TRANSFORMATION OF *PINUS RADIATA* BASED ON SELECTION WITH HYGROMYCIN B

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ABSTRACT

A method was investigated for transforming *Pinus radiata* D.Don embryogenic tissue based on the *aph*IV gene from *Escherichia coli* coding for aminocyclitol phosphotransferase, which confers resistance to the antibiotic hygromycin B. Embryogenic tissue captured from immature *P. radiata* embryos was transformed using the DuPont Biolistic® particle delivery system. Transformation of embryogenic tissue was confirmed by PCR amplification of the transferred *aph*IV gene and enzymatic activity of the *uid*A gene product (β-glucuronidase) in transgenic material. Transformed embryogenic lines could be recovered at a frequency of approximately 0.14 transformants per bombarded petri dish, an efficiency which is suitable for routine transformation experiments.

Keywords: Biolistic® transformation; *aph*IV resistance gene; *uid*A reporter gene; hygromycin B; embryogenic tissue; *Pinus radiata*.

INTRODUCTION

Genetic engineering is a very attractive technique for basic and applied forestry science, since it allows the investigator to modify a specific trait of the target organism, without altering the genetic background of the selected clone. Furthermore, this technology has the potential to add traits to the target organism which are not readily accessible to conventional breeding techniques. These are some of the reasons for the enormous effort in recent years directed at the establishment of transformation protocols for forest trees of economic importance, such as *Populus tremula* L. (Leplé *et al.* 1992), *Eucalyptus grandis* Hill ex Maid. (Maunders 1997), and conifers such as *Larix decidua* Miller (Huang *et al.* 1991), *Picea abies* (L.) Karsten (Robertson *et al.* 1992), and *Pinus sylvestris* L. (Häggman 1997). Recently we described a transformation system for *P. radiata* and other conifers based on a Biolistic® transformation protocol using *npt*II as a selective marker (Walter *et al.* 1998). An *Agrobacterium*-based transformation system for *P. radiata* is also under development (Holland *et al.* 1997).

Selection genes, such as the *aph*IV gene from *E. coli*, which confer resistance to the aminoglycoside antibiotic hygromycin B, have been successfully used in the past to transform a number of angiosperm species such as tobacco (van den Elzen *et al.* 1985), *Arabidopsis* (Damm *et al.* 1989), *Brassica* (Golz *et al.* 1990), rice (Meijer *et al.* 1991), maize

(Walters *et al.* 1992), and wheat (Ortiz *et al.* 1996). Recently the first stable transformation of a conifer species (black spruce—*Picea mariana* B.S.& P.) using a hygromycin-B-based selection protocol was reported in a conference abstract (Tian *et al.* 1997).

We were able to show that hygromycin-B-based selection is also applicable to pine species such as *Pinus radiata*. The establishment of a selection system based on the *aph*IV resistance marker using hygromycin B as the selective agent will widen our potential for genetic engineering of *P. radiata*.

MATERIAL AND METHODS

Vector Construct

The pUC-derived vector pCW121 (Fig. 1) used for this study was constructed by insertion of the *aph*IV cassette from pJIT109 (*Kpn* I) into pJIT166, which contains the *uid*A reporter gene controlled by the double *35S* promoter (both pJIT109 and pJIT166 were kindly provided by John Innes Institute, Norwich, UK). All cloning procedures were done according to standard protocols (Sambrook *et al.* 1989). DNA for vector construction and bombardment of plant tissue was isolated using the Wizard Plus DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA).

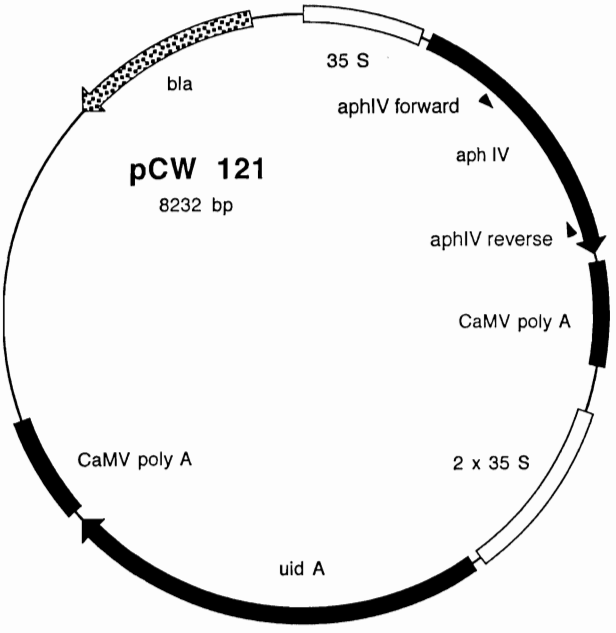

FIG. 1–Schematic of the plasmid vector pCW121 used in this study. This pUC-based vector contains the *bla* resistance gene coding for β-lactamase, the *aph*IV resistance gene coding for aminocyclitol phosphotransferase driven by the *35S* promoter, and the *uid*A reporter gene coding for β-glucuronidase controlled by the double *35S* promoter. The positions of the primers *aphIV forward* and *aphIV reverse* used to amplify a 776bp fragment of the *aphIV* gene are indicated by arrows.

Tissue Material

Embryogenic tissue (clone D93-199) of *P. radiata* used in this study was originally initiated from immature zygotic embryos as described by Smith (1996). Tissue was subsequently maintained and propagated on solid Embryogenesis Medium (EM) containing no plant growth regulators, or solid Embryo Development Medium (EDM6) containing 5 μM 2,4-dichlorophenoxyacetic acid and 2.5 μM benzylaminopurine (Smith 1996) prior to dose-response and transformation experiments with subculture on fresh medium every fortnight.

Dose-response Experiments

The effect of hygromycin B (Sigma, St. Louis, USA) on non-transformed *P. radiata* tissue propagated on solid EDM6 was tested with both dispersed and undispersed tissue. To test dispersed material, 200 mg (fresh weight) of embryogenic calli were suspended in 1 ml of liquid EM (Smith 1996), according to a standard protocol (Walter *et al.* 1998), then spread on to solid EDM6 containing hygromycin B at various concentrations (0–30 μg/ml). When testing undispersed embryogenic tissue, small pieces of calli (approximately 300 mg fresh weight) were placed on the same series of $EDM6_{hygromycin\ B}$ media as above. The tissue was maintained on this selection medium for a period of 5 weeks with subculture at 2-weekly intervals. Growth of the tissue suspension or tissue pieces was measured by determining the fresh weight of the samples weekly.

Biolistic® Transformation and Selection Procedure

All transformation experiments were performed using the DuPont Biolistic® particle delivery device (PDS 1000He) and according to bombardment conditions described previously (Walter *et al.* 1998). Embryogenic tissue was prepared for bombardment experiments as described by Walter *et al.* (1994). The tissue was bombarded with 1.6- to 3-μm-sized gold particles (Aldrich Chemicals, Milwaukee, USA), sterilised, and coated with DNA according to a standard protocol (Sanford *et al.* 1993). A plasmid DNA concentration of 1.5 μg/μl was used for the coating procedure to saturate the surface of the gold particle with DNA material (C.Walter *et al.* unpubl. data). Approximately 10% of the tissue samples were stained for *uid*A activity according to Jefferson (1987) 3 days after bombardment to estimate the efficiency of the transformation experiments by counting the number of blue spots per plate. Bombarded tissue was transferred to EDM6 containing 25 μg/ml hygromycin B 3 days after bombardment and further incubated on this medium for 12–14 weeks with a fortnightly subculture. Proliferating pieces of tissue were removed and further propagated for several weeks on EDM6 plus hygromycin B to obtain enough transgenic tissue for molecular analysis. Selected transgenic lines were transferred to Embryo Maturation Medium (EMM1) for 2 weeks and then cultured on EMM2 (Smith 1996) for a period of 6 weeks to promote embryo formation.

Histochemical and Fluorometric *uid*A Analysis

Histochemical staining and fluorometric analysis of *uid*A activity in transformed embryogenic *P. radiata* tissue were carried out as described previously (Walter *et al.* 1988).

Isolation of Genomic DNA and PCR Analysis

Genomic DNA from embryogenic tissue of *P. radiata* was isolated using the FastDNA Kit in combination with the FastPrep FP120 apparatus according to instructions of the manufacturer (BIO 101 Inc., La Jolla, California, USA). PCR reactions were carried out in 1 × Taq PCR buffer containing 1.5 mM $MgCl_2$ (Boehringer Mannheim, Germany), 250 μM dNTPs, 0.4 μM primers, 1 unit Taq polymerase (Boehringer Mannheim, Germany), and 50 ng genomic DNA using a Techne Cyclogene Thermal Cycler (Techne Ltd, Cambridge, England). The reaction consisted of 40 cycles with 30 s annealing at 65°C, 30 s elongation at 72°C, and 30 s denaturation at 94°C. The reaction was started by hot start and terminated by an elongation step of 3 min at 72°C. The primers used were: 5′ TGT AGC CCT TGA CTA TGA GC 3′ and 5′ AGG GAC CTG ACT CCT CAT AC 3′ for actin and 5′ ATA GCT GCG CCG ATG GTT TCT AC 3′ and 5′ GAT TTG TGT ACG CCC GAC AGT C 3′ for *aph*IV.

RESULTS AND DISCUSSION

Dose-response experiments demonstrated a severe inhibitory effect of hygromycin B, at relatively low concentrations, on growth and vitality of suspended embryogenic tissue of *P. radiata*. The growth of this material was, compared to control tissue growing on EDM6 without antibiotic, substantially reduced at 10 μg/ml and completely inhibited at ≥15 μg/ml hygromycin B in the medium (Fig. 2). Transfer of the tissue to antibiotic-free EDM6 after 5 weeks of selection confirmed that hygromycin B concentrations ≥15 μg/ml were lethal rather than inhibitory for the suspended embryogenic material.

The growth of unsuspended embryogenic material was effectively inhibited at hygromycin B concentrations ≥20 μg/ml (Fig. 3), indicating that the antibiotic affected not just tissue which was in direct contact with the medium. These results indicate that hygromycin B concentrations ≥20 μg/ml are likely to prevent the production of escapes during selection for transformants. Consequently, a concentration of 25 μg/ml hygromycin B was used for all subsequent transformation experiments. Interestingly, the antibiotic concentrations found to be suitable for a selection procedure for *P. radiata* were similar to those used for angiosperm species such as tobacco (20 μg/ml hygromycin B, van den Elzen *et al.* 1985) and wheat (25 μg/ml hygromycin B, Ortiz *et al.* 1996). The concentration of hygromycin B used for stable transformation experiments with black spruce was not specified in the published conference abstract (Tian *et al.* 1997).

The transferred plasmid pCW121 (Fig. 1) contained the *uid*A reporter gene so that histochemical and fluorometric gene expression assays could be used to estimate the efficiency of the transformation experiments and determine the continued presence of the transferred gene in the subsequently proliferating tissue. Ten bombarded tissue samples stained for *uid*A activity 3 days after transformation showed an average of 543.5 ±228.9 transient transformation events, which compared favourably to earlier transformation experiments (Walter *et al.* 1994).

All embryogenic cell lines recovered on EDM6 containing 25 μg/ml hygromycin B were transgenic based on the combined evidence of *uid*A analyses (Table 1, Fig. 4), PCR data (Fig. 5), and the prolonged survival of the tissue on selective media. Transgenic lines showed, as in previous transformation experiments, considerable variation in *uid*A activity levels (Table 1), which most likely reflects the different integration pattern of the transferred

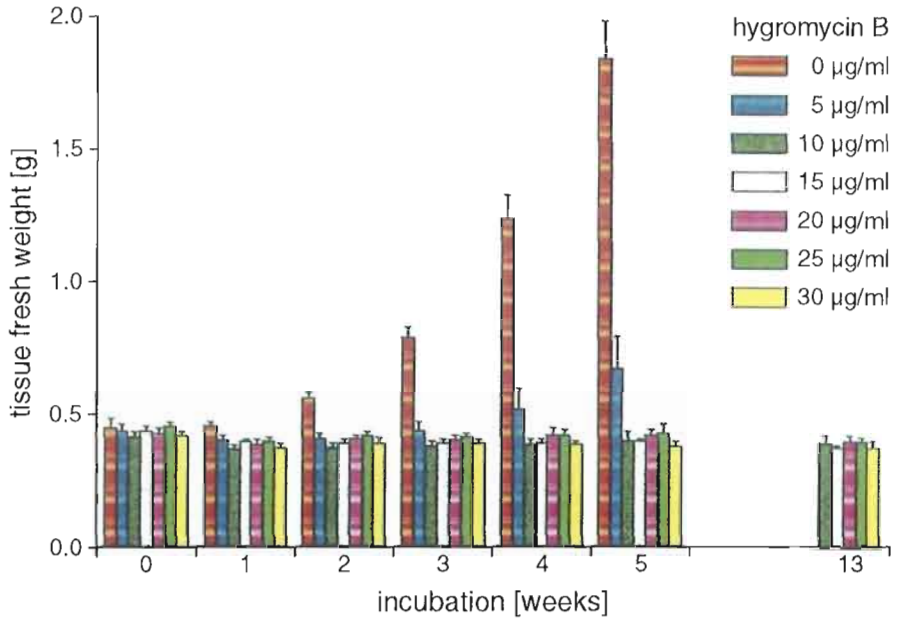

FIG. 2–Effect of different hygromycin B concentrations on growth of non-transformed embryogenic *P. radiata* tissue placed as dispersed calli on to solid EDM6 medium. The growth of the tissue was measured weekly by weighing. Each bar represents the average fresh weight of •six tissue samples including standard deviation σ_n. Tissue without significant growth on hygromycin-B-containing medium (10–30 µg/ml) was transferred to antibiotic-free EDM6 after 5 weeks and subsequently incubated for a further 8 weeks. Only tissue samples originally maintained on medium with hygromycin B at 10 µg/ml showed signs of growth on week 13, but this growth was minimal.

TABLE 1–Fluorometric and histochemical *uid*A analysis of regenerated *P. radiata* lines after transformation and selection

Tested cell lines	Fluorometric *uid*A activity*	Histochemical *uid*A staining
Untransformed control	0.05 ±0.02	–
ln2/1	26.3 ±1.6	+
ln3/1	5.2 ±2.0	+
ln5/1	0.09 ±0.05	–
ln6/1	28.2 ±7.3	+
ln6/2	37.7 ±9.4	+
ln6/3	32.2 ±8.4	+
ln6/4	33.8 ±7.0	+
ln7/1	48.4 ±6.2	+
ln8/1	34.8 ±7.5	+
ln9/1	36.6 ±3.3	+
ln10/1	23.0 ±2.3	+
ln11/1	2.8 ±1.0	+

* *uid*A activity in nmol MU ∞ min^{-1} ∞ mg $protein^{-1}$. Fluorometric data are mean values of at least three independent measurements including standard deviation σ_n; – = no detectable staining. + = clearly visible staining.

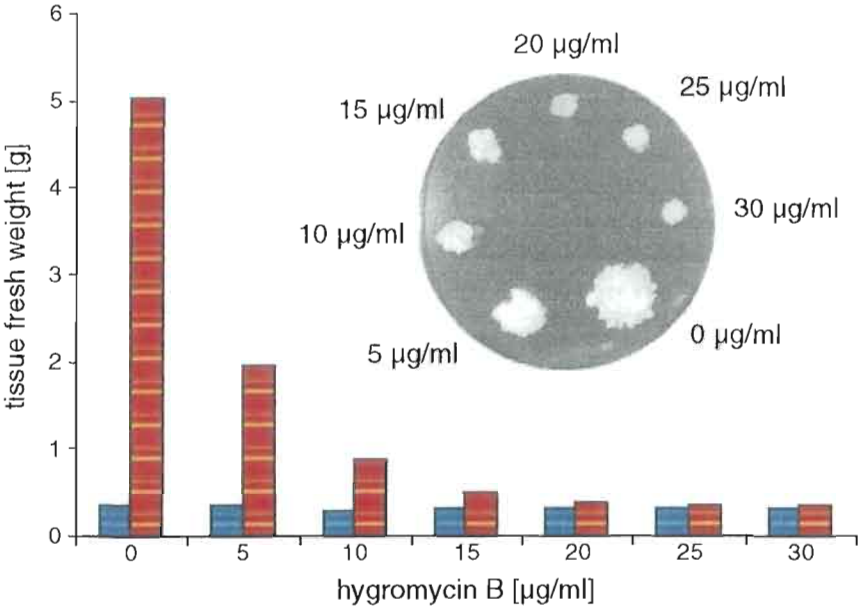

FIG. 3–Effect of different hygromycin B concentrations on growth of non-transformed embryogenic *P. radiata* tissue placed as undispersed calli on to solid EDM6 medium. Each bar represents the combined fresh weight of six embryogenic calli measured before (blue bars) and after (red bars) 5 weeks of incubation on selection medium. Essentially no increase in weight of the calli could be observed at hygromycin B concentrations •20 µg/ml. The development of the embryogenic material exposed to different hygromycin B concentrations for a period of 5 weeks is illustrated above.

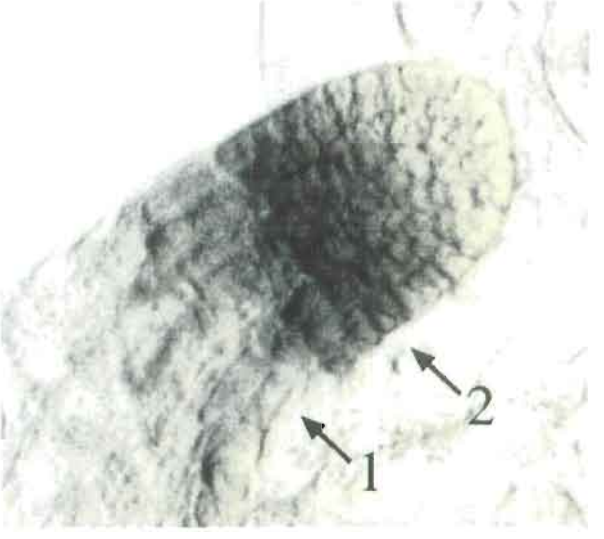

FIG. 4–Histochemical *uid*A staining in an embryo of the transgenic, hygromycin-B-resistant line D93/199 ln6/4. The blue precipitate of the histochemical staining reaction is clearly visible in the suspensor cells (1) and in the basis of the embryo head (2).

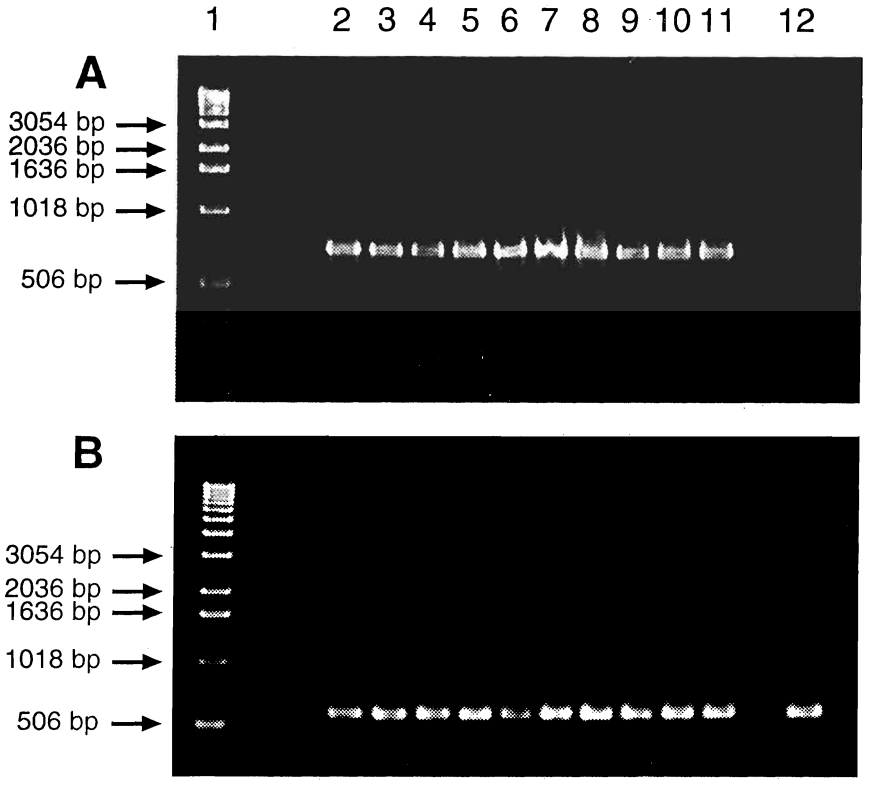

FIG. 5–PCR analysis of embryogenic lines recovered after transformation and selection experiments. Ethidium bromide stained agarose gel showing a 776bp amplification product of the *aph*IV resistance gene (A). Amplification of a 590bp fragment of a constitutive actin gene was carried out as a loading and quality control (B) using the same DNA preparations as in (A).
Lane 1: 1kb DNA ladder (Gibco BRL); lanes 2–11: hygromycin-B-resistant lines in the following order: ln2/1, ln3/1, ln5/1, ln6/1, ln7/1, ln8/1, ln9/1, ln10/1, ln11/1, ln6/2, lane 12: non-transgenic control line D93-199.

genes in each line (Walter *et al.* 1998). The observation that the isolated line 5/1 shows only background *uid*A activity (Table 1), although putatively transgenic for *aph*IV as demonstrated by PCR (Fig. 5), could also be an indication for suppression or disruption of the *uid*A construct.

The lack of histochemical *uid*A staining in the apical part of transformed embryos (Fig. 4) was most likely a consequence of the expression pattern of the *35S* promoter in *P. radiata* (Walter *et al.* 1998).

Transgenic lines were obtained only from tissue which was maintained on EM prior to transformation. On this medium tissue remains in a more primitive, undifferentiated state than on EDM6 (Smith 1996). This finding underlines earlier observations that for the production of stable *P. radiata* transformants it is beneficial to bombard material in a more primitive, undifferentiated state (C.Walter *et al.* unpubl. data).

We were able to isolate 12 transgenic lines from 96 plates bombarded with the vector pCW121, resulting in an overall frequency of approximately 0.14 transformants per plate. This transformation frequency was somewhat lower than the 0.5 transformants obtained per plate when using geneticin selection (Walter *et al.* 1998). In black spruce, however, the transformation frequency was similar with either the hygromycin B or the geneticin selection procedure (Tian *et al.* 1997).

One reason for the difference in the transformation efficiency between the two selection systems used for *P. radiata* could be the existence of a Kozak consensus sequence around the ATG start codon of the *npt*II resistance gene in the vector used for transformation with geneticin as a selectable agent. This modification significantly enhances translation efficiency in eukaryotic organisms (Kozak 1989), including *P. radiata* (Walter *et al.* 1994). The *aph*IV construct did not contain a Kozak or Kozak-like sequence around the ATG start codon, probably resulting in a lower expression level of *aph*IV than the *npt*II construct. Further experiments designed to increase the expression level of the *aph*IV resistance gene are in progress.

Attempts to regenerate hygromycin-B-resistant plantlets were not undertaken in this study, since our focus was to retransform geneticin-resistant embryogenic lines to carry out gene suppression studies in *P. radiata*. Regeneration experiments in black spruce, however, showed no adverse effect of hygromycin B on the plant-forming potential of the embryogenic lines used (Tian *et al.* 1997).

Current experiments are also directed at investigating the possibility of extending the established selection protocol to other conifer species such as *Picea abies*.

To date, histochemical *uid*A staining of recovered *P. abies* lines indicates that the established hygromycin-B-based selection procedure can also be used for transformation of this conifer species.

CONCLUSION

Our results demonstrated that hygromycin B is a potent growth inhibitor of non-transformed embryogenic tissue of *Pinus radiata* even at relatively low concentrations of 10–15 μg/ml. The *aph*IV resistance gene can be used, together with hygromycin B as a selective agent, to transform embryogenic *P. radiata* tissue. The selection procedure is very reliable, since no escapes were observed using 25 μg/ml hygromycin B during the selection process. The selection protocol can also be used for transformation of other conifer species such as *Picea abies*.

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REFERENCES

DAMM, B.; SCHMIDT, R.; WILLMITZER, L. 1989: Efficient transformation of *Arabidopsis thaliana* using direct gene transfer to protoplasts. *Molecular & General Genetics 217*: 6–12.

GOLZ, C.; KOHLER, F.; SCHIEDER, O. 1990: Transfer of hygromycin resistance into *Brassica napus* using total DNA of a transgenic *B. nigra* line. *Plant Molecular Biology 15*: 475–483.

HÄGGMAN, H.M. 1997: Tissue culture and genetic transformation of nordic conifers. Joint IUFRO & UIORF meeting, 12–16 August, Quebec city, Canada.

HOLLAND, L.; GEMMELL, J.E.; CHARITY, J.A.; WALTER, C. 1997: Foreign gene transfer into *Pinus radiata* cotyledons using *Agrobacterium tumefaciens*. *New Zealand Journal of Forestry Science 27(3)*: 289–304.

HUANG, Y.; DINER, A.M.; KARNOFSKY, D.F. 1991: *Agrobacterium rhizogenes* mediated genetic transformation and regeneration of a conifer: *Larix decidua*. *In Vitro Cellular & Developmental Biology 27*: 201–207.

JEFFERSON, R.A. 1987: Assaying chimaeric genes in plants: The *uid*A gene fusion system. *Plant Molecular Biology Reporter 5*: 387–405.

KOZAK, M. 1989: The scanning model for translation: An update. *Journal of Cell Biology 109*: 229–241.

LEPLÉ, J.C.; BRASILEIRO, A.C.M.; MICHEL, M.F.; DELMOTTE, F.; JOUANIN, L. 1992: Transgenic poplars: expression of chimaeric genes using different constructs. *Plant Cell Reports 11*: 137–141.

MAUNDERS, M.J. 1997: Genetic Transformation of *Eucalyptus* Species towards the Modifications of Fibre Characteristics. *Second Wood Biotechnology Symposium*, 10-12 March, Canberra, Australia.

MEIJER, E.G.; SCHILPEROORT, R.A.; RUEB, S.; VAN OS-RUYGROK, P.E.; HENSGENS, L.A. 1991: Transgenic rice cell lines and plants: expression of transferred chimeric genes. *Plant Molecular Biology 16*: 807–820.

ORTIZ, J.P.A.; REGGIARDO, M.I.; RAVIZZINI, R.A.; ALTABE, S.G.; CERVINGI, G.D.L.; SPITELLER, M.A.; MORATA, M.M.; ELIAS, F.E.; VALLEJOS, R.H. 1996: Hygromycin resistance as an efficient selectable marker for wheat stable transformation. *Plant Cell Reports 15*: 877–881.

ROBERTSON, D.; WEISSINGER, A.K.; ACKLEY, R.; GLOVER, S.; SEDEROFF, R.R. 1992: Genetic transformation of Norway spruce (*Picea abies* L. Karst) using somatic embryo explants by microprojectile bombardment. *Plant Molecular Biology 19*: 925–935.

SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T. 1989: "A Laboratory Manual". Cold Spring Harbor Laboratory Press, New York, USA.

SANFORD, J.C.; SMITH, F.D.; RUSSELL, J.A. 1993: Optimizing the biolistic process for different biological applications. *Methods in Enzymology 217*: 483–509.

SMITH, D.R. 1996: Growth medium US patent number: 5, 565, 355.

TIAN, L.-N.; CHAREST, P.; SEGUIN, A.; RUTLEDGE, R. 1997: Hygromycin resistance as an efficient selectable marker for black spruce transformation. Joint IUFRO & UIORF meeting, August 12–16, Quebec city, Canada.

VAN DEN ELZEN, P.J.M.; TOWNSEND, J.; LEE, K.Y.; BEDBROOK, J.R. 1985: A chimaeric hygromycin resistance gene as a selectable marker in plant cells. *Plant Molecular Biology 5*: 299–302.

WALTER, C.; SMITH, D.R.; CONNETT, M.B.; GRACE, L.J.; WHITE, D.W.R. 1994: A biolistic approach for the transfer and expression of a *gusA* reporter gene in embryogenic cultures of *Pinus radiata*. *Plant Cell Reports 14*: 69–74.

WALTER, C.; GRACE, L.J.; WAGNER, A.; WHITE, D.W.R.; WALDEN, A.R.; DONALDSON, S.S.; HINTON, H.; GARDNER, R.C.; SMITH, D.R. 1998: Stable transformation and regeneration of transgenic plants of *Pinus radiata* D Don. *Plant Cell Reports 17*: 460–468.

WALTERS, D.A.; VETSCH, C.S.; POTTS, D.E., LUNDQUIST, R.C. 1992: Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. *Plant Molecular Biology 18*: 189–200.