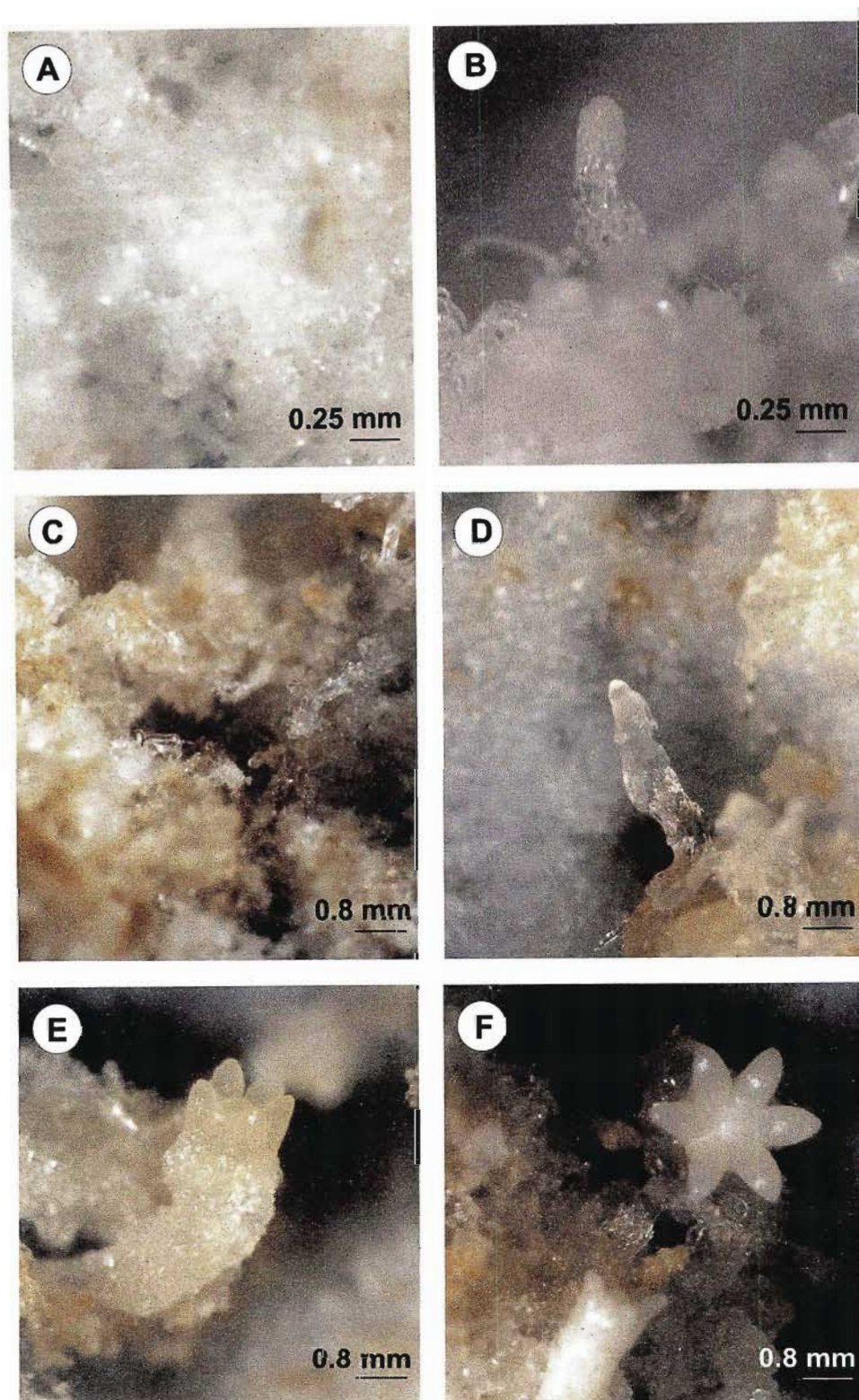


Figure 5.6 Regenerating mature somatic embryos from bombarded material. (A) Proliferating ESM mass (Line 1); **(B)** Stage I (Line 1); **(C)** Proliferating ESM mass (Line 2); **(D)** Stage II (Line 2) indicated by opaque head; **(E)** Stage III (Line 2) indicated by cotyledonary projections; **(F)** Stage IV of Line 2 indicated by full development of cotyledons

CHAPTER 6

AGROBACTERIUM-MEDIATED TRANSFORMATION OF PINUS PATULA

6.1 Introduction

Historically *Agrobacterium*-mediated transformations were unable to overcome species recalcitrance, especially in monocotyledonous plants and gymnosperms, which used biolistic transfer. The early standard *Agrobacterium* strains could only be used for angiosperm trees (PEÑA and SÉGUIN 2001). However, improvements in tissue culture regimes, regeneration protocols and vectors with promoters of plant origin (versus bacterial origins), which are easily recognized by the plant transcription machinery (BIRCH 1997), are some of the factors now enabling researchers to return to using genetic manipulation via *Agrobacterium*. They included firstly, developed disarmed strains (the deletion of the oncogenes from the Ti-plasmid) circumventing gall formation and therefore regeneration problems. Secondly, binary vectors with versatile T-DNA borders between which selectable markers could be introduced (CHRISTOU 1995). Advanced knowledge of the molecular biology of *Agrobacterium*, especially on the role of virulence (*vir*) genes on the Ti-plasmid has further increased the frequency of *Agrobacterium*-based transformations. Further significant advances have been made within conifer protocols using other modifications such as: the additive coniferyl alcohol during co-cultivation (LEVÉE *et al.* 1997), modified *Agrobacterium* strains, noting responses between species and genotypes, and using extra virulence genes involved in virulence and T-DNA transfer to disarmed strains (constitutively active *virG* or extra copies of *virG* and *virB* from pTiBo542) to enhance *Agrobacterium*-mediated transformation in Norway spruce and loblolly pine embryogenic cultures (WENCK *et al.* 1999).

The universal gene transfer bombardment technique has disadvantages such as (1) the high cost of consumables, (2) variable transformation efficiency with increasing evidence that gene silencing occurs (possibly due to the high number of gene

integrations), (3) transgene fragmentation (Section 5.3) and (4) decreasing the fertility of transgenic plants, such as rice (DAI *et al.* 2001; CHARITY *et al.* 2005). The *Agrobacterium* method has the advantage of high co-expression of introduced genes, less fragmentation of the transferred gene, single or lower copy number of integrated transgenes (though tandem copies of a few T-DNA are common at a single locus), a more predictable integration pattern (SMITH and HOOD 1995; HADI *et al.* 1996, GELVIN 2003) and preferentially into transcriptionally active sites of the host genome (GELVIN 2003) but not at a defined locus (BHAT and SRINIVASAN 2002). Presently, T-DNA is randomly integrated into the genome by illegitimate recombination (Section 2.5), which can cause position effects (GELVIN 2003). However, use of *Agrobacterium* for homologous (or site-directed) recombination into known transcriptionally active sites, which occurs at 10^{-5} frequency of illegitimate recombination, (reviewed in GELVIN 2000; 2003) would enhance the ability for plant gene targeting and gene replacement experiments in molecular biological research. Inherent difficulties are also associated with plasmid preparation from *A. tumefaciens* as both binary vector plasmids and the large (>200kb) Ti plasmids are maintained only at low copy number in *A. tumefaciens* (LI *et al.* 1995).

Since the mid-1990s, this indirect method of transfer has been used across many coniferous species and explants with successful regeneration following *Agrobacterium* transfer in conifers were reported for *P. radiata* (CHARITY *et al.* 2005), *Larix decidua* (HUANG *et al.* 1991; SHIN *et al.* 1994), *Larix kaempferi* X *L. deciduas* (LEVÉE *et al.* 1997), *P. strobus* (LEVÉE *et al.* 1999) and *P. taeda* (TANG *et al.* 2001) (Section 2.8). For a comprehensive list of *Agrobacterium*-mediated investigations (including regenerated plantlets) reported in conifers refer to Table 2.2. Many *Agrobacterium* transformation reports have relied on somatic embryogenesis as it provides rapidly dividing tissue, often using immature zygotic embryos for culture initiation, resulting in thousands of mature somatic embryos that can be regenerated into plantlets, from a single transformation event (TANG *et al.* 2001). Regeneration of *P. radiata* plantlets *in vitro* from mature zygotic embryos, such as adventitious shoots from cotyledons of mature seed, is reportedly higher than production of somatic

embryos from immature embryogenic tissue, allowing a greater number of genotypes to be transformed (GRANT *et al.* 2004). Further advantages lie in all-year availability of mature seed, which can be stored at 4 °C, unlike embryogenic tissue that loses embryogenic potential through long-term tissue culture (TANG *et al.* 2001). An organogenic protocol for *P. patula* has not yet been formulated. Although gene transfer into plant cells (without regard to particular organ or complex tissue targeting) and plant regeneration are no longer limiting factors with the array of techniques currently available (BIRCH 1997; LE *et al.* 2001), genetic transformation of conifers still has major limitations (LE *et al.* 2001; TANG and NEWTON 2003). These difficulties probably arise with type and physiological status of explants, vector systems, the *Agrobacterium* strains and the selection scheme for transformants (LE *et al.* 2001). Studies such as those performed by STOMP *et al.* (1990) where gall growth and opine production were monitored after transformation of nine *Pinus* species with five different *A. tumefaciens* strains, are a useful means of assessing the most suitable *Agrobacterium* strain for a particular species. The study did not include *P. patula* or the *A. tumefaciens* LBA4404 strain that was used in the present study.

The aim of this particular study was to establish an indirect method of gene transfer to *Pinus patula* tissue using the *A. tumefaciens* LBA4404 strain transformed with the pAHC25 plasmid containing the *bar* and GUS genes. To our knowledge, this is the first application of *Agrobacterium*-mediated transformation in *P. patula*.

6.2 Materials and Methods

6.2.1 Transfer of pAHC25 vector into *Agrobacterium*

Disarmed *Agrobacterium* LBA4404 cultures were transformed with the ampicillin resistant pAHC25 plasmid using the calcium chloride and heat shock method described in Section 4.3.1. Transformed cells were plated on YMA medium (5 g l⁻¹ yeast, 0.5 g l⁻¹ casein hydrolysate, 8 g l⁻¹ mannitol, 2 g l⁻¹ ammonium sulphate, 5 g l⁻¹ NaCl at [pH 6.6]) supplemented with 150 µg ml⁻¹ rifampicin (rif^r) and 100 µg ml⁻¹

ampicillin (amp^+) plates, in order to select for cells containing the pAHC25 vector. Selected transformed *Agrobacterium* cells were later maintained on YMB medium (0.4 g l^{-1} yeast extract, 10 g l^{-1} mannitol, 0.1 g l^{-1} NaCl, 0.2 g l^{-1} Mg_2SO_4 , 0.5 g l^{-1} KH_2PO_4 [pH 7.0]) supplemented with the appropriate antibiotics.

6.2.2 Triparental Mating of *Agrobacterium*

In addition, *Agrobacterium tumefaciens* containing pAL4404 (HOEKEMA *et al.* 1983) was mobilized via triparental mating with both pAHC25 (CHRISTENSEN and QUAIL 1996) and the helper pRK2013 (DITTA *et al.* 1980) plasmids. Separate overnight cultures of *E. coli* containing pAHC25 and pRK2013 were grown overnight at $37 \text{ }^\circ\text{C}$ in LB medium supplemented with appropriate antibiotics ($100 \text{ } \mu\text{g ml}^{-1}$ each of ampicillin and kanamycin). The *Agrobacterium* cultures were grown in $150 \text{ } \mu\text{g ml}^{-1}$ rifampicin supplemented YMA (5 g l^{-1} yeast, 0.5 g l^{-1} casein hydrolysate, 8 g l^{-1} mannitol, 2 g l^{-1} ammonium sulphate, 5 g l^{-1} NaCl [pH 6.6]) at $28 \text{ }^\circ\text{C}$ and were shaken at approximately 60 rpm for 2 d.

Aliquots of 1 ml inoculated cultures were centrifuged at 16 000 rpm (SIGMA 113 tabletop microfuge). Bacterial cells were washed twice in 1 ml water and the final pellet was resuspended in 1 ml Luria broth. Control cultures were established by plating each culture onto LB and AB medium containing appropriate antibiotics, with a sterile glass hockey stick. Mating was performed by vortexing equal portions of culture in an Erlenmeyer flask. Three hundred μl of mating samples were concentrated onto sterile $0.22 \text{ } \mu\text{m}$ Millipore filters by filtration. The bacteria-containing filters were incubated on Luria agar plates for 6 h at $30 \text{ }^\circ\text{C}$. The cells were resuspended with 1 ml suspension medium (SM) diluent (2.05 g l^{-1} KH_2PO_4 , 1.45 g l^{-1} K_2HPO_4 , 0.5 g l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g l^{-1} NaCl, 0.02 g l^{-1} CaCl_2 , 3.0 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$, 4.0 g l^{-1} glucose).

Transconjugates were selected from a 100-fold dilution series on AB minimal media (3.0 g l^{-1} KH_2PO_4 , 1.0 g l^{-1} $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.0 g l^{-1} NH_4Cl , 0.3 g l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,

Agrobacterium-mediated transformation of *Pinus patula*

0.15 g l⁻¹ KCl, 0.15 g l⁻¹ CaCl₂, 2.5 mg l⁻¹ FeSO₄.7H₂O, 2 g l⁻¹ glucose [pH 7] solidified with 15 g l⁻¹ agar) supplemented with 100 µg ml⁻¹ ampicillin, 100 µg ml⁻¹ kanamycin and 150 µg ml⁻¹ rifampicin. The exconjugates were initially streaked onto YMA (5 g l⁻¹ yeast, 0.5 g l⁻¹ casein hydrolysate, 8 g l⁻¹ mannitol, 2 g l⁻¹ ammonium sulphate, 5 g l⁻¹ NaCl, 12 g l⁻¹ agar [pH 6.6]) and subsequently maintained on LB medium with the same concentrations of all antibiotics.

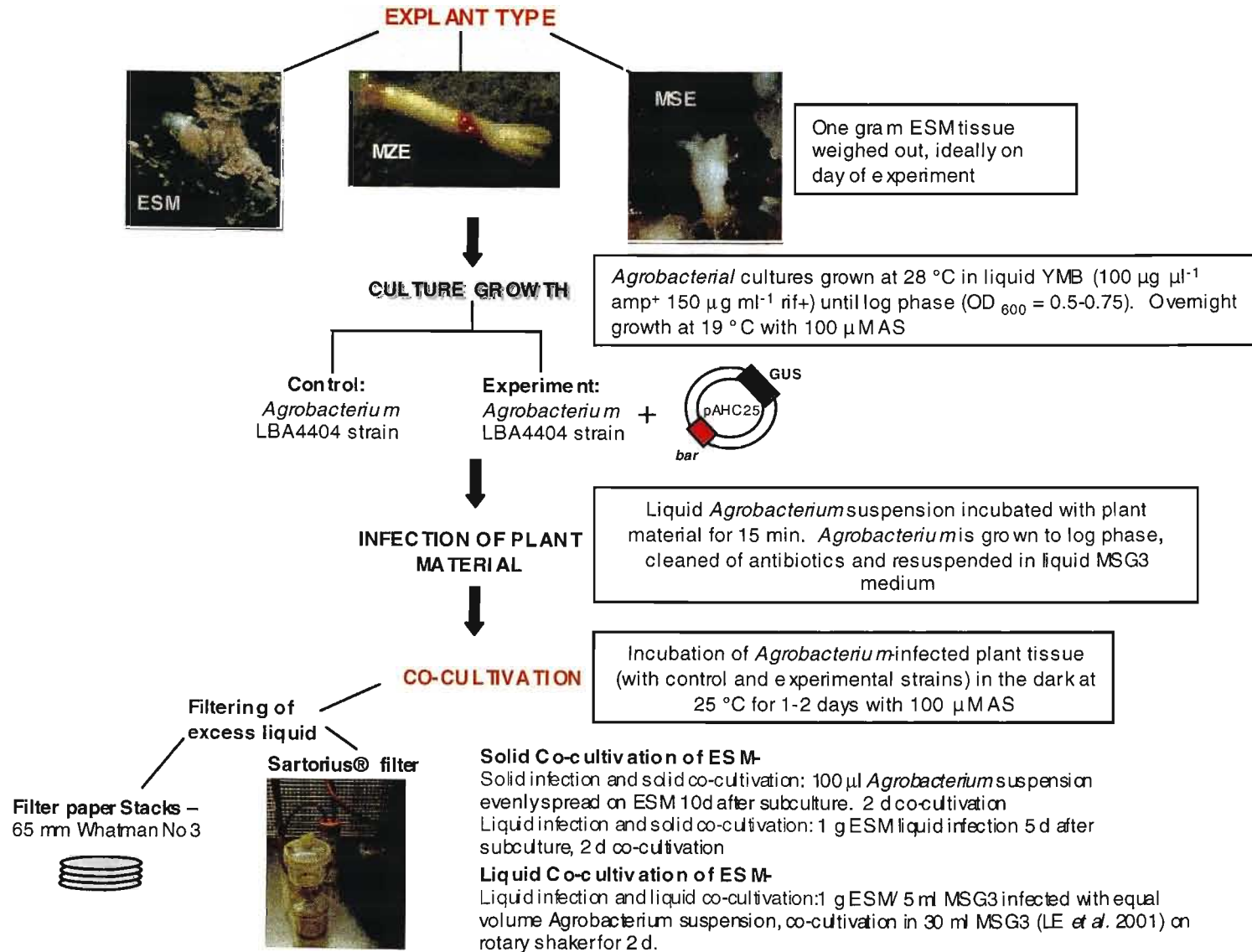
Of the two methods, the *Agrobacterium* cultures transformed with pAHC25, using the heat shock method, were maintained on solid YMA (rif⁺ and amp⁺) medium as stock cultures for gene transfer experiments. Freezer stocks of the LBA4404 *Agrobacterium* strain containing pAHC25 were made (as described in Section 4.3.1).

6.2.3 Outline of *Agrobacterium*-mediated transformation of *Pinus patula*

The efficacy of *Agrobacterium*-mediated transformations was tested using various types of explants (refer to Figure 6.1): excised zygotic embryos (open pollinated (OP1) and open pollinated (OP2)), mature somatic embryos, and solid-medium derived ESM. The *Agrobacterium* transformation methods described by LEVÉE *et al.* (1999), LE *et al.* (2001) and TRONTIN *et al.* (2002) were followed for *Pinus patula* ESM. The protocol described by CHARITY *et al.* (2002), was attempted with excised zygotic embryos maintaining the following three conditions: desiccation of cotyledons prior to inoculation; acetosyringone (AS) present in the co-cultivating media to induce virulence (BOLTON *et al.* 1986) and *A. tumefaciens* was resuspended in liquid plant medium (MSG3) instead of bacterial medium (HOLLAND *et al.* 1997). The same protocol was also used for mature somatic embryos, with some amendments, which were bombarded prior to transformation. Log phase-grown *Agrobacterium* inocula were purified from antibiotics (Section 6.2.4.2) before use. Acetosyringone was supplemented to bacterial growth media as well as all media used during co-cultivation (Figure 6.1). All *Agrobacterium* transformation experiments were carried out on media appropriate for the target tissue in 65 mm plates: MSG3 for ESM (supported on 42.5 mm Whatman No. 1 filter papers), ½DCR for MZE's and 240

medium (and derivatives) for somatic embryos. Putatively transformed tissue was placed onto media supplemented with 500 mg l⁻¹ Claforan® (Hoechst, South Africa) containing the active ingredient of cefotaxime to inhibit growth of bacterial cells, before transfer to a two-phase selection (BASTA®-containing medium) step (Figure 6.1). GUS assays for transient expression were performed at the selection stage. Samples for stable gene analysis were also taken and stored at -70 °C until further use (DNA extraction for stable integration analysis, Section 7.2.1.2). Experiments were undertaken with the *A. tumefaciens* LBA4404 strain transformed with the pAHC25 plasmid (Section 4.3.1), grown to log phase, while negative controls used an untransformed *A. tumefaciens* LBA4404 strain (Figure 6.1). The pAHC25 plasmid was also used in the biolistic transformation of *P. patula* tissue (refer to Chapter 5).

Lines 1 and 2 (derived from different *P. patula* families) used in the previous (biolistic) transformation study were continued in the *Agrobacterium*-mediated study. These lines were retained due to their successful regeneration after manipulation *in vitro* and ability to undergo genetic transformation. A further line, 3, also from a different *P. patula* family, selected for its prolific embryogenic growth was included in the study. Superior embryogenic ability of the selected lines were exhibited and Lines 1 and 3 successfully underwent plant regeneration after biolistic bombardment (Section 5.5.4). A number of other ESM lines were also tested for their amenability to *Agrobacterium*-mediated transformation. These included: Line 5, Line 6, and Line 7 (derived from the same family as Line 3). Mature somatic embryos (MSE) from Line 5, Line 6 and Line 8 also underwent *Agrobacterium*-mediated transformation.



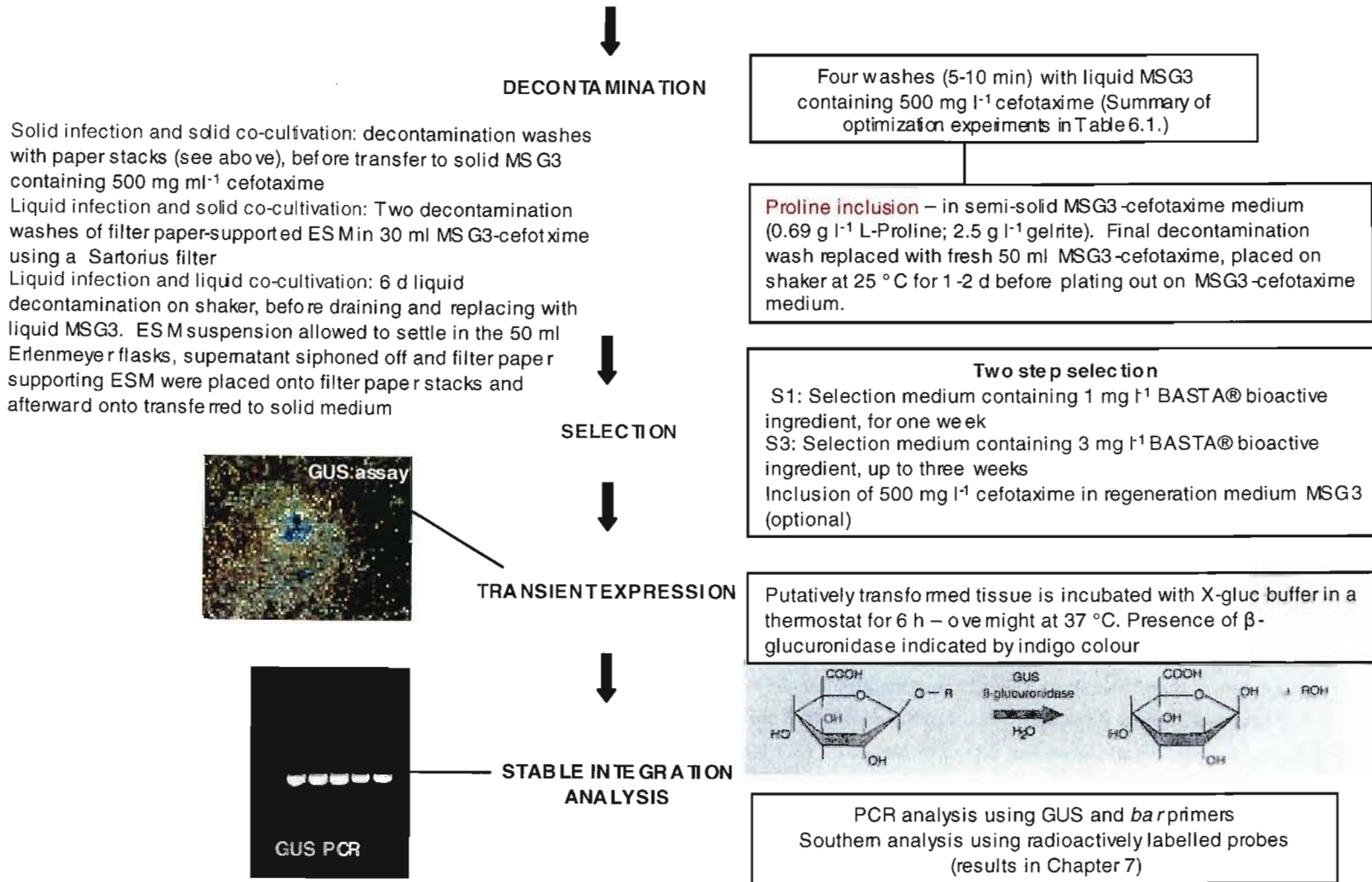


Figure 6.1 Summary of *Agrobacterium*-assisted transformation procedures using ESM as an example. A ESM – embryonal suspensor masses, B MZE – mature zygotic embryo, C MSE – mature somatic embryo, MSG3-cefotaxime – liquid MSG3 containing 500 mg l⁻¹ cefotaxime. Coloured titles indicate changes to standard protocol

6.2.4 Standard *Agrobacterium*-mediated Procedure

6.2.4.1 Preparation of Transformation Material

Rapidly growing solid medium-derived ESM were subcultured 5-7 d before the transformation event. ESM suspended in liquid MSG3 medium was filtered-off onto filter paper supports using a Sartorius® filter unit and tapered pipette tips to minimize damage to embryonal heads. After maturation, partially dried (Section 3.2.5) mature somatic embryos were bombarded (to create a wound for entry of the *Agrobacterium*) on filter paper at -0.4 bar vacuum and at 40 bar shooting pressure of the nitrogen gas before *Agrobacterium* infection on the same day. Mature zygotic embryos were dissected aseptically from surface sterilized seeds (Section 3.2.10) and placed on ½ DCR medium (half the salt concentration) supplemented with BA and TDZ.

6.2.4.2 Preparation of *Agrobacterium* Inoculum

One colony of *Agrobacterium* per ml media was grown at 28 °C in liquid YMB (amp⁺ and rif^r) until log phase, thereafter at 19 °C with 100 µM acetosyringone, 3'5'-Dimethoxy-4'-hydroxy-acetophenone, (Sigma). Bacterial suspensions were purified of antibiotics by centrifugation at 5860 rpm Hettich Universal/ K25 for 25 min at 4 °C. YMB medium was aseptically poured off before resuspending the bacterial pellet in 10 ml liquid MSG3 media. However, *Agrobacterium* pellets that were used for mature somatic embryo transformations were resuspended in YMB media instead of MSG3 liquid medium. This was to avoid a re-introduction of the plant growth hormones BA and 2,4-D to the somatic embryos during transformation as these mature structures use ABA during maturation stages (discussed in Section 2.1). Transformations were performed using *Agrobacterium* cultures grown to an optical density of 0.5 – 0.75 at 600 nm (OD₆₀₀) (Figure 6.1).

6.2.4.3 *Agrobacterium*-mediated transformation of ESM

6.2.4.3.a *Trial Agrobacterium*-mediated Experiments

Two methods of trial *Agrobacterium* transformations were attempted at the beginning of the study. Two 65 mm plates containing ESM (2 weeks after subculture) were incubated with *Agrobacterium* (transformed with pAHC25 by triparental mating) for 16 h and then filtered using Sartorius® filter units or pipetted without filtering onto MSG3 maintenance medium for 2 d co-cultivation (kept in constant dark at 25 °C). In the second method, 1 ml of *Agrobacterium*-containing pAHC25 was pipetted onto a plate containing ESM and co-cultivated for 2 d. All *Agrobacterium*-infected ESM samples were transferred to 500 mg ml⁻¹ cefotaxime-containing media. Severe overgrowth of *Agrobacterium* motivated optimization of the transformation process.

As the study progressed it became clear that the co-cultivation and most importantly the decontamination steps of the *Agrobacterium*-mediated transformations had to be optimized before a working protocol could be implemented in *Pinus patula* (ESM in particular) (see the following sections 6.2.4.3.b - 6.2.4.3.d).

6.2.4.3.b *Liquid and Solid Infection and Co-cultivation Conditions*

Different infection and co-cultivation environments were tested (see Figure 6.1) using ESM as target material. Earlier experiments or treatments used Sartorius® filter units to drain off excess liquid from the ESM-containing filter paper supports. Whatman No 3 (65 mm) filter paper stacks were later implemented to avoid excess filtering and stress to the immature embryos present in ESM.

6.2.4.3.c *Standard Decontamination Conditions*

The infected material was first washed with sterile distilled water followed by three more washes with liquid MSG3 supplemented with 500 mg l⁻¹ cefotaxime to inhibit growth of bacterial cells (refer to Figure 6.1). Decontamination washes were repeated between each treatment or subculture, for somatic embryos and MZE's in particular. Selection was implemented on solid media in a stepwise fashion with

Agrobacterium-mediated transformation of *Pinus patula*

inclusion of 1 mg l⁻¹ then 3 mg l⁻¹ BASTA® bioactive ingredient (glufosinate ammonium) for one month which was then increased to 3 mg l⁻¹ in the second month or after subculture. GUS assays were performed at this stage. Maturation and germination of putative transformed tissue continued without antibiotics or BASTA® active ingredient, at which point tissue was selected for stable integration analysis for PCR and/ or Southern hybridization.

6.2.4.3.d Optimization of Decontamination Conditions (DeCo)

Decontamination experiments (DeCo) tested the effect of antibiotics on the control LBA4404 *Agrobacterium* strain, ESM tissue only or both as performed in a transformation event (Table 6.1). The *Agrobacterium*-mediated transformation protocol was followed as closely as possible (Figure 6.1).

Table 6.1 Summary of decontamination (DeCo) experiments using solid-derived ESM

| Experiments | DeCo 1 (no ESM) | DeCo 2 (no <i>Agrobacterium</i>) | DeCo 3 (no <i>Agrobacterium</i>) | DeCo 4 | DeCo 5 |
|---|---|---|---|---|--|
| Treatment | Effect of antibiotic on <i>Agrobacterium</i> strain, full-strength and diluted | Effect of antibiotic on ESM in overnight liquid conditions | Effect of antibiotic on ESM in liquid conditions for one week | Transformation of entire ESM mass with solid media selection | Liquid co-cultivation of ESM tissue pieces with liquid selection |
| Method | Cefotaxime (500 mg l ⁻¹) evenly spread onto 10 MSG3 plates, 2 h absorption. <i>Agrobacterium</i> inoculums filtered onto filter paper supports & placed onto 5 plates each for 5 d dark incubation. | Rapidly growing tissue (1 g) placed in 30 ml MSG3-cefotaxime. Liquid suspension shaken vigorously on rotary shaker before ESM suspension plated onto solid MSG. | Treatment prepared as previously described (DeCo 2). | Solid-derived ESM masses (20 X 0.2 g)* liquid infected for 15 min in 15 ml Sterilin tubes. Agitation by gently drumming fingers on tubes. Excess suspension filtered off. | Filter papers supporting ESM pieces were placed into wide-necked 50 ml Erlenmeyer flask, infected with 5 ml <i>Agrobacterium</i> suspension (15 min on rotary shaker). |
| Time after subculture | N/A | 4 d | 4 d | 7 d (Line 5); 10 d (Line 7) | 1 d |
| Exposure to antibiotic | 5 d | 24 h | 6 d | Washes and solid decontamination media (2 weeks) | 6 d |
| Co-cultivation | Dark incubation | Incubated on rotary shaker at 140 rpm. | Rotary shaker at 140 rpm. | 2 d | 2 d on rotary shaker |
| Decontamination regime (500 mg l⁻¹ cefotaxime) | N/A After observation the filter papers were transferred to plain MSG3 medium for a further 5 d. | N/A | N/A | Liquid MSG3-cefotaxime poured over filtered ESM on filter unit (4x). Transfer to solid DeCo media before selection. Additional DeCo washes between subcultures. | 10 min sterile dH ₂ O wash on shaker, excess water filtered off, before 6 d DeCo wash in 30 ml liquid MSG3-cefotaxime on shaker. |
| <p>Selection medium - MSG3 supplemented with 3 mg l⁻¹ BASTA®; 500 mg l⁻¹ cefotaxime. MSG3-cefotaxime - MSG3 medium supplemented with 500 mg l⁻¹ cefotaxime. *Additional tissue from the periphery of another mass was taken to make up 0.2 g, if necessary. †Taken from the periphery of the ESM mass.</p> | | | | | |

6.2.4.4 Transformation of Mature Somatic Embryos

In trial experiments (Table 6.3) partially dried embryos (Section 3.2.5) were bombarded on filter paper supports before complete immersion in 5 ml *Agrobacterium* suspension supplemented with 100 μ M acetosyringone (AS) overnight. Controls were left to dry or infected with the native *Agrobacterium* strain (refer Figure 6.1). Following co-cultivation, mature somatic embryos were washed 2-3 times in sterile dH₂O supplemented with 500 mg l⁻¹ cefotaxime before transfer onto semi-solid 240 medium supplemented with 0.5% activated charcoal [w/v] and 500 mg l⁻¹ cefotaxime. In later experiments, somatic embryos were decontaminated, transferred to germination medium without hormones but still supplemented with 500 mg l⁻¹ cefotaxime and then kept in the dark for two subcultures, before transfer to 240 medium supplemented with activated charcoal to induce elongation as per usual practice.

Subsequent experiments, using partially dried embryos from Lines 5 and 6, streamlined the protocol in the following way:

- i) tissue was incubated for 15-30 min infection period in 5 ml *Agrobacterium* suspension;
- ii) embryos were blotted dry and placed on MS (MURASHIGE and SKOOG 1962) or 240 media supplemented with 100 μ m AS for a 2 d co-cultivation period;
- iii) tissue was washed three times with a sterile water solution of 500 mg l⁻¹ cefotaxime;
- iv) embryos were placed on 240 medium (without hormones or charcoal) for elongation during the first week; and,
- v) provided embryos were free of *Agrobacterium* contamination, they were then placed onto 240 charcoal containing media for the second week of elongation.

Plant growth regulators and activated charcoal were left out of the media, on the first subculture, to allow embryo elongation and to prevent the adsorption of the antibiotic to the charcoal respectively.

6.2.4.5 Transformation of Mature Zygotic Embryos (MZE's)

Fifty MZE's were initially pricked (to mimic wounding) with a sterile scalpel or needle and then air-dried for 15 min under laminar flow conditions prior to 30 min co-incubation with 10 ml *Agrobacterium* suspension. The wounding attempts were later abandoned as the piercing implements were not fine enough, and it was difficult to accomplish without support on filter paper. Control *Agrobacterium*-mediated transformations used the LBA4404 bacterial strain without the pAHC25 plasmid (refer to Figure 6.1). Thereafter the infected MZE's were blotted dry and transferred onto hormone-free ½DCR medium for a 2-3 d co-cultivation period. On the second or third day the MZE's were decontaminated with sterile dH₂O supplemented with 500 mg l⁻¹ cefotaxime as previously detailed (Section 6.2.4.3.c) and transferred onto selection medium (Figure 6.1, Section 5.4.6), decontamination washes between treatments were repeated if necessary. GUS assays were performed 3-6 d after the transformation event. A series of trial, qualitative experiments were undertaken with 2 d co-cultivation (Table 6.4), experiments were repeated using MZE's from genotypes OP2 and OP1 and recorded in Table 6.5.

6.3 Results and Discussion

6.3.1 Trial ESM *Agrobacterium*-mediated Transformations

Mobilization of *Agrobacterium tumefaciens* with the pAHC25 vector was successful as indicated by *Agrobacterium* growth on selective media containing ampicillin, kanamycin and rifampicin antibiotics at limiting concentrations. Rifampicin inhibits bacteriostatic RNA synthesis through binding and inhibition of the β-subunit of RNA polymerase. Growing *E. coli* cells are killed by inhibiting wall synthesis by

suppressed formation of the peptidoglycan crosslink using ampicillin, and protein synthesis is arrested by kanamycin binding to the L6 protein of the 50S ribosomal unit (AUSUBEL *et al.* 1988).

Initial transformations of ESM with the transformed *Agrobacterium* strain produced the following results: *Agrobacterium* infection was efficient as the target material consisted of rapidly dividing cells (GELVIN 2000) but *Agrobacterium* growth was not eliminated on solid media supplemented with cefotaxime. Subsequent amendments to the co-cultivation and decontamination conditions (DeCo) improved recovery of infected material.

6.3.2 Optimization of Decontamination and Co-cultivation Conditions for ESM

Optimization of decontamination conditions concluded the use of 500 mg l⁻¹ cefotaxime antibiotic to be effective inhibition of *Agrobacterium* growth of LBA4404 strain containing the pAHC25 vector. No further growth of *Agrobacterium* was observed on the filter paper supports either using MSG3 medium supplemented with cefotaxime or when transferred to plain MSG3 (DeCo1 of Table 6.2). The antibiotic did not seem to adversely affect ESM cultures (DeCo 2 and 3 of Table 6.2). As a result, no other antibiotics such as Timentin (CHENG *et al.* 1998) were tested. Problems experienced regarding removal of residual *Agrobacterium* cells after transformation, were related to the tissue type (friability of the ESM), and the possibility that the antibiotic was not evenly distributed on the target tissue. In the case where ESM was filtered off onto filter paper - only the embryogenic cells in direct contact with the filter paper and therefore the decontamination medium had the benefit of the antibiotic effect. Embryogenic cells in the outer layers of the ESM mass did not have access to the full concentration of cefotaxime and were at risk of being overgrown by the contaminating *Agrobacterium*.

It was further concluded that the timing of transformation (d after subculture) affected the regeneration potential. Transformation of incubated ESM material from Lines 5 and 7 (DeCo 4 in Tables 6.1 and 6.2) took place near subculture time, normally 10-4 d (see Section 3.2.3), therefore not when regeneration potential was at the optimum.

A liquid decontamination step (using rotation), replacing liquid MSG3-cefotaxime every 1-2 d, was put into practice for successful removal of *Agrobacterium* (DeCo 5 in Table 6.2) and was employed for subsequent transformation experiments. The preliminary experiments indicated that the 2 d liquid co-cultivation period should be reduced to 1 d, due to re-growth of *Agrobacterium* (DeCo 5 in Table 6.2) after cultures appeared clean in liquid decontamination, and did not regenerate. Yellow colouration of cultures (DeCo 2 and 4 in Table 6.2) during decontamination could be attributed to cefotaxime breakdown, which should be prepared on the day of use.

Decontamination washes were continued for at least three weeks in experiments that used combinations of liquid medium and solid medium for infection and co-cultivation of ESM (Figure 6.1). Although most filter-supported ESMs were observed to be free of contaminating bacteria during decontamination, varying levels of re-contamination occurred across all experiment and control treatments once all the filter discs were transferred onto plain MSG3 to aid regeneration. No further proliferation of tissue was observed once ESM discs were transferred to selection MSG3 solid medium (1 mg l^{-1} BASTA®), indicating that selection conditions were hampering the regeneration process. Samples were taken for Southern analysis (Section 7.3.3).

Table 6.2 Summary of outcomes for optimized decontamination experiments using solid-derived ESM

| | DeCo 1 no ESM | DeCo 2 no <i>Agrobacterium</i> | DeCo 3 no <i>Agrobacterium</i> | DeCo 4 | DeCo 5 |
|-------------------|---|---|--|---|---|
| Treatment | Effect of antibiotic on <i>Agrobacterium</i> strain | Effect of antibiotic on ESM in overnight liquid conditions | Effect of antibiotic on ESM in liquid conditions for one week. | Transformation of entire ESM with solid media selection | Liquid co-cultivation of ESM pieces with liquid selection |
| Outcome | No further <i>Agrobacterium</i> growth observed. | Good recovery of ESM material. Initially small areas of tissue growing with some senescence seen as browning and yellowing areas. | Growth observed though not as prolific as 1 d exposure (DeCo 2). Very few brown spots. | Persistent <i>Agrobacterium</i> on explants. No successful regeneration. | Tissue appeared brown and dead. After plating onto solid MSG3 medium, growth of <i>Agrobacterium</i> resumed. |
| Suggestion | - | - | Add 5 μ M ABA in selection to aid regeneration of stressed tissues | Inclusion of cefotaxime in selection media should be made on the day of transfer. Selection media should only have 3 mg l ⁻¹ BASTA®. Use ESM at actively diving stage, 4-6 d after subculture. | Drain and change liquid decontamination medium on shaker every 2 nd d. |

ESM – embryonal suspensor masses

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Several novel steps were implemented to optimize efficiency of the *Agrobacterium*-mediated transformation process. They included the use of sterile filter paper stacks to drain off excess liquid, a liquid decontamination phase, better consideration of the ESM regeneration capability by subculturing 1 g of tissue on the day of transformation, and the inclusion of L-proline to aid regeneration. The capillary action employed by filter stacks was a less aggressive approach to draining off excess *Agrobacterium* suspension from infected ESM compared to suction (from Sartorius® filter units), thereby aiding regeneration.

Proline seemed to aid regeneration of decontaminated material (experimental tissue appeared to proliferate) and is an optional inclusion that could be further investigated to improve the regeneration ability of putatively transformed tissue as free proline accumulates in response to stress such as drought (KEMBLE and MACPHERSON 1954). Although the control tissue appeared to senesce (brown) by the end of the experiment, it is possible that the starting target tissue was not at an optimal growth stage as these samples had appeared yellow during the washing step. Unfortunately, the experiment using proline had to be terminated due to recontamination of the material. DENNEHEY *et al.* (1994) had included L-proline to benefit recovery of overall maize callus lines but it was shown to significantly interfere with BASTA® selection. Further investigation (using other selection agents such as bialaphos) would be required to test whether the inclusion of L-proline benefits regeneration for stressed explants or if improved growth is due to interfered selection.

The two primary challenges encountered in this work were: (1) the elimination of infecting *Agrobacterium* cells; and (2) additional work needed to keep the ESM healthy enough for regeneration. Steps to improve recovery have been investigated by CHARITY *et al.* (2005) using nurse cultures to alleviate tissue damage after *Agrobacterium* inoculation and improve response to target cells during transformation. New tissue does not grow readily after co-cultivation due to the introduced stress of infecting *Agrobacterium* cells. Frequent subculture (twice weekly) of decontaminated ESM onto selection medium is suggested to avoid emergence of any residual

Agrobacterium cells. Implementation of a liquid decontamination step would greatly reduce re-contamination by *Agrobacterium*.

Another consideration with ESM as a target tissue would be the transformation ability of lines used. Few genotypes of conifer species support regeneration into somatic embryos (only 10% in *Pinus radiata*) and of these only a small percentage can be genetically transformed (GRANT *et al.* 2004).

6.3.3 Outcomes of Transformation of Mature Somatic Embryos

In general, somatic embryos have been used as target explants in particle bombardment transformations, and cotyledons have often been used in *Agrobacterium*-mediated studies (refer to Table 2.2). This study combined the wounding effect of bombardment with the *Agrobacterium* infection process on mature somatic embryos. Particle bombardment creates micro-wounds due to the impact of particles over the tissue surface area, creating possible sites for *Agrobacterium* infection (HUMARA *et al.* 1999b).

The effect of transformation on mature somatic embryos was monitored in terms of percentage germination viability and transgene analysis (Table 6.3). Line 6 had the highest percentage viable experimental embryos at 75%, with Line 8 having the lowest, but the highest percentage of surviving germinating control embryos. The percentage viability of experimental and control embryos were not similar in the lines tested. Varying lengths of co-cultivation did not enhance transient expression (or stable integration of the GUS transgene, refer to Table 6.3), nor did it influence the rate of re-contamination. Subsequent washes were necessary between subcultures, this despite rigorous decontamination washes after the transformation experiments.

Cefotaxime was maintained in all media at 500 mg l⁻¹, including the germination medium, despite an *Agrobacterium*-free appearance of mature somatic embryos. It was felt that immediate transfer to 240 germination (charcoal-supplemented) medium, as per

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normal maturation protocol (Section 3.2.6), could result in *Agrobacterium* re-growth because the charcoal was likely to absorb the cefotaxime antibiotic, eliminating effective decontamination conditions. Therefore, prior transfer to cefotaxime-supplemented germination medium, without hormones, was deemed necessary (Section 6.2.4.4). A similar precaution was discussed in Section 5.5.1 where activated charcoal masked the effect of BASTA®.

Although mature somatic embryos were readily available for *Agrobacterium*-mediated transformations, their suitability for target tissue was a concern, as it was in the biolistic study (Section 5.5.3). Transformation of this tissue type risks incomplete transformation of the whole explant as a primary transgenic (T₀) (BHAT and SRINIVASAN 2002) and therefore the production of chimaeras upon regeneration, as do MZE's (Section 6.3.4).

Table 6.3 Survival (%) and transgene analysis of mature somatic embryos following *Agrobacterium*-mediated transformation

| Genotype | Initial no. somatic embryos infected (E/C) | Treatment | Co-cultivation regime | Decontamination regime | Notes on surviving embryos | Viable germinating embryos (%) | Transgene analysis of somatic embryos* | |
|----------|--|--|-------------------------------------|---|--|--------------------------------|--|------------------------|
| | | | | | | | GUS assay | Southern hybridization |
| Line 8 | 15 E 14 C | Experimental embryos bombarded, covered with <i>Agrobacterium</i> suspension and overnight shaking. Control embryos only bombarded | 1 d on filter paper. | 3 Washes before transfer to AC. Washes necessary between transfer to different selection media. | Heavy contamination. Embryos appeared dead with only a few becoming elongated. | 0 E 57C | 4 Tested, no expression. | Negative |
| Line 6 | 8 E 5 C | Bombardment preceding <i>Agrobacterium</i> infection | 2 d | 2 Washes before placing onto HF - germination medium, no light. Subsequent washing necessary. | No control embryos elongated on germination medium with AC. | 75 E 0 C | 2 Tested, no expression. | Negative |
| Line 5 | 83 E 40 C | Bombardment preceding infection | 5 d Co-cultivation on filter paper. | 3 Washes before HF germination medium, no light. Subsequent washing necessary. | 4 E and 3 C emblings developed root hypocotyls. | 22.9 E 10 C | None tested. | Negative |

E - experiment; C - control; AC activated charcoal; HF - hormone free; washes were performed with sterile dH₂O and 500 mg l⁻¹ cefotaxime; *refer to Chapter 7

6.3.4 Transformation of Organogenic Tissue (MZE's)

Initial transformations with mature zygotic embryos (Table 6.4) dealt with two facets: the length of hormone exposure needed to induce adventitious budding, and the implementation of a decontamination regime for MZE's. Exposure to BA and TDZ ranged from 1 d to 3 weeks, but no significant budding was observed. Severe contamination was also encountered in these trials. In subsequent experiments (Table 6.5), MZE's were exposed to hormones ($\frac{1}{2}$ DCR supplemented with BA and TDZ) for 24 h – 36 h, as described in Section 3.2.10, unless otherwise specified.

Table 6.4 Outcome of trial *Agrobacterium*-mediated transformations of MZE's

| Family | OP1 | | OP2 | |
|--|--|--|---|--|
| Experiment No. | Experiment | Control | 1 | 2 |
| Exposure to hormones | 23 d | 10 d | 1 d | 3 weeks, relieved from hormones on selective media. |
| Decontamination regime (as described in Section 6.2.4.5) | Additional washes (250 mg l ⁻¹ cef) between S1 & S3 + 250 mg l ⁻¹ cef. | As described in Section 6.2.4.5. | As described in Section 6.2.4.5. | As described in Section 6.2.4.5. |
| Outcome | Cotyledonary opening but no characteristic adventitious budding. Some embryos browning on side in contact with selective medium. Severe contamination noted. | Contaminated or dead embryos on S3 supplemented with 250 mg l ⁻¹ cefotaxime. No significant budding observed. | Persistent <i>Agrobacterium</i> contamination observed. Random MZE's assayed for GUS expression. No budding observed. | Severe confluence of <i>Agrobacterium</i> . No significant budding observed. |

OP1 - Open pollinated, family coded 1; OP2 - Open pollinated, family coded 2; S1 - selection medium containing 1 mg l⁻¹ glufosinate ammonium; S3 - selection medium containing 3 mg l⁻¹ glufosinate ammonium; cef – cefotaxime used at 500 mg l⁻¹ unless otherwise described

Throughout the *Agrobacterium*-mediated transformations decontaminated MZE's were transferred to a fresh plate, separate from originally infected MZE's, where they remained decontaminated. Contaminated embryos, even after subsequent efforts of extra decontamination washes, could not be cleared of *Agrobacterium* growth, in which case they were assumed dead.

Table 6.5: Assessment (% budding, decontamination, viability) of MZE transformation observed after selection

| Genotype | Total inoculated | Total % budding | Total % decontaminated | Total % viable |
|--|------------------|-----------------|------------------------|----------------|
| Family OP1^{S3} (Experiment) | 55 | 16.36 | 60 | 27.27 |
| Family OP2^{S3} (Experiment) | 55 | 12.73 | 38.18 | 43.64 |
| Family OP2^{S1} (Experiment) | 50 | 10 | 6 | 14 |
| Family OP2^{S1} (Control) | 50 | 22 | 18 | 48 |

S3 – assessment of MZE transformation observed at S3 selection; S1 – assessment after S1 selection

The highest percentage of successful decontamination (60%) was obtained with the family OP1^{S3}, although viability was low and most embryos were dead by the S3 selection stage. Hormones were included in media up to the S3 stage (equivalent of 2-3 weeks or up 2 months as with OP2^{S3} exposure), which was later considered to be too long and exposure reduced to 1 d. Relatively high viability of 43.64% was attained with OP2^{S3}, where MZE's were placed on ½DCR supplemented with hormones for 5-6 d before wounding (Section 6.2.4.5) and subsequent infection. Although a budding response was exhibited, most MZE's turned brown by S1, and were assumed dead. Decreased viability occurred across both genotypes (Table 6.5), the longer MZE's were kept on selection media. Subsequent experiments (Experiment and Control OP2^{S1}) were assessed at the S1 selection stage.

The lowest organogenic-type responses (10%), viability (14%) and decontamination (6%) were shown in the Experiment OP2^{S1} (Table 6.5). Surprisingly its counterpart, the control experiment, exhibited the highest percentage budding (although only 10% higher) and almost 50% viability. GUS expression was tested in Experiment OP2^{S1} (Table 6.5): portions from five random MZE's were stained and incubated at 37 °C overnight (for details on methods and results refer to Section 7.2.1.1 and Section

7.3.1, respectively) and frozen for PCR analysis. Success rates of MZE decontamination or preliminary problems, such as characteristic adventitious budding, encountered by other *Pinus* researchers when implementing a new organogenesis protocol was not available in current literature (unsuccessful scientific reports are rarely published), so it was difficult to compare work generated from this study with that of other laboratories.

However, the main constraint with regards to *Agrobacterium*-mediated transformation of *P. patula* MZE's was the implementation of an organogenetic method that promoted characteristic adventitious budding. It would be expected that regeneration capability would vary between the open-pollinated families as reported in *P. radiata* zygotic embryos (TANG *et al.* 2001). As an adventitious *in vitro* protocol has not been determined for *P. patula* species in this study or reported in the literature, it was deemed impractical to continue with the organogenetic route (MZE's) for transformation material. Research into *P. patula*, genetic engineering in particular, is new and ground-breaking research. This is unlike for other species such as *P. radiata* where methods for the regeneration of plantlets from mature zygotic embryos have been available for the last 20 years (GRANT *et al.* 2004). Although MZE's were an easier explant to transform (they were easier to decontaminate than ESM), no characteristic budding was observed, as reported by PULIDO *et al.* (1992), refer to discussion on budding in Section 3.3.3. Other reported limitations to using organogenetic tissue for transformation are the labour intensive protocol, the development of an effective selection regime, as well as the regeneration of undesirable chimeric tissue (CHARITY *et al.* 2002). In the case of *Pinus radiata* adventitious shoots were chimeric, indicating that only some of the cells of origin were transformed (CHARITY *et al.* 2002), this could be the case for somatic embryos (Section 6.3.3).

There are further teething problems with the organogenetic route: organogenesis is considered to be a good system for shoot production in *Pinus* species but the low rooting frequency of the adventitious shoots hampers its application for commercial

large-scale production. VILLALOBOS-AMADOR *et al.* (2002) tried to circumvent this problem by providing a protocol for both adventitious shoot generation and rooting by *A. rhizogenes*.

Efficiency of *Agrobacterium*-mediated transformation is dependant on many different factors: *Agrobacterium* strains and vector system, concentration of bacteria, duration of co-cultivation, selection marker genes, - agents and promoters, cell concentration, explant type and quality, pre-culture, physiological state and developmental stage of inoculated tissue, wounding procedure, temperature, pH, temporal competence following wounding, and presence of *vir* gene inducers such as acetosyringone (AS) (BIRCH 1997; HUMARA *et al.* 1999b; LE *et al.* 2001; reviewed in GELVIN 2000; TANG and NEWTON 2003). Conifer genera, family and genotypes are particularly important in *Agrobacterium*-mediated gene transfer (TANG and NEWTON 2003). Acetosyringone is a phenolic compound released from wounded plant cells that specifically induces the entire *vir* region in the *Agrobacterium* and forms intermediate T-DNA molecules (STACHEL *et al.* 1985). The effects of different AS concentrations (used at 100 µM in this study) were not tested on *P. patula* tissue, but were included in both bacterial growth media and the co-cultivation medium. TANG *et al.* 2001 (who used 50 µM AS for *P. radiata* MZE transformation) suggested that addition of AS to co-cultivation medium is more important for transformation efficiency than its presence in bacterial growth medium. Growth of *Agrobacterium*, supplemented with cefotaxime, was maintained at 19 °C (FULLNER *et al.* 1996) as lower temperatures ensure that the *Agrobacterium* T-pilus remains stable (GELVIN 2003). A lower co-cultivation temperature could be considered although *vir* gene expression is optimal between 25–27 °C (reviewed in GELVIN 2003). Cefotaxime has been used in other studies to stimulate embryogenesis and regeneration in hexaploid wheat (MATHIAS and BOYD 1986). Infectivity is also dependant upon the type of organs or tissue used (TANG *et al.* 2001). Although gene transfer of DNA in plant cells has become standard practice (BIRCH 1997), genetic engineering of conifers including transformation with selection and regeneration is still difficult and can be a slow and tedious process (LE *et al.* 2001; JAYASHREE *et al.* 2003).

Efficient *Agrobacterium* methodology is well established for dicotyledonous plants, with the list of coniferous species increasing. This indirect method of gene transfer has been applied to approximately 25 species from the genera *Abies*, *Larix*, *Libocedrus*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga* (refer to Table 2.2; TANG and NEWTON 2003). Successful transfer has been carried out in *Larix*, *Picea* and *Pinus*, although transformation efficiency (irrespective of transient expression) has varied within species (TANG and NEWTON 2003). Despite efforts from laboratories worldwide, low transformation efficiency is still common. This is evident with silver birch where only a few per thousand were transformed (KEINONEN-METTÄLÄ *et al.* 1998), and 1-2 transformation events per co-cultivated embryogenic tissue with hybrid larch (LEVÉE *et al.* 1997). Low transformation efficiency was also reported in *P. radiata* detached cotyledons (CHARITY *et al.* 2002) with only one stably transformed *P. radiata* transgenic shoot derived from apical domes, shown by Southern hybridization (CHARITY *et al.* 2002). Based on these examples it might be an unrealistic assumption to expect a working *Agrobacterium* protocol that can formulate and produce stable integration from this study. Efficiency of transformation and selection are still considered as limitations to *Agrobacterium*-mediated transformations. Genotype-independent transformations in forest species remain challenging (GELVIN 2003).

The concluding thoughts resulting from this work with *Pinus patula* have shown cefotaxime to be an effective antibiotic. Post co-cultivation procedures need to be optimized and developed before an *Agrobacterium*-mediated transformation procedure can be implemented in this species of pine. Decontamination problems were related to tissue type: ESM material was more resistant to decontamination than MZE's, but inclusion of L-proline showed promise for regeneration as were the implementation of liquid decontamination steps and use of filter paper stacks. MZEs were discontinued as target material because no true-to-type adventitious budding was observed. Somatic embryos carry the concern of chimeric tissue production. Perhaps more diluted bacterial cultures should be used during co-cultivation for friable ESM tissue to ensure successful removal of *Agrobacterium* cells. A mixture of

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antibiotics could also prove effective such as the use of cefotaxime (300 mg l⁻¹) combined with vancomycine (200 mg l⁻¹) as suggested by BURGOS (*pers comm.*, burgos@CEBAS.CSIC.ES) or cefotaxime combined with augmentin (posted by maniplasmids@YAHOO.CO.IN on PLANT-TC@LISTS.UMN.EDU). Alternative antibiotics for further transformation studies could be novobiocin or timentin (LE *et al.* 2001). It is also best to establish suitable regenerable genotypes for gene transfer as no assurance can be made that transformable cell types are regenerable, despite expert handling (BIRCH 1997).

Progress, worthy of consideration, into the *Agrobacterium*-mediated transformation of *P. patula* has been described and would serve well towards producing an indirect transfer protocol for this key forestry species.

CHAPTER 7

MOLECULAR ANALYSIS OF PUTATIVE TRANSFORMANTS

7.1 Introduction

Plant transformation, over the last few decades, has become a core tool in basic and applied plant biology that can be used for crop improvement and to understand the regulation and organization of eukaryotic genes (POTRYKUS 1991; BIRCH 1997; BHAT and SRINIVASAN 2002). Transgenic technology has also been successfully applied to a variety of other biological arenas, including genetics, virology and biochemistry (BHAT and SRINIVASAN 2002).

The success of a transformation event can be monitored through Southern hybridization analysis (SOUTHERN 1975) or indicated by the Polymerase Chain Reaction (PCR) (MULLIS *et al.* 1986). The size of a PCR product can establish whether the original template contains an insertion or deletion produced in the amplicon (BROWN 1995) and provides indicative evidence for transgenic plants (POTRYKUS 1991). Southern analysis shows the integrity of the plasmid DNA following transfer into a cell (HADI *et al.* 1996) as well as the number of independent insertions of the transgene in the designated genome (NEGRUTIU 1995; BHAT and SRINIVASAN 2002).

In his assessment of plant transformation methodologies POTRYKUS (1991) warned researchers about misleading artifactual data which mask apparent successes in transformation reports. An explicit definition of proof of integrative transformation was offered based on a comprehensive list of combined prerequisite physical, genetic and phenotypic data including: (1) controls for treatment and analysis; (2) Southern hybridization showing hybridization between the host genome and the transgene, as well as evidence for absence of contaminating DNA; and (3) molecular and genetic analysis of offspring

populations. This is, however, a limitation for trees as they are slow to reach sexual maturity (BIRCH 1997). Subsequently, these criteria were revised by BIRCH (1997). A pertinent amendment is that Southern hybridizations of multiple independent transformants should use a family of generated hybridizing fragments in order to confirm that sizes of hybridizing fragments and flanking DNA are reproducible within a line, and also differ between transformed lines (illustrated in BHAT and SRINIVASAN 2002).

Significant differences in transgene expression levels have been noted in transgenic plants under identical conditions and vectors; as well as showing phenotypic variation associated (not directly) with the transgene. In some cases the selection agent has been responsible for somaclonal variation. Many of these differences have been a result of copy number and position effect of the transgene in the transformed plant (BHAT and SRINIVASAN 2002).

At present there is no gene transfer technique that is able to accurately place a transgene at a defined locus nor with any degree of control over the number of transgene copies that become integrated (BHAT and SRINIVASAN 2002). The presence of multiple transgene copies is generally believed to trigger silencing, suppressing the expression of exogenous DNA (MATZKE *et al.* 1994). The number of introduced transgene copies, determined in a Southern hybridization, would thus influence the expression of the transgene. The transformation vector may also be rearranged prior to or during integration into the genome which could lead to a loss of integrity resulting in abnormal transcripts (KOHLI *et al.* 1999). Reproducible and predictable transgene expression levels with intact transgene transfer are fundamental aims in plant transformation studies (KOHLI *et al.* 1999).

Despite improvements in the bombardment technique, the fate of introduced DNA during biolistic transformations can include gene fragmentation, multiple integrations and head-to-tail concatemers (indicative of homologous recombination during integration). Transgene rearrangements are common in transgenics derived from a biolistic route (DAI *et al.* 2001), as it is the physical force used to discharge the DNA-coated microprojectiles; which may cause these rearrangements (BHAT and SRINIVASAN 2002). Plasmid DNA could also

recombine through illegitimate recombination or be linearized and ligated to other plasmid fragments during transformation (HADI *et al.* 1996). Maximum purity and DNA integrity of introduced DNA is required to ensure successful transformation of the explant (SANFORD *et al.* 1993) and subsequent regeneration.

Visual reporter genes are a very useful means of monitoring the efficiency of the transformation protocol so putatively transformed regenerants can be distinguished amongst a heterogeneous pool of samples (MOLINIER and HAHNE 2002). Perhaps a more useful application of transient expression patterns is found in the study of gene regulation (POTRYKUS 1991). Ideally this should be done without reduced viability of the regeneration tissue as occurs with the selectable markers for herbicides and antibiotics. The GUS reporter system was predominantly used for this purpose until the green fluorescent protein (GFP) became an alternative selectable marker in the 1990's (CHALFIE *et al.* 1994). The GUS system has facilitated routine experimental manipulation and detailed gene analysis in two and three cell dimensions of many transgenic crops, bacteria and fungi of agricultural importance (JEFFERSON 1992). A primary limitation of the GUS assay is the required use of exogenous substrates that are toxic to the tissue. It is necessary to use it *in vitro* due to highly water-soluble GUS substrates (β -D-glucuronides) that are unable to permeate lipid bilayers of living membranes (JEFFERSON 1992). Development of non-destructive assays (such as GFP) allows for *in campo* molecular biology (gene analysis under field conditions) which has been successfully used in *Picea mariana*, *Pinus strobus* and *Populus* spp. (TIAN *et al.* 1999). GFP, from the jellyfish *Aequorea victoria*, can be directly visualized as emitted green fluorescence (due to an intrinsic chromophore structure) when excited with long UV or blue light (CHALFIE *et al.* 1994) allowing for non-destructive and non-invasive analysis (TIAN *et al.* 1999). It is a small protein that only requires oxygen, no substrates or co-factors, as an additional factor for its detection (MOLINIER and HAHNE 2002) and is useful for *in situ* and *in vivo* gene expression assays (TIAN *et al.* 1999). The second limitation of the GUS assay is the unreliability of using quantitative transient analysis (counting blue foci). Internet-based newsgroups (for instance plant-tc@tc.umn.edu) and various researchers agree on these limitations of the assay. Although many early transformation studies used this method to quantify the success of a

transformation event, GUS expression, displayed as blue foci per unit mass or volume of recipient cells, does decrease over 6-14 days (YIBRAH *et al.* 1994) rendering studies on transiently expressing cells meaningless (CHRISTOU 1995). Transient expression is useful as a rough guide of transformation (CHRISTOU 1995) as no expression would indicate experimental failure. However, enhancement of transient expression requires optimal conditions that are too harsh for stable transformants (CLAPHAM *et al.* 1995). Expression varies with the line used, time of subculture and the type of promoter used (NEWTON *et al.* 1992) as well as the physiological state (growth and age) of suspension-cultured cells (KUI and MORRIS 1995). The GUS assay is therefore best used as an indicator of possible transformants in experimental design and optimization.

The objective of the ensuing work was to determine, using molecular analytical methods, the transgenic state of genetically-manipulated *Pinus patula* tissue through analysis of transient expression, PCR and Southern hybridization.

7.2 Materials and Methods

7.2.1 Characterization of *Pinus patula* Transformants

7.2.1.1 β -Glucuronidase (GUS) Assay

A histochemical GUS assay was performed as a test for putative transformants. Random samples of bombarded or *Agrobacterium*-infected material were routinely tested by staining a spatula-full of ESM or embryo (somatic or MZE) with 30 μ l X-gluc buffer (0.3% X-gluc [v/v] Sigma Germany, 5 mM K-ferrocyanide, 5 mM K-ferricyanide, 0.005% Triton X-100 [v/v], 100 mM Na-phosphate buffer [0.5 M NaH₂PO₄, 0.5 M Na₂HPO₄·2H₂O], [pH 7], dissolved in methanol) during the course of the study. Samples were incubated with the X-gluc buffer in a thermostat overnight at 37 °C. Expression of the transgene resulted in a deep blue coloured precipitate (Figure 7.2; Figure 7.3). Portions of five random MZE's of Experiment OP2^{S1} (Table 6.5) were stained and frozen with liquid nitrogen, stored at -70 °C prior to PCR analysis (Figure 7.3).

7.2.1.2 DNA Extraction for Stable Gene Integration Analysis

Genomic *P. patula* DNA was extracted using a urea-based extraction protocol. Approximately 0.1 g ESM or target material was frozen using liquid nitrogen to arrest cellular activity and then ground to a fine powder using a pestle and mortar. The cellular powder was transferred, prior to thawing, to sterile 1.5 ml microfuge tubes in which 500 µl urea extraction buffer (7 M urea crystals, 5 M NaCl, 1 M Tris/Cl [pH 8.0], 0.5 M EDTA, 20% sarkosyl [v/v]) was placed and then vortexed for 10 sec. Phenol: chloroform (1:1) [v/v] was added to the cell extract and shaken on a table top shaker at 120 rpm for 1 h at room temperature. After centrifugation (15 min at 15 000 rpm in a desktop SIGMA 113 microfuge), the supernatant, containing the nucleic acids, was transferred to fresh microfuge tubes. The DNA was precipitated with a tenth volume 4.4 M ammonium acetate [pH 5.2] and an equal volume of ice-cold isopropanol, mixed well by inversion and placed at -20 °C for 15 min. Nucleic acids were collected by a 15 min centrifugation at 15 000 rpm (desktop SIGMA 113 microfuge) and subsequently purified using 70% ethanol [v/v] and air-dried. Isolated genomic DNA was stored in 20 µl ultra pure water (BDH, England) at -20 °C.

Later, DNA extractions for stable gene integration analysis were performed using a DNeasy Plant Mini isolation kit (Qiagen, Germany), according to the manufacturer's instructions.

7.2.1.3 Polymerase Chain Reaction (PCR)-Mediated Gene Detection

Stable integration of the *bar* and GUS genes in *Pinus patula* tissue was tested for by using PCR-mediated amplification of the desired gene. The GUS primer sets 5'-GGTGGGAAAGCGCGTTACAAG-3'/5'-GTTTACGCGTTGCTTCCGCCA-3', donated by Dr D. Berger (Agricultural Research Council, Roodeplaat, South Africa), yielded a fragment of 1.2 kb after electrophoretic analysis on a 1.5% agarose gel [w/v]. HPLC-grade water was used in all instances to either make up desired PCR volumes or primer concentrations. The *bar* primer sets 5'-CATCGAGACAGCACGGTCAACTTC-3'/5'-ATATCCGAGCGCCTCGTGCATGCG-3' (LEMAUX *et al.* 1996) were synthesized by Roche Products (South Africa) and

yielded a 0.34 kb *bar* fragment if template was present. The homology of *bar* primers was confirmed using the Basic Local Alignment Search Tool (BLAST®, <http://www.ncbi.nlm.nih.gov/BLAST/>). MZE samples were tested individually, while mature somatic embryos (MSE's) were exclusively tested for stable expression using Southern analysis (see Section 7.2.1.4).

7.2.1.3.a Amplification of the *GUS* gene

The GUS amplification protocol as described by HARE (1998) consisted of 50 µl total volume reaction using 100 ng genomic template DNA, 2.5 units of *Taq* DNA polymerase (Roche, Germany), 0.5 µM of each primer or 1 µl of each primer at 25 µM, 1 µl of each dNTP: dATP, dTTP, dCTP and dGTP at 10 mM for a final concentration of 200 µM, and 5 µl PCR buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin [w/v]). Dimethyl sulphoxide (DMSO), 10% [v/v] addition, was also included in the reaction mix to enhance the efficiency of the PCR (WINSHIP 1989) (Figure 7.4 A). The PCR contents were mixed well and all samples (codes listed in Table 7.2) were overlaid with an equal volume of paraffin oil prior to undergoing 36 amplification cycles using a Hybaid Thermal Reactor thermal cycler (England). An extension step of 5 min was employed during the last cycle and a modification of 1 min at 94 °C was held at the beginning of the PCR reaction. The first 35 cycles each comprised a 94 °C denaturing temperature (30 sec), a 60 °C annealing step (30 sec) and a 72 °C primer extension step (45 sec).

7.2.1.3.b Amplification of the *bar* gene

Several PCR regimes were tested in order to detect the *bar* gene in transgenic lines and these included:

- i. The same reaction mix and cycling regime as described by HARE (1998) for the GUS gene (using the *bar* primer sets) (Section 7.2.1.3.a). Further modifications included the use of a single primer (5'-GTTTACGCGTTGCTTCCGCCA-3') and further PCR reactions used a double concentration of a single primer in 50 µl volume;
- ii. The reaction setup of FALCO *et al.* (2000) was followed. PCR conditions used were 94 °C (4 min) followed by 30 cycles of 94 °C denaturing

temperature (1 min), 55 °C annealing step (1.5 min) and 72 °C primer extension step (2 min). A 7 min extension at 72 °C was the final step of the cycling regime;

- iii. Three hundred nanograms DNA in a 50 µl volume were amplified using the PCR regime described by LEMAUX *et al.* (1996), who used a DNA concentration range of 250-500 ng. Another two experiments were run in a 25 µl volume, amplifying 250 ng DNA: one using the PCR regime described by LEMAUX *et al.* (1996), the other using half the amount of dNTP's and primer concentration. Formamide, a low molecular weight amide was included in both reactions to enhance PCR amplification (SARKAR *et al.* 1990).
- iv. Further experiments were designed to optimize *bar* PCR reactions by determining the effect of PCR enhancers, Mg⁺⁺ and the amount of the *Taq* enzyme (0.5 or 1 unit) (Table 7.1). Each amplification reaction (25 µl total volume) contained 250 ng genomic DNA or plasmid DNA for the control, 10 mM of each of dATP, dGTP, dCTP, dTTP mM, 1 µM *bar* primer (5'-ATATCCGAGCGCCTCGTGCG-3') and 1X PCR buffer.

Table 7.1: Optimization of PCR reactions to elucidate the presence of the *bar* gene

| | 1x PCR buffer | Mg ⁺⁺ (mM) | Taq enzyme units | PCR Enhancers | | |
|-------------|---------------|-----------------------|------------------|---------------|------------------|---------------|
| | | | | DMSO % [v/v] | Glycerol % [v/v] | Formamide (M) |
| Control 1 | Roche | 1.5 | - | 10 | - | - |
| Control 2 | Roche | 1.5 | 0.5 | 10 | - | - |
| Control 3 | Roche | 1.5 | 0.5 | 10 | - | - |
| Reaction 1 | Roche | 1.5 | 1 | 10 | - | 0.7 |
| Reaction 2 | Roche | 1.5 | 1 | 10 | - | - |
| Reaction 3 | Roche | 1.5 | 0.5 | - | 5 | 0.7 |
| Reaction 4 | Roche | 1.5 | 0.5 | - | 5 | - |
| Reaction 5 | Roche | 1.5 | 1 | 10 | 5 | - |
| Reaction 6 | Roche | 1.5 | 1 | 10 | - | - |
| Reaction 7 | Roche | 1.5 | 0.5 | - | - | 0.7 |
| Reaction 8 | Perkin Elmer | 0.75 | 0.5 | - | - | 0.7 |
| Reaction 9 | Perkin Elmer | 1.5 | 0.5 | - | - | 0.7 |
| Reaction 10 | Perkin Elmer | 3 | 0.5 | - | - | 0.7 |
| Reaction 11 | Perkin Elmer | 0.75 | 1 | 10 | - | - |
| Reaction 12 | Perkin Elmer | 1.5 | 1 | 10 | - | - |
| Reaction 13 | Perkin Elmer | 3 | 1 | 10 | - | - |

Control 1 excluded *Taq* enzyme; Control 2 excluded DNA; Control 3 used unbombarded genomic DNA. Roche 10X buffer (100 mM Tris-HCl [pH 8.3]; 15 mM MgCl₂, 500 mM KCl; 0.1 % gelatin [w/v]); Perkin Elmer buffer (100 mM Tris-HCl [pH 8.3]; 100 mM KCl)

- v. Amplification of the *bar* gene was finally achieved using PCR conditions described by VICKERS *et al.* (1996): 3 min denaturation period at 94 °C followed by 35 cycles of 2 min at 55 °C, 2 min at 72 °C, and 1 min at 94 °C, with a final extension period of 72 °C for 5 min. The protocol consisted of a 25 µl total volume reaction using 2.6 units Expand High Fidelity *Taq* DNA polymerase (Roche, Germany), 1 µg genomic DNA, 2 µl of each primer at 25 µM, 200 µM of each dNTP (dATP, dTTP, dCTP and dGTP) 1.5 mM MgCl₂, 2.5 µl Expand High Fidelity DNA polymerase buffer (20 mM Tris-HCl [pH 7.5] 25 °C, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween® 20 [v/v] 0.5% NonidetP40 [v/v], 50% glycerol [v/v]). A similar reaction was set up to amplify control DNA (pAHC25 plasmid) and then PCR products were electrophoresed on 1.5% agarose gel [w/v] (Figure 7.4 D).

Upon completion, of the PCR amplification, reaction mixes were stored at -20 °C until electrophoresis on 0.8% or 2.0% agarose gels [w/v]. For each PCR, positive control (isolated pAHC25 plasmid) and negative control reactions (no DNA or untransformed tissue) were included.

7.2.1.4 Southern Analysis with Downward Transfer

The physical presence of the *bar* or GUS transgenes in samples of genetically modified tissue was tested by Downward Southern hybridization (KOETSIER *et al.* 1993). The *bar* probe was generated through double digestion of the pAHC25 plasmid for the *bar* gene only (Section 4.3.5) or through PCR for both the *bar* and GUS probes (Section 7.2.1.3) as was the case with later hybridization experiments. Genomic *P. patula* DNA (10 µg) was digested with *Hind*III at 37 °C for 2 h, electrophoresed in a 0.8% agarose gel [w/v] (CHOWDHURY and VASIL 1992; YAO *et al.* 1997), and transferred to a Zeta-Probe® Genomic Tested (GT) blotting membrane (BIO-RAD, USA) or Hybond™-N+ (Amersham, United Kingdom) (methodology of Southern analysis outlined in Figure 7.1).

In order to ensure that no remaining backbone of the pAHC25 plasmid was transferred to the bombarded ESM, a control Southern hybridization experiment was included (results not shown). The transformation pAHC25 vector was digested using the double endonuclease method described in Section 4.3.5 to release the fragments of the vector backbone as well as the selectable *bar* gene and reporter GUS gene.

Post-hybridization washes for the Zeta-Probe® blotting membrane were carried out according to the membrane manufacturer's instructions, with a few amendments (Figure 7.1). Further optimization for post-hybridization washes were tested using the Roche Instruction manual (DIG High Prime DNA labelling and detection starter Kit I) (Figure 7.1).

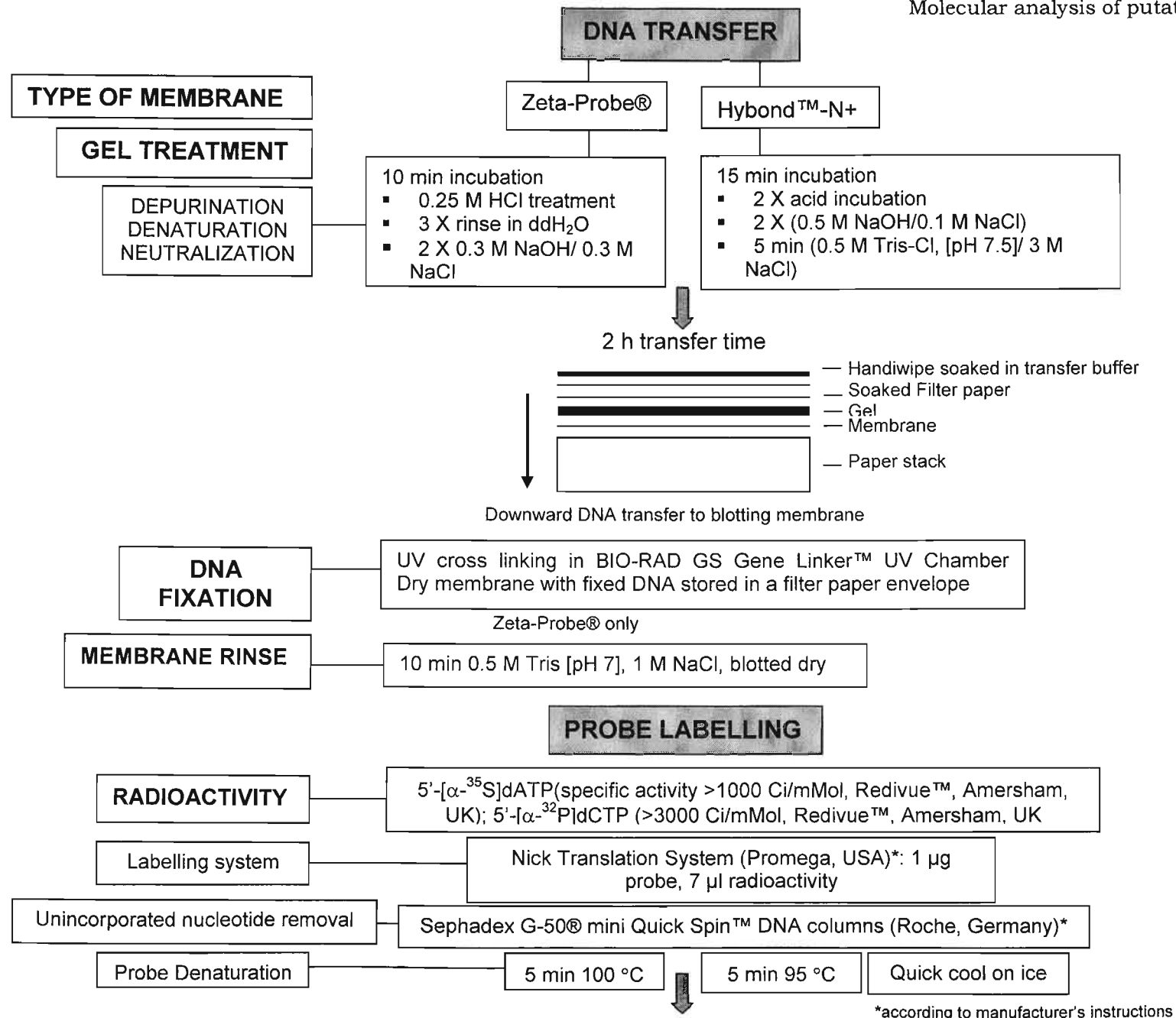
After preliminary Southern hybridizations, it became apparent that the process needed to be optimized. Attempts to optimize the Southern hybridizations were carried out using the following amendments:

- i. Both the *bar* and GUS genes (prepared through PCR) were used as probes;
- ii. The probe was labeled with 5'-[α -³²P]dATP instead of 5'-[α -³⁵S]dATP;
- iii. The Hybond™-N+ membrane substituted the use of Zeta-Probe® membrane (Figure 7.1);
- iv. Stringency of post-hybridization washes (controls particularly) were increased;
- v. Roche post-hybridization wash conditions were tested (Figure 7.1);
- vi. Less probe was used during hybridization with a longer development period.

Positive controls (isolated pAHC25 plasmid or purified *bar* or GUS probe) and negative controls (untransformed tissue) were included in each Southern hybridization experiment. Five to eight somatic embryos or MZE's, from the same transformation event, were randomly pooled for DNA extractions in order to yield enough DNA (approximately 0.01 g) to test stable expression in the putatively transformed tissue (Figure 7.8). Germinated somatic embryos were tested individually for stable expression.

A series of control hybridizations were performed to test the efficacy of the optimized Southern hybridization protocols (results not shown): GUS and *bar*

probes (generated through PCR) were transferred to Hybond™-N+ membranes with pAHC25 positive controls and subsequently hybridized.



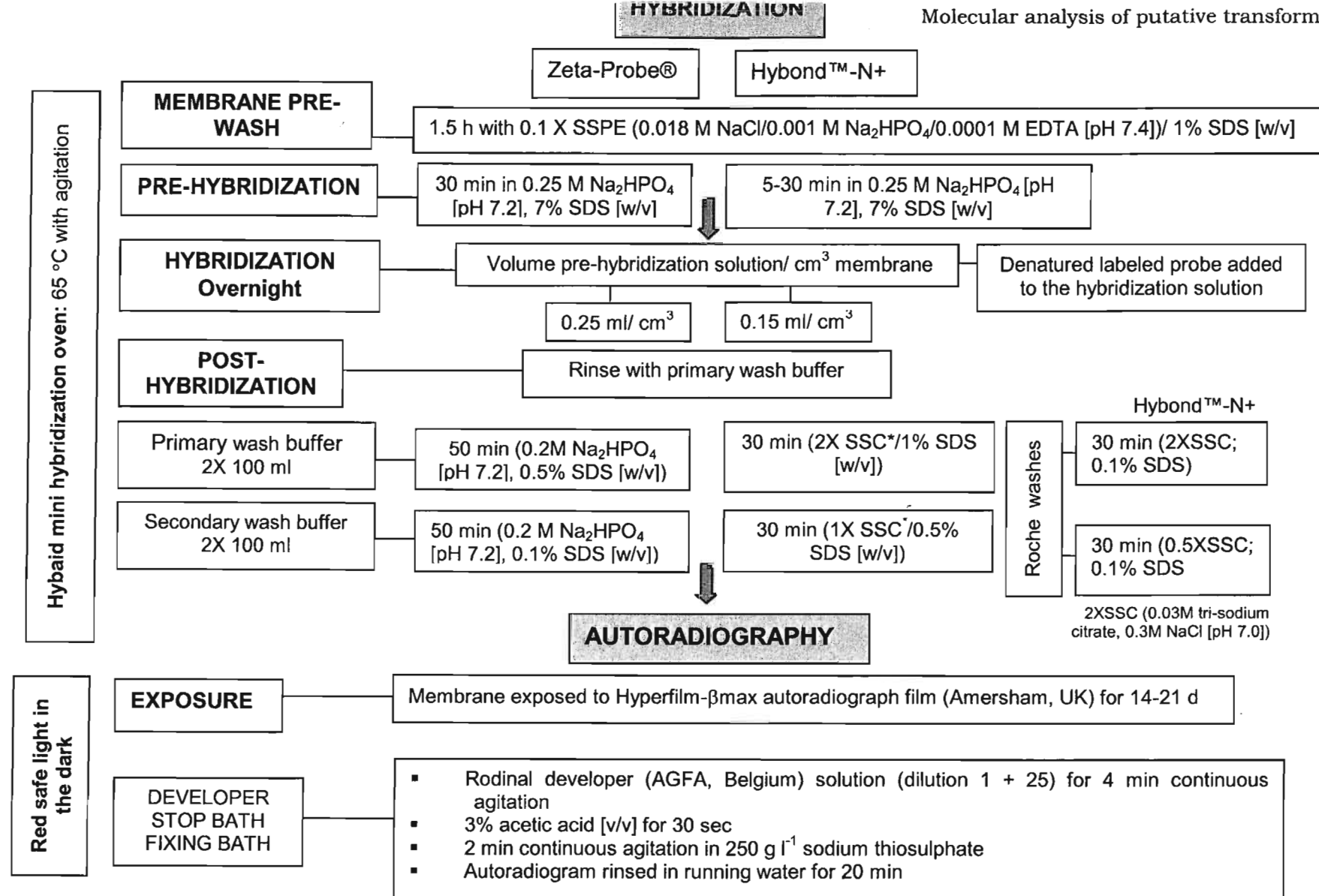


Figure 7.1 Schematic methodology for Southern hybridization analysis

7.3 Results and Discussion

7.3.1 β -Glucuronidase (GUS) Assay

The use of the GUS assay as an indicator of transient transformation for ESM and MZE samples was successful. No GUS activity was observed in non-transformed cultures or control transformations that did not have DNA.

Histochemical GUS expression in bombarded cultures resulted in entire blue ESM cultures (Figure 7.2 A-D). Higher magnification revealed that the embryonal head (Figure 7.2 C and D) had expressed the β -glucuronidase enzyme as they had turned a turquoise-blue colour, as expected. This was consistent with the findings of BOMMINENI *et al.* (1994), who observed that most GUS foci were present in embryonal heads of immature embryos. Bombarded samples exhibited a range of expression strength of the β -glucuronidase enzyme, seen in the range of chromogenic blue stain (dark to light or clear indicating no expression) as in wells 3, 9 and 10 (Figure 7.2 A).

Portions of MZE's were stained to test GUS activity 6 d after the *Agrobacterium* transformation event of Experiment OP2^{S1} (refer to Table 6.5). No initial GUS expression was observed. However, latent expression (2 weeks storage at -70°C) or intercellular diffusion of X-gluc products (GUIVARC'H *et al.* 1996) resulted in 40% positive GUS expression (Figure 7.3). No GUS activity was detected in *Agrobacterium* as this construct (intron-GUS) is not expressed in bacterial cells (VANCANNEYT *et al.* 1990; BIRCH 1997). Of the samples (2/5 portions of MZE) tested, 40% were positive for GUS transgenes. According to these transgenic tests, some of the portions of MZE's clearly contain the GUS reporter gene, indicating possible transgenic status.

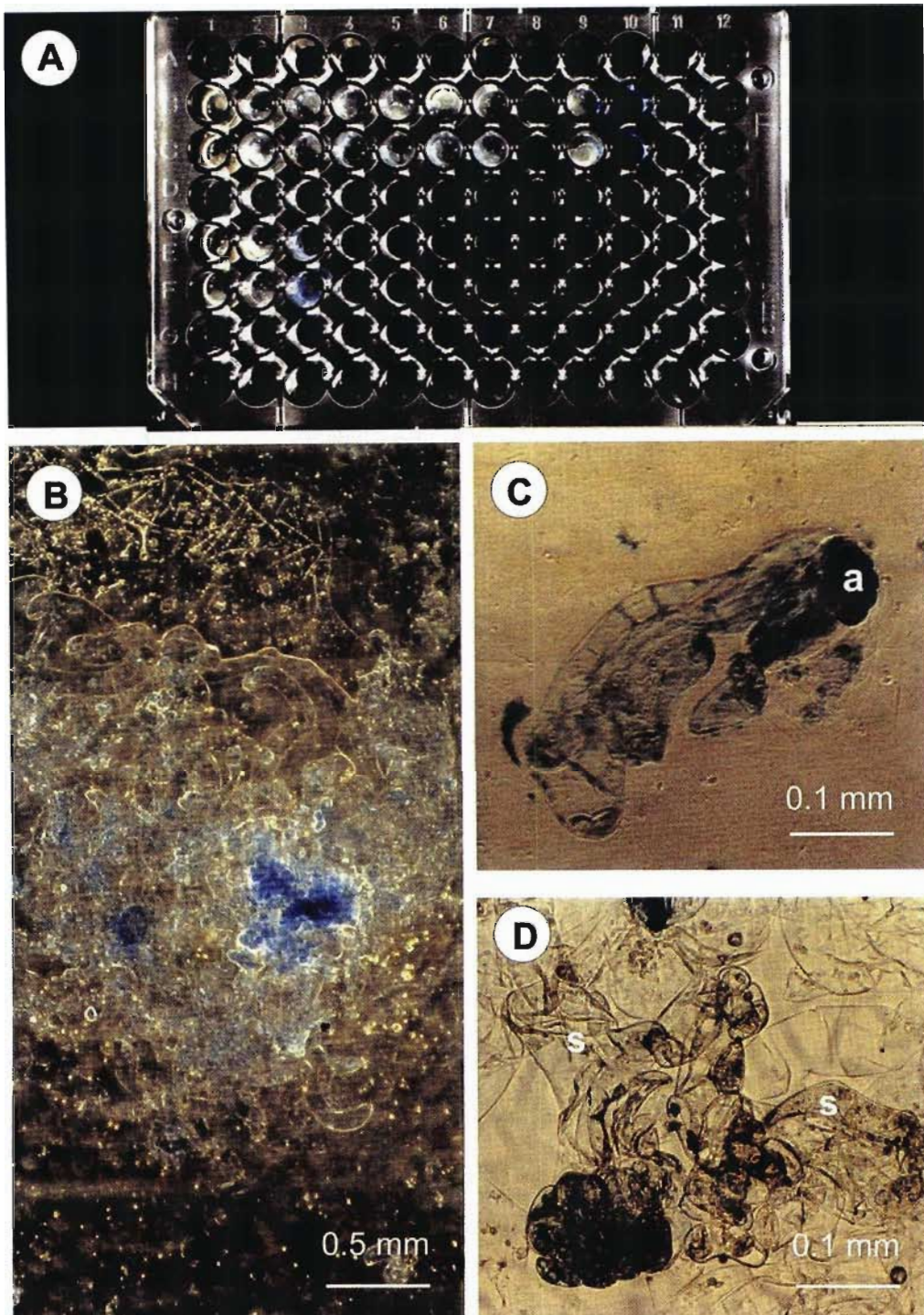


Figure 7.2 Histochemical analysis of bombarded material using the GUS assay. Transient expression of the β -glucuronidase enzyme encoded by the GUS reporter gene is indicated by a turquoise-blue colour. (A) Microtitre plate containing bombarded samples expressing the GUS reporter gene in wells 1-10. (B) Microscopic analysis of putatively transformed ESM. (C) Single embryo indicating successful uptake of GUS construct into DNA packed embryonal head as apex (a) of proembryo is stained blue. (D) A transformed somatic embryo, suspensor cells (s) contain scattered nuclei which stained blue as GUS was incorporated into the cell

GUS staining of other mature structures included mature somatic embryos (refer to Figure 5.2 D) where peripheral blue spots were observed reflecting that only the outer cells (see discussion Section 5.5.3) were putatively transformed. GUS expression is typically expressed in certain parts of somatic embryos (ARONEN *et al.* 2002), and probably MZEs too. Similarly, ELLIS *et al.* (1994b) also found GUS expression patterns were uniform in all cells of embryonic callus in *Picea glauca* but generally concentrated in outer vascular and epidermal regions of somatic embryos. However, Southern hybridization tests are a necessary part of transgenic analysis to conclusively prove the presence of the transgene (see Sections 7.2.1.4 and 7.3.3).

A transient GUS assay or screening of the number of GUS foci, though useful, was not relied upon, during the biolistic or *Agrobacterium*-mediated transformations, as an indication of successful transgene transfer, since β -glucuronidase expression was not always evident or it was faint. Unreliable or inhibited *uidA* expression in pine needles was also reported by GRANT *et al.* (2004), due to the presence of unidentified compounds. Low levels of GUS activity could be due to gene silencing mechanisms (as with DAI *et al.* 2001). Other problems that can occur with the GUS assay are non-specific localization of GUS expression and blue precipitates at sites other than those of GUS activity, which can be corrected with increased potassium ferricyanide concentration (GUIVARC'H *et al.* 1996). These spurious expressions manifest when tissues contain high levels of peroxidase activity (GUIVARC'H *et al.* 1996). In addition high transient GUS expression is not linear to stable integration (HUMARA *et al.* 1999b; CHARITY *et al.* 2002) and thereby cannot be taken as a true indication of successful gene transfer.

Differences in transient expression levels can be expected in different tissue types, cell cycles and size of receptor cells (CHAREST *et al.* 1993; DUCHESNE and CHAREST 1991; TZFIRA *et al.* 1996; TANG *et al.* 2001) and, in the case of biolistic transfer, the type of microparticles used. Further biological factors important to transient expression are: promoters and time after subculture (YIBRAH *et al.* 1994). Higher levels of GUS expression have been detected using the MUG assay in embryogenic cell lines bombarded with gold rather than

tungsten particles (CHAREST *et al.* 1993). Furthermore the fluorescent MUG assay has been reported to be more sensitive for the detection of GUS activity than the histochemical GUS assay (CHAREST *et al.* 1993).

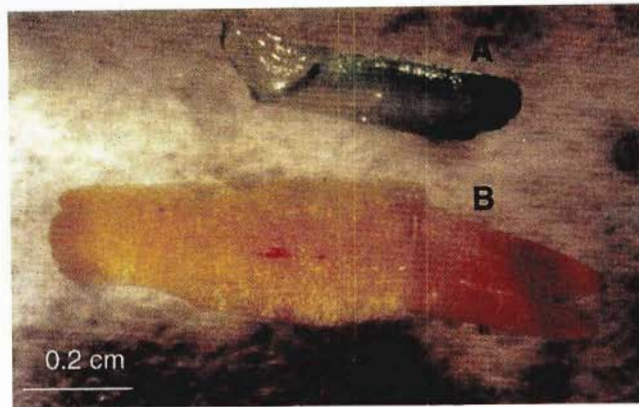


Figure 7.3 Positive GUS expression (indigo colour) of MZE portion (A), control MZE (untransformed) without expression (B)

Improved transient GUS expression has also been noted with longer *Agrobacterium* co-cultivation periods (up to 2 weeks) as with *Pinus taeda* zygotic embryos (TANG *et al.* 2001). These authors also increased sonication time (more than 60 sec). However, this led to a substantial decrease in survival.

7.3.2 PCR-Mediated Gene Detection

7.3.2.1 PCR of bombarded samples

DNA extractions from ESM or other plant material successfully yielded DNA samples of 0.5–2 $\mu\text{g g}^{-1}$ fresh weight, with the urea-based protocol, or an average of 10 μg using the Qiagen kit in a 20 μl volume protocol. DNA extractions for pooled samples of mature somatic embryos or MZE's yielded 1 μg –3 μg genomic DNA. The PCR reaction for amplification of the GUS gene was successful once DMSO was included to improve reaction efficiency. The GUS transgenes, (Figure 7.4 A), were resolved on 0.8% agarose gels [w/v] at 1.2 kb. An estimated 40% transformation efficiency was concluded from the first samples tested (14/36 samples) with a total transformation efficiency of 45% (18/40 including Line 5 samples). Biolistic experiments was carried out for Line 5 ESM masses, these

samples were tested for stable gene incorporation through Southern hybridization analysis but no hybridization was observed to any of the genomic samples using a GUS probe. Four samples were taken for GUS PCR analysis to reveal their transgenic status and all elucidated GUS amplicons, at 1.2 kb (Figure 7.4 C). Only a few of Line 5 samples tested positive for GUS activity, these low expression levels could be due to gene silencing mechanisms (as with DAI *et al.* 2001).

These results indicated that embryogenic tissue of this species was amenable to genetic transformation and the GUS reporter gene could be incorporated into, and expressed in, the *P. patula* genome. This also indicated efficient splicing of the eukaryotic intron during transcription.

The initial difficulty obtaining amplification of the *bar* gene concurred with VICKERS *et al.* (1996) and CHARITY (*pers comm.* 2001) who, like other laboratories in the world, experienced difficulties in obtaining a PCR-based assay sensitive enough to screen for the *bar* gene. Problems of *bar* amplification were attributed to the high GC content (68.3%) present in the gene. Erratic results of *bar* amplification were best confirmed as PCR products by probing a Southern blot with a *bar* probe (VICKERS *et al.* 1996), (similarly executed in this study, see Figure 7.6). Southern hybridization was used to establish stable integration of introduced DNA (the *bar* gene in particular) as well as the determination of the number of copies introduced that would influence the level of transgene expression.

Optimization of *bar* PCR reactions (Table 7.1) were not successful and Reaction 5 (1.5 Mg⁺⁺ mM, 1 unit *Taq*, 10% DMSO, 5% glycerol) was the only experiment that produced faint DNA smears. The *bar* amplicon was resolved at 0.34 kb (Figure 7.4 D) using the regime described by VICKERS *et al.* (1996). Of the 36 samples initially tested, 17 contained positive *bar* amplicons, 25/47 in total (including Line 5 samples), resulting in a higher transformation efficiency (47% or 53% total) than GUS. Perhaps the smaller gene was easier to incorporate into the genome and was expressed at a higher rate during selection. This indicated that co-integration of both the reporter GUS gene and the herbicide resistant *bar* gene did not always occur.

Impact distribution of DNA-coated tungsten particles in bombarded ESM was usually observed as an uneven dispersal, with a tendency towards central microparticle dispersal in ESM. The lack of uniformity in bombardment could have led to cell death in some zones and a total lack of gene transfer in others (KLEIN 1995). This was reflected in the GUS PCR results in Figure 7.4 A when two samples from the same bombarded tissue mass did not both amplify the desired transgene; as in the case of samples 1a, 2b, 5a and 7b. This could be a potential disadvantage to using particle bombardment as a transformation vehicle leading to partial and non-uniform gene transfer to explants.

One of the advantages of using PCR for transgene analysis is the quick delivery of reliable results. With this in mind, PCR should ideally be used as a rapid diagnostic and identification tool in transformation studies (TANG *et al.* 2001) circumventing selection that can be toxic towards regenerating tissue (noted in Section 5.5.1) and samples are likely to exhibit transient expression. Cross-protection by secreted products of contaminating micro-organisms or selection-resistant mutants are further deterrents to using selection as sole confirmation of transformation (BIRCH 1997). Furthermore, the outcome of the biolistic and *Agrobacterium*-mediated transformations has been a decreasing amount of available tissue, due to selection and decontamination steps, for analysis and regeneration – a constraint already apparent when it comes to Southern hybridization analysis that requires 10 µg DNA (see Section 7.2.1.4). Intervention of PCR earlier in the regeneration regime would aid tissue conservation and be more cost-effective, as less preparation of media would be needed.

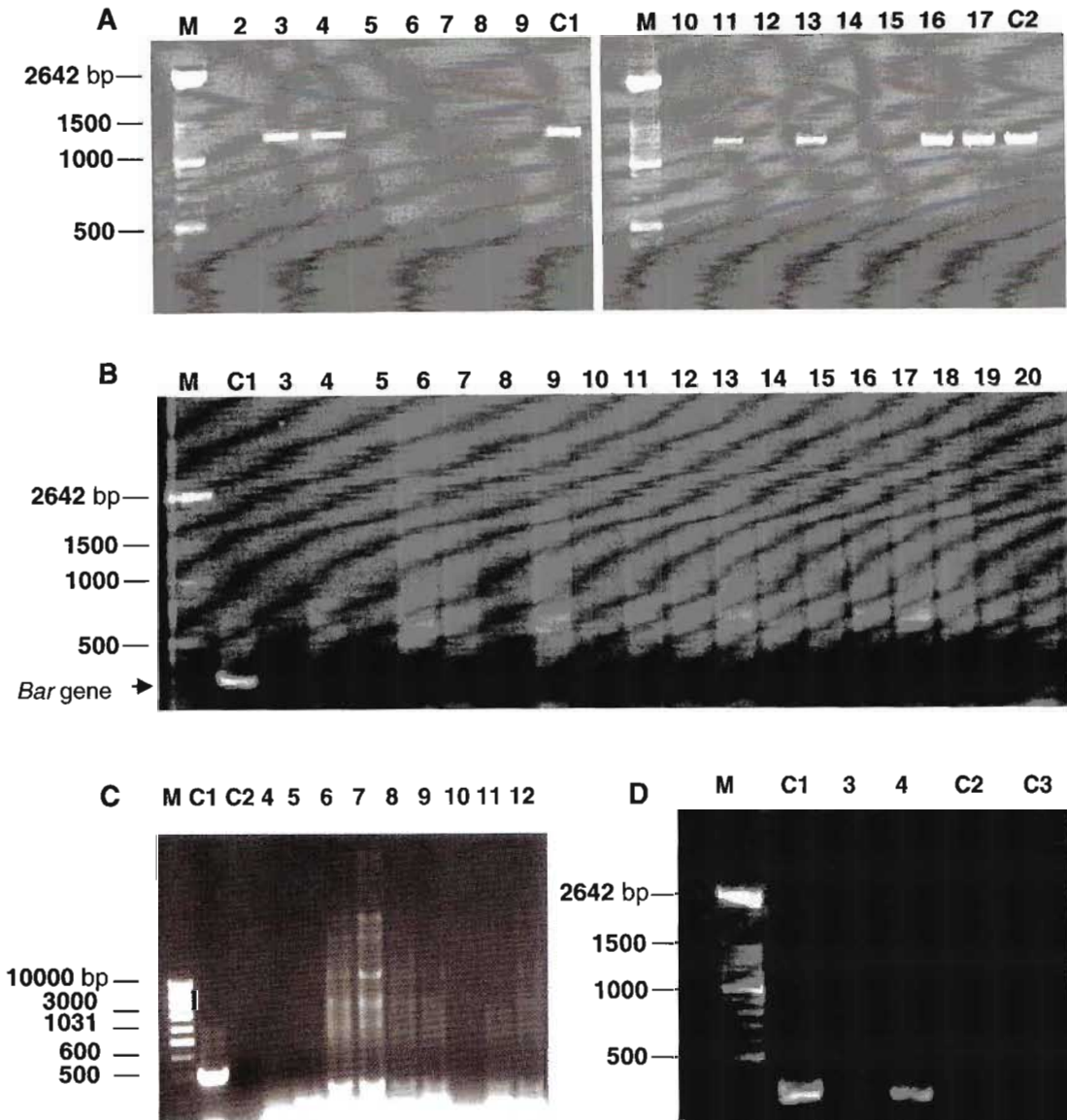


Figure 7.4 Analysis of putative transformants using PCR. (A) GUS products (1.2 kb) from bombarded ESM (HARE 1998). The DNA contents of the lanes are: lane M – MXIV (Roche); lanes 2-17 are samples of genomic DNA bombarded tissue (1a, 1b–8a, 8b) (see Table 7.2); C1 and C2 indicate positive controls of pAHC25. (B) Spurious amplification of the *bar* gene (VICKERS *et al.* 1996) in preliminary bombarded samples. Lane C1 - positive control; lane C2 – negative control lanes 3-20 are samples of genomic bombarded DNA (samples 1a-9b). (C) Non-specific *bar* amplification of bombarded Line 5 ESM masses. Lane M - DNA Ladder Mix (100-10000 bp range, GeneRuler™ Fermentas, USA); lane C1 – positive control, *bar* probe; lane C2 – negative control, no DNA; lanes 4–12, 17-18 – genomic *P. patula* DNA, bombarded Line 5 ESM samples. (D) Positive PCR analysis using the method of VICKERS *et al.* (1996). Lane M – MXIV (Roche); lane C1 - positive control of pAHC25; lanes 3 and 4 - genomic samples of bombarded *P. patula* (1b; 5b); lane C2 - negative control, non-bombarded *P. patula* genomic DNA; lane C3 - negative control, no genomic DNA included in the reaction

Although spurious *bar* amplification was observed in Figure 7.4 B and Figure 7.4 C, the addition of formamide or investigations into practical recommendations to optimize PCR's by INNIS and GELFAND (1990) could ameliorate artifactual amplification. Evidence from these GUS and *bar* PCR results, and indications from histochemical analysis (Section 7.3.1), confirm that tissue from lines 1, 2, 3 and 5 contain the GUS and *bar* transgenes derived from the pAHC25 plasmid. This indicative gene transfer verification suffices to show that ESM is an appropriate target for genetic manipulations. This therefore revealed the transgenic status of *P. patula* ESM using the biolistic protocol described by NIGRO *et al.* (2004).

Table 7.2: Qualitative analysis of genomic samples of bombarded *Pinus patula* ESM

| Coding number | ESM Genotype and Treatment | Stable Integration Analysis | |
|---------------|----------------------------|-----------------------------|----------|
| | | PCR | Southern |
| 1a – 5b | Line 3 untreated | GUS <i>bar</i> | - |
| 6a – 10b | Line 3 untreated | GUS | - |
| 11a – 12b | Line 1 myo-inositol | <i>bar</i> | - |
| 13a – 15b | Line 2 sorbitol | <i>bar</i> | - |
| 16a – 19b | Line 3 myo-inositol | GUS <i>bar</i> | - |
| 20a – 23b | Line 3 unsealed overnight | - | 0 |
| 24a – 25b | Line 3 untreated | - | 0 |
| 26a – 29b | Line 3 untreated | - | 0 |
| 30a – 34b | Line 3 sorbitol | - | 0 |
| 35a – 46b | Line 3 sorbitol + mannitol | - | 0 |
| 47a – 51b | Line 3 sorbitol | - | - |
| 52a – 56b | Line 3 untreated | - | - |
| 1 – 10 | Line 5 sorbitol | GUS <i>bar</i> | 0 |

Untreated – ESM is bombarded without osmoticum on plain MSG3 medium, (-) - not tested; 0 negative; Southern – Southern hybridization; GUS *bar* - presence of these transgenes in samples. Description of ESM treatments found in Section 5.4.3. Refer to Figures 7.4 for representation of PCR results and Figures 7.6 and 7.7 for Southern hybridization results

7.3.2.2 PCR of *Agrobacterium*-infected samples

Portions of MZE's underwent GUS analysis (Section 7.3.1) before PCR analysis (Figure 7.6), followed by blotting. In this case *bar* amplification correlated to the positive GUS expression previously observed in MZE samples. Non-specific bands slightly larger than the *bar* fragment were amplified in all samples which correspond to results reported by VICKERS *et al.* (1996). This PCR evidence shows that MZE target tissue (as well as ESM) is also amenable to gene transfer.

Further experimental analysis was necessary to determine if the transgenes (*GUS* and *bar*) were stably incorporated into the MZE explants.

As is often the case with transformation studies, large numbers of explants are required to be transformed to verify the transgenic status of a few individuals, as a low transformation frequency is a usual occurrence. A more expedient method of determining physical presence (without transfer of vector backbone), stable integration and number of transgene copies for large sample numbers with certainty would be Southern hybridization analysis (Section 7.3.3).

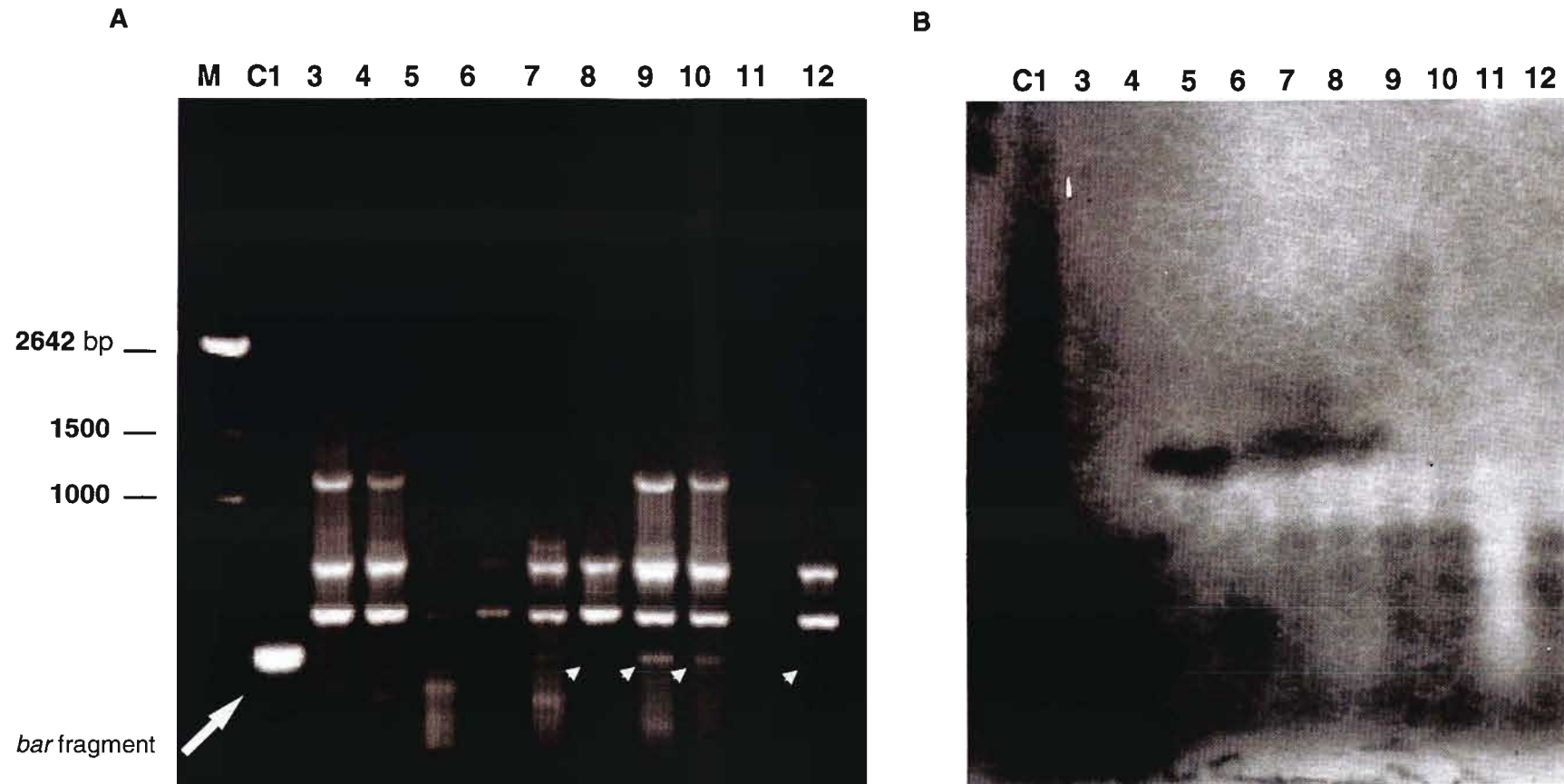


Figure 7.5 *Bar* PCR of MZE's transformed by *Agrobacterium* and subsequent Southern blot (A) DNA contents of the lanes are: lane M – MXIV (Roche); lane C1 – positive control of pAHC25 plasmid (indicated by arrow); Lanes 3-10 and 12 - MZE samples that underwent histochemical analysis. *Bar* transgenes shown in samples loaded in lanes 8-10 and 12 (indicated by arrows). The samples had also shown positive GUS expression. (B) Southern hybridization blot of *bar* PCR products using a Zeta-Probe® membrane

7.3.3 Southern Analysis with Downward Transfer

7.3.3.1 Southern analysis of bombarded samples

Transgenic status has, in the past, been deduced through PCR amplification of the marker gene or transgene (BHAT and SRINIVASAN 2002). However, it is essential to confirm the physical and stable integration of the transferred gene into the host genome (POTRYKUS 1991). Sustained use of transgenic trees requires long-term stable integration and expression of introduced genes (PEÑA and SÉGUIN 2001). Preliminary efforts to clarify the physical presence (and therefore stable integration) of the transgene transferred during preliminary bombardments, revealed hybridization to the positive control only (Figure 7.5 B), which displayed chimaeras. No hybridization between bombarded samples and the *bar* probe was observed. It was expected that digestion of a single copy of pAHC25 with *Hind*III would yield two fragments: the *bar*-containing fragment of 5.5 kb and a fragment of 4.1 kb containing the GUS gene (refer to pAHC25 restriction map Figure 4.2), and that an even distribution of *P. patula* restriction fragments would be generated from *Hind*III digestion of *P. patula* genomic DNA.

Preliminary Southern hybridization analysis yielded faint bands only observed in the positive control of pAHC25 in lane 1. This was attributed to inefficient probe labeling and a weak signal generated by old radioactivity samples. The use of 5'-[α -³⁵S]dATP can be used for nick translation reactions, as is the case in this study, though incorporation levels may be decreased (VOSBERG and ECKSTEIN 1977) or probes may have low specific activity (FÜTTERER *et al.* 1995). Weak signals may further be attributed to poor DNA transfer during blotting, poor crosslinking to the membrane, extended washing at high temperatures or too high a temperature during washing (FÜTTERER *et al.* 1995). Poor DNA transfer was not applicable in this study as in all cases the gel was checked under UV light after transfer to ensure that gel products were completely transferred to the membrane.

Further Southern hybridizations were carried out directly (without prior PCR analysis) to try and elucidate the transgenic status of the remaining bombarded material (samples pt 20a-40b) using the *bar* probe, see Figure 7.7 as an

example. Hybridization to the *bar* probe was again observed in the positive controls (and none in the negative as expected), but no hybridization was observed in the bombarded ESM samples.

Perhaps fragmented copies of the *bar* gene, especially regarding biolistic transfer, had integrated into the *P. patula* genome and therefore were not able to be detected through Southern hybridization analysis. Initially the *bar* probe was generated from double endonuclease digestion (resolved at 569 bp). However, the *bar* probe generated from PCR amplification (an expedient probe source) results in a 340 bp fragment using the primer set described by LEMAUX *et al.* (1996) and in combination with the PCR regime of VICKERS *et al.* (1996). VICKERS *et al.* (1996) were able to show Southern hybridization to one pg pPATO, equivalent to one copy of *bar* per barley genome. Southern controls using *Bam*HI restriction of pAHC25 using *bar* and GUS probes (results not shown), also resulted in no hybridization (negative), which confirmed the need for optimization (listed in Section 7.2.1.4) in any further Southern blot analysis.

Further bombardments using Line 5 ESM masses (refer to Section 5.4.3) were undertaken, of which 3 samples showed positive GUS expression before stable integration analysis (results not shown) using an optimized Southern hybridization process (discussed in Section 7.2.1.4) and GUS fragment as a probe. No hybridization was observed with any of the genomic samples during Southern analysis. During the numerous efforts taken to achieve hybridization with transgenic samples for this gene transfer protocol it became evident that more optimization was required for this Southern blot technique. This would perhaps require a more efficient labeling system or stringency protocol. However, the time taken for this type of optimization was out of the scope of this study. Larger transformation experiments could increase the likelihood of the transgene being passed onto the offspring (POTRYKUS 1991). It is realistic to expect a low conversion of transgenic samples as illustrated in Southern analysis by CHARITY *et al.* (2002) where only one transgenic shoot derived from an apical dome that had been co-cultivated with *A. tumefaciens* showed stable integration of the transgene.

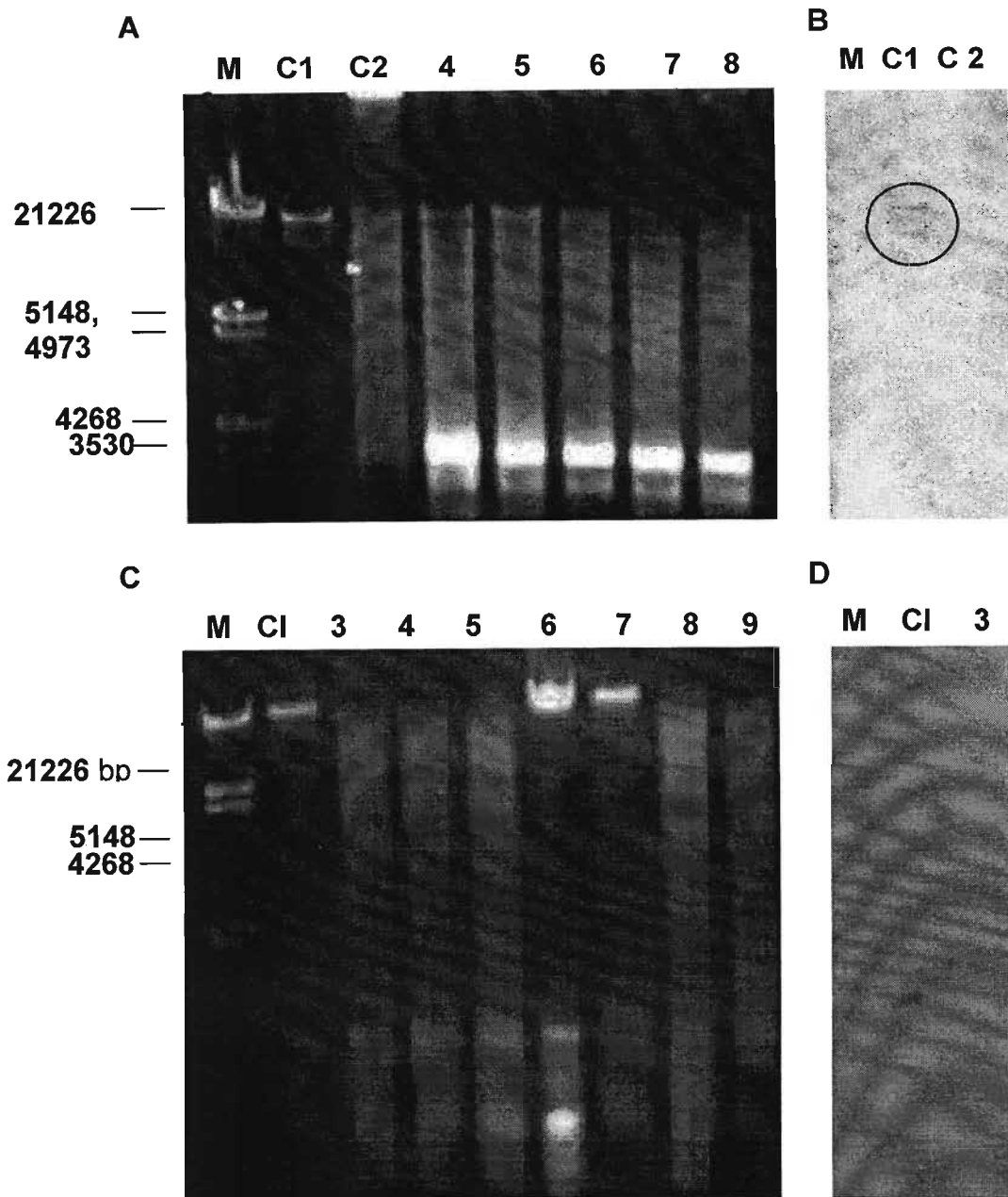


Figure 7.6 Preliminary Southern hybridization analysis of *Hind*III digested bombarded ESM samples (A) Digestion of bombarded ESM samples with *Hind*III prior to Southern transfer. Lane contents are: lane M – MIII (Roche); lane C1 - positive control of pAHC25 plasmid; lane C2 - negative control, unbombarded *Pinus patula* sample; lanes 4-8 – bombarded genomic DNA digested samples (1a; 2a; 5b; 18a; 18b) (Table 7.2) (B) Two bands (indicated by the circle) showing positive hybridization of the *bar* probe to pAHC25 on Zeta-Probe® membrane (C) Restriction digestion of bombarded ESM samples 25a–28a. Samples loaded in the following order: lane M - MIII (Roche), lane C1 - negative control of unrestricted genomic DNA sample 32a; lanes 3-9 restricted DNA (D) Unsuccessful Southern analysis using a Hybond™-N+ membrane

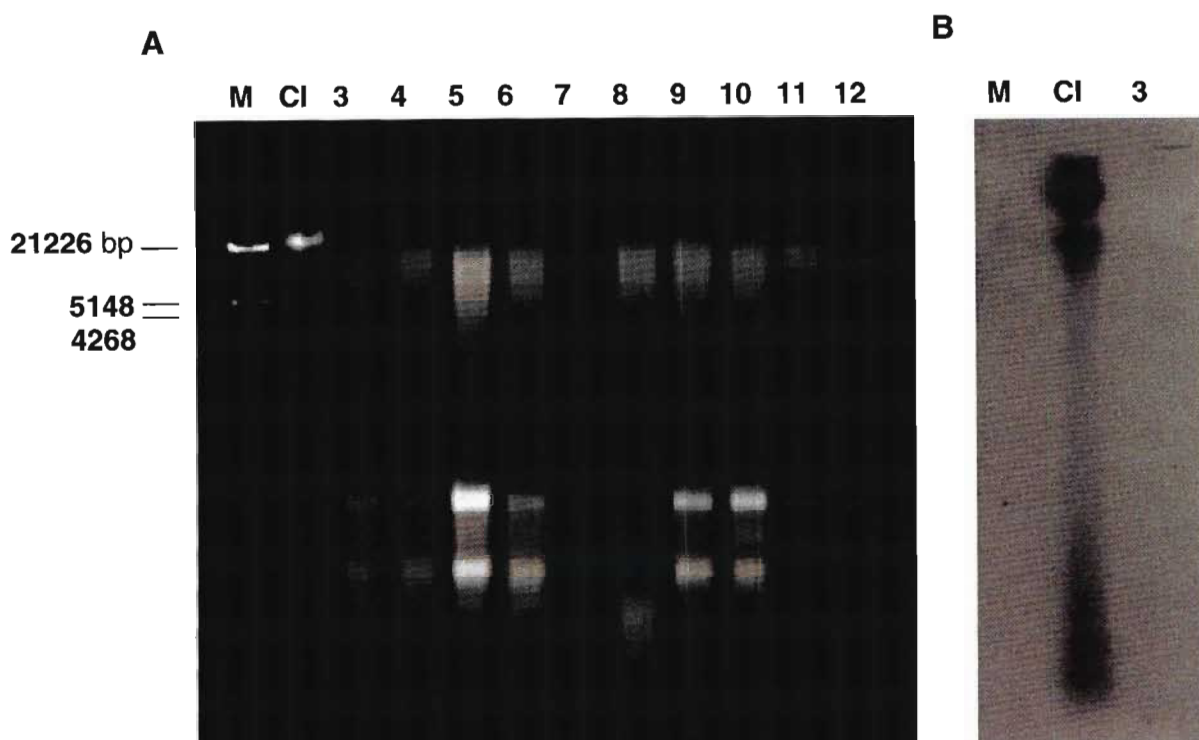


Figure 7.7: Stable integration analysis of bombarded ESM samples 20a–24b (A) Lanes were loaded in the following order: lane M – Mill (Roche); lane C1 - uncut pAHC25 plasmid, lanes 3-12 - genomic *P. patula* samples (20a-24b) restricted with *HindIII* endonuclease. **(B)** Southern hybridization of gel showing positive hybridization (Hybond™-N+ membrane) using the GUS probe to the uncut plasmid control only

The control (uncut and double digested pAHC25 plasmid DNA) tested the premise that no part of the pAHC25 backbone plasmid was inadvertently transferred with the *bar* and GUS transgenes during the biolistic gene transfer process. Hybridization was observed to these fragments only (results not shown). However, as the Southern hybridization technique was not optimized, this evidence was inconclusive rendering it difficult to negate or confirm vector backbone integration. The effort is concurrent with guidelines set for stable integrative data (POTRYKUS 1991; BIRCH 1997) giving evidence for the absence of contaminating DNA fragments.

7.3.3.2 Southern analysis of *Agrobacterium*-infected samples

In some cases, insufficient DNA could be extracted for Southern hybridization as with Line 5 ESM transformed with *Agrobacterium* (results not shown). However, this brings to light a practical consideration and possibly a limitation of Southern analysis for stable integration analysis: the amount of tissue required (10 µg DNA for *Hind*III restriction, refer to Section 7.2.1.3). This analysis is intended for regenerated plants, however, use with tissue samples (as seen above) before regeneration, could be a limited DNA source.

Ideally each individual transformed embryo should be tested for its stable transgenic properties, but this has practical constraints. The results presented below (Figure 7.8) are Southern analysis of pooled samples of transformed embryos, which would serve as a reflection of the success of the stable integration event (of the GUS transgene in this instance) into the *P. patula* genome. Hybridization to all the positive controls was achieved, but the probes are homologous whereas the transgene might be fragmented or rearranged and not completely complementary to the probe. Further optimization to the Southern technique is recommended. Regrettably the physical evidence to validate transgenesis was not obtained at the time of producing this thesis but *in lieu* of the fact that optimization is needed it is shortsighted to conclude that all of the putative transformants were not stably transformed.

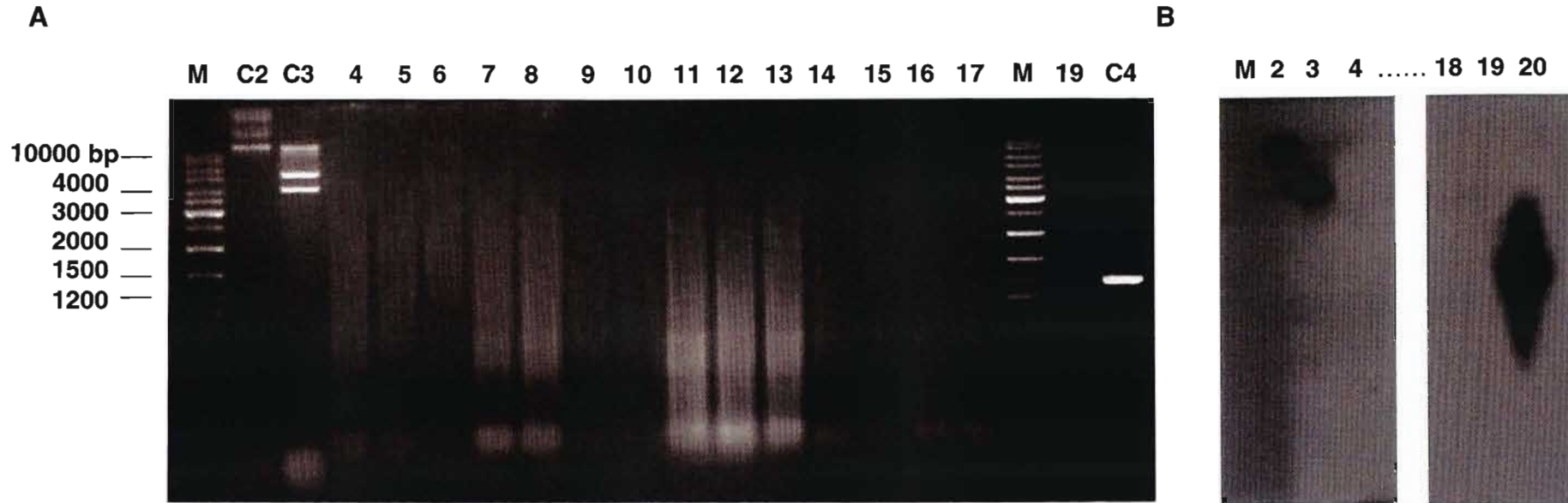


Figure 7.8 Stable integration analysis of Line 5 embryos after *Agrobacterium*-mediated transformation. (A) Digestion of plant DNA samples with *Hind*III enzyme in preparation for Southern blotting. DNA Contents for lanes are: M – DNA ladder mix (Fermentas), lane C2 - positive control, uncut pAHC25 plasmid DNA; lane C3 - positive control, restricted plasmid DNA with *Bam*HI endonuclease, lane 4-8 negative controls, embryos not inoculated with *Agrobacterium*; lanes 9-17 embryos inoculated with *Agrobacterium*; lane C4 - positive control (GUS fragment, generated through PCR, resolved at 1.2 kb). (B) Southern hybridization analysis of *Pinus patula* embryos after *Agrobacterium*-mediated transformation using 5'-[α - 32 P]dATP. Hybridization of GUS probe to positive controls of restricted (lane 2) and uncut pAHC25 (lane 3) samples; and GUS fragment (lane 20) observed on HybondTM-N+ membrane

Previous PCR-mediated analysis for the *bar* gene indicated gene transfer from both biolistic and *Agrobacterium*-mediated means, but evidence for stable integration has not been forthcoming. Inconsistent results of positive PCR with negative Southern hybridization could be accounted for by mosaic transgene expression as observed with *Pinus radiata* shoots derived from *Agrobacterium*-transformed cotyledons (CHARITY *et al.* 2002).

The success of the biolistic and *Agrobacterium*-mediated gene transfer methods have been presented from a molecular (analytical) perspective. Transient assays gave positive results, which indicated gene transfer using both methods across all tissue types. This was confirmed by PCR analysis providing assurance that both procedures are useful for transformation of *P. patula*. Confidence in initial Southern analyses was compromised. However, after some optimization steps were implemented subsequent data could not conclusively prove the physical presence of either the GUS or *bar* transgenes in transformed material.

BHAT and SRINIVASAN (2002), advises that backcross-progenies (BC₁F₁ plants) would be the best form of analysis for transgenic plants as they allow for (1) greater comparison between progeny plants to distinguish the transgene effect from effect of other influences; and (2) better prospects of attaining single copy insertions. However, as BIRCH (1997) noted this is an impractical form of analysis in trees. Current literature reporting transgenic trees suffices with Southern blot analysis for confirmation, with additional techniques such as T-DNA junction analysis (example discussed in TANG *et al.* 2001). Fluctuations in transgene expression in trees are often related to the metabolic state of cells and tissues which can be prevented using matrix attachment regions (MARs) flanking the introduced gene sequence (reviewed in PEÑA and SÉGUIN 2001). Present field trials for transgenic trees are extensive and aim to test transgene integration and expression over years under various environmental conditions (PEÑA and SÉGUIN 2001).

CHAPTER 8

FINAL THOUGHTS

The combined tools of *in vitro* plant propagation and genetic engineering have facilitated the genetic modification of *Pinus patula*, a central softwood species for pulp production. Pre-existing *in vitro* and cryopreservation protocols were a platform on which genetic modification of selected lines could be attempted in order to produce stocks of high genetic value. Of particular interest is the long-term goal of disease resistance to pathogens like pitch canker fungus and the modification of wood properties. This study undertook to provide genetic engineering protocols through biolistic and *Agrobacterium*-mediated transfer to embryogenic and organogenic tissue of *P. patula*.

A working *in vitro* system is often a pre-requisite for a transformation investigation. *In vitro* somatic embryogenesis, useful for gene transfer as tissue is juvenile and rapidly dividing, was reproduced with successful embryo maturation steps implemented.

Important biological parameters for target tissue, to be taken into consideration during the transformation studies, were identified as: (1) placing time after subculture prior to bombardment or *Agrobacterium*-mediated infection, and (2) the physiological state of tissue. Phenotypic and morphological variation was seen in harvested cones of different families and in the different rates of ESM proliferation from the different lines. The fast growing lines 2, 3 and 5 were selected for gene transfer experiments. All lines showed a phase of active growth at 4-8 d due to cleavage polyembryogenesis. Results from both studies reflected this as an ideal stage at which to conduct gene transfer.

Regeneration was one of the most significant challenges of both transformation studies regardless of technique used to introduce the transgene. With particular

reference to the biolistic study, regeneration did not always correlate with embryogenic potential. *Agrobacterium*-mediated transformations are genotype-dependent while bombardment is considered to be genotype-independent. Thus there is a need to select lines that not only have good embryogenic potential but have an inherent ability to undergo genetic transformation and subsequent regeneration. Relative experience and familiarity with lines in tissue culture ensure successful selection, but also bear the risk of selecting against characteristics associated with other genes, not present with embryogenic potential. Success with regeneration was achieved once a stepwise selection protocol was established. This study has also culminated in an improved regeneration protocol which encouraged maturation by using outer cell layers that had the greatest potential for maturation. Osmotica-supplemented treatment of tissue helped to maintain tissue integrity during bombardments and also improved transient expression. Age of target material was another variable for consideration, as tissue should not be used after a year from initiation date, in which case tissue could be cryopreserved to maintain juvenility. The *Agrobacterium*-mediated approach for ESM implemented a liquid decontamination regime (with rotation), use of filter paper stacks (in place of a filter unit) to avoid excess filtering and stress to the immature embryos present in ESM and the inclusion of L-proline in an effort to aid regeneration. It was also suggested that tissue (1 g) should be subcultured on the day of *Agrobacterium* infection, to promote better recovery.

Elimination of *Agrobacterium* was tissue-dependent and was a persistent problem during the indirect transfer study. Mature somatic embryos (given additional biolistic micro-wounding) and MZE's, were more convenient explants in this respect. However, both mature explants risked incomplete transformation resulting in chimaera production and, as mentioned, MZE's did not have a reliable regeneration protocol in place. Furthermore cefotaxime could not be included in maturation media where charcoal was present reducing the efficacy of decontamination regimes for MSE's and creating the likelihood for escapes.

Transient GUS assays gave positive results, which indicated gene transfer using both methods across all tissue types. This was confirmed by PCR analysis

providing assurance that both procedures are useful for transformation of *P. patula*. PCR amplification for the GUS transgene needed minimal amendments, requiring only the addition of DMSO for its resolution. The *bar* gene proved to be more elusive but, after a plethora of different protocols being investigated, was amplified. Positive transient assays across various tissue types were confirmed by PCR when both transgenes were resolved in transformed samples of ESM and MZE's using biolistic and *Agrobacterium*-mediated methods. Also observed in the PCR results were differing biolistic impact distributions as well as transformation efficiencies of the respective transgenes, which are not linked on the pAHC25 plasmid. Current research and public thinking has shifted the trend of selection towards other alternatives. PCR could be implemented earlier during a regeneration regime circumventing lengthy selection as well as conserving tissue.

Stable integration is crucial to confirm the physical presence of a transgene in order to validate a transformation event. Much tissue was lost during selection, leaving little for stable integration analysis. Physical evidence of either transgene could not be provided, but it was felt that the Southern hybridization technique needed to be optimized before any conclusions about their integration into the plant genome could be surmised. The ultimate confirmation of stable transformation would be the survival of regenerated pine plantlets when sprayed with a selective concentration of BASTA® herbicide.

The efficacy of both styles of transformation was reliant on a range of factors: from operator skill to Petri-dish micro-environment for biolistic transfer; or pH, bacterial strains and particularly conifer family, genera and genotype for *Agrobacterium*-mediated transfer. A typical transformation experiment requires large numbers of target explants (1) to survive the transformation process and begin healthy regeneration; (2) account for low transformation frequencies despite established transformation protocols and; (3) test many cell lines.

Future experimentation could explore different pre-treatments using different osmotica of various concentrations; use of L-proline for post-transformation

regeneration and to determine, if any, its effect on selection; different co-cultivation temperatures; different antibiotics and concentrations for decontamination; effect of boron on tissue establishment and implementation of protoplasts as another platform for genetic modification.

Several successes were achieved in the biolistic study particularly after the establishment of an optimized regeneration regime for bombarded *P. patula*, transgenic lines 2 and 3 were successfully regenerated. Much progress was made towards the implementation of an *Agrobacterium*-mediated transformation approach, which is a novel study in this key forestry species. PCR analysis provided confidence that both methods of transformation were useful for the transformation of *P. patula*. The biolistic study was able to show that *P. patula* embryonal suspensor masses (ESM) were amenable to genetic transformation.

This project has served as a pilot study for the genetic modification of *Pinus patula* using transgenic technology, culminating in the first successful report published for this species (NIGRO *et al.* 2004).

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