Transgenic Plant Journal ©2007 Global Science Books

Recent Progress in Genetic Transformation of Four *Pinus* spp.

Jean-François Trontin[1] • Christian Walter[2] • Krystyna Klimaszewska[3] • Yill-Sung Park[4] • Marie-Anne Lelu-Walter[5*]

[1] Technological Institute FCBA, Biotechnology Laboratory, Domaine de l'Étançon, F-77370 Nangis, France
[2] Cellwall Biotechnology Center, SCION Research, Rotorua, New Zealand
[3] Natural Resources Canada, Canadian Forest Service - Laurentian Forestry Centre, 1055 du P.E.P.S., P.O. Box 10380, Stn. Sainte-Foy, Quebec, Quebec, G1V 4C7, Canada
[4] Natural Resources Canada, Canadian Forest Service - Canadian Wood Fibre Centre, PO Box 4000, Fredericton, New Brunswick E3B 5P7, Canada
[5] INRA, Research Unit on Breeding, Genetic and Physiology of Forest Trees, BP 20 619, Ardon F-45166 Olivet Cedex, France

Corresponding author: * **leluwa@orleans.inra.fr**

ABSTRACT

Advances in conifer tissue culture and genetic transformation offer new opportunities in the field of genetic engineering. Genetic transformation is an important tool for breeders because it allows them to introduce valuable genes that might otherwise be difficult to integrate into elite genotypes. Combined with mass vegetative propagation, such as somatic embryogenesis, regeneration of transgenic conifers could result in accelerated tree improvement. Genetic transformation requires the development of an efficient gene delivery system. Extensive work has been done in *Pinus* spp. to improve transformation methods, either by DNA-coated particle bombardment or by co-culture with *Agrobacterium tumefaciens*. This review describes the most recent progress made in genetic transformation in the genus *Pinus*, with emphasis on four important forest and plantation species grown in Europe (*P. pinaster*), New Zealand, Australia and South America (*P. radiata*), and North America (*P. taeda* and *P. strobus*). The biosafety issues associated with potential deployment of transgenic pine varieties in commercial forestry are highlighted.

Keywords: *Agrobacterium tumefaciens*, biosafety, deployment strategy, embryonal mass, gene transfer, organogenesis, particle bombardment, pines, somatic embryogenesis, stable transformation, transgenic trees, zygotic embryos
Abbreviations: **AT**, *Agrobacterium (tumefaciens)*-mediated transformation; **EM**, embryonal mass; **H+**, hygromycin-resistant line; **K+**, kanamycin-resistant line; **MAS**, marker-assisted selection; **MVF**, multi-varietal forestry; **P+**, phosphinothricin-resistant line; **PB**, microprojectile (particle) bombardment (or biolistic); **PTGS**, post-transcriptional gene silencing; **SE**, somatic embryogenesis; **RNAi**, RNA interference; **T-DNA**, transfer DNA

CONTENTS

INTRODUCTION

One of the alternatives to meet the growing world demand for wood is to farm genetically improved trees in plantations (Victor and Ausubel 2000; Fenning and Gershenzon 2002). However, a long generation time, high background polymorphism, and self-incompatibility mechanisms are serious impediments to forest research and tree breeding. Compared with other plants, the process of domesticating trees to human needs is time-consuming and expensive. As a result, most trees are essentially wild because genetic improvement programs are less than 100 years old. This is especially the case for complex multigenic traits of major commercial importance, such as wood quality and processing (lignin and cellulose contents), and control of the juvenile phase and flowering, which are difficult to breed using conventional techniques.

Innovative biotechnologies can offer real and cost-effective opportunities to accelerate genetic improvement of forest trees (Sedjo 2001). Impressive discoveries and developments have been made in the past 20 years in the fields of molecular biology and genomics (i.e., genome sequencing, candidate gene identification), large-scale clonal propagation using somatic embryogenesis (SE) and genetic

Received: 25 May, 2007. Accepted: 25 August, 2007.

Invited Review

transformation of elite clones.

Species belonging to the genus *Pinus* cover vast forest areas of Europe, North and South America, and Asia. Pines are among the most important forestry species worldwide, providing timber, pulp, and other products. A few of them, such as loblolly pine (*Pinus taeda* (L.)), maritime pine (*P. pinaster* (Ait.)), radiata or Monterey pine (*P. radiata* (D. Don)), and to a certain degree, eastern white pine (*P. strobus* (L.)) have been extensively used as plantation species. For this reason, most research effort has been focused on these pine species, and progress in optimizing SE protocols and cryopreservation has been greatest for these species (see review by Klimaszewska *et al*. 2007). Similarly, other biotechnologies such as genetic transformation and identification of DNA-based markers for marker-assisted selection (MAS) are also being developed with the plantation species.

Genetic engineering is potentially very useful in forestry because inter- and intra-species gene transfers could be achieved in a relatively short period of time compared with conventional breeding. In addition, it would facilitate introduction of economically important genes that may otherwise be difficult to integrate into elite genotypes because of mating barriers or low heritability of these genes. Combined with SE, regeneration of transgenic trees is likely to result in accelerated tree improvement by delivering the best genetic stock from the breeding program at each breeding cycle. Transgenic trees thus have the potential to enhance the commercial competitive advantage of high-yield plantation forests (Sedjo 2006), preferably within the framework of multi-varietal forestry (MVF; Klimaszewska *et al*. 2007). Furthermore, genetic engineering (reverse genetics) is a very attractive alternative to association studies (forward genetics) in demonstrating direct links between candidate gene function and phenotype (Busov *et al*. 2005). Such a powerful tool for dissection of adaptive traits would greatly improve our basic knowledge of plant physiology and contribute to the practical development of MAS.

Several general overviews of tree biotechnologies, including genetic transformation of both hardwoods and conifers, have been published in recent years (Tang and Newton 2003; Giri *et al*. 2004; Nehra *et al*. 2005). This review describes the most recent advances in protocol development for genetic transformation in the genus *Pinus*, focusing on the above-mentioned four pine species. Biosafety issues associated with deployment of transgenic trees in commercial MVF are also discussed.

GENETIC TRANSFORMATION: GENERAL CONSIDERATIONS

To produce stable transformed plants, foreign DNA has to be first introduced into plant cells and second, integrated into the cell genome. These transgenic cells must then be selected and multiplied, and finally regenerated into a plant. Therefore, development of efficient gene delivery systems based on efficient *in vitro* plant regeneration protocols is a prerequisite for application of genetic transformation in any species.

Strong evidence for stable transfer and expression of foreign genes in the pine genome was first obtained more than 20 years ago by Sederoff *et al*. (1986). Extensive research has since been carried out with a total of 30 pine species (**Table 1**) to improve transformation methods. Excluding gall formation, foreign gene transfer and stable expression in pine cells and tissues have been demonstrated in 17 species. The most frequently used gene delivery methods for stable transformation of pines are co-culture of cells or explants with disarmed strains (i.e., without tumor-inducing genes) of the ubiquitous, pathogenic, soil-borne bacterium *Agrobacterium tumefaciens* (AT, *Agrobacterium*-mediated transformation; **Table 2**), and DNA-coated particle bombardment (PB, **Table 3**). Particle bombardment is a direct transformation method that entails bombarding the plant cells with small metallic particles coated with the gene(s) of interest. After the particles enter a cell (i.e., without detrimental effects), the foreign DNA "randomly" integrates into

Table 1 Genetic transformation studies in *Pinus* spp. (first and most significant references).

Species	Method[a]	Result[b]	Reference
aristata	PB	T	Fernando *et al.* 2000
ayacahuite	AT	Gall	Saborio *et al.* 1999
banksiana	AR, EP	T	Tautorus *et al.* 1989; McAfee *et al.* 1993
contorta	AR	S	Magnussen *et al.* 1994; Lindroth *et al.* 1999
densiflora	AT, PB	T	Choi *et al.* 1988; Taniguchi *et al.* 2004
eldarica	AT	SP	Stomp *et al.* 1990; Gould *et al.* 2002
echinata	AT	Gall	Huang and Tauer 1994
elliottii	AT, LISW, PB	S	Stomp *et al.* 1990; Newton *et al.* 1993; Tang *et al.* 2006b
griffithii	PB	T	Fernando *et al.* 2000
halepensis	AR	S	Tzfira *et al.* 1996
jeffreyi	AT	Gall	Stomp *et al.* 1990
lambertiana	AT	S	Loopstra *et al.* 1990
maximartinezii	AT	S	Villalobos-Amador *et al.* 2002
monticola	AR, PB	T	McAfee *et al.* 1993; Fernando *et al.* 2000
nigra	AR, AT, EP, PB	S	Mihaljevic *et al.* 1996; Lopez *et al.* 2000a; Salaj *et al.* 2005
palustris	AR, AT, PB	T	Diner 1999
patula	PB	SP	Nigro *et al.* 2004
pinaster	AT, EP, PB	SP	Gomez-Maldonado *et al.* 2001; Trontin *et al.* 2002
pinceana	AT	S	Villalobos-Amador *et al.* 2002
pinea	AT, PB, SAAT	T	Humara *et al.* 1999a, 1999b
ponderosa	AT	Gall	Morris *et al.* 1989; Stomp *et al.* 1990
radiata	AT, EP, PB	SP	Stomp *et al.* 1990; Campbell *et al.* 1992; Walter *et al.* 1998; Charity *et al.* 2005
rigida x *taeda*	AT, PB	SP	Connett-Porceddu *et al.* 2003, 2007
roxburghii	PB	SP	Parasharami *et al.* 2006
strobus	AT, PB	SP	Tian *et al.* 1997; Levée *et al.* 1999
sylvestris	AT, AR, PB	SP	Stomp *et al.* 1990; Aronen *et al.* 2003
taeda	AT, PB, SAAT	SP	Sederoff *et al.* 1986; Tang *et al.* 2001; Connett-Porceddu *et al.* 2003, 2007
taeda x *elliottii*	AT	Gall	Huang and Tauer 1994
thunbergii	PB	T	Taniguchi *et al.* 2004
virginiana	AT, PB	SP	Stomp *et al.* 1990; Tang and Newton 2004b, 2005

[a] PB: particle bombardment; AT/AR: *A. tumefaciens*/*rhizogenes*-mediated transformation; EP: electroporation; SAAT: sonication-assisted *Agrobacterium*-mediated transformation; LISW: nanosecond pulsed laser-induced stress wave.
[b] Best result obtained: gall/tumor formation, transient (T) or stable expression (S) including plant regeneration (P).

Table 2 Stable AT transformation and plant regeneration in *Pinus* spp.

Species	Explant (source)[a]	Gene (promoter) transferred[b,c]	RM[d]	Reference
eldarica	AS (MZE)	*npt*II (nos), *uid*A (35S, RbcS)	O	Gould *et al.* 2002
pinaster	EM (IZE)	*uid*A, *hpt* (35S)	SE	Trontin *et al.* 2002
radiata	AS (MZE)	*npt*II (nos), *uid*A (35S, RbcS)	O	Gould *et al.* 2002
radiata	EM (IZE)	*npt*II (nos), *uid*A (35S),	SE	Cerda *et al.* 2002
radiata	Cot, MD (MZE)	*npt*II (nos), *uid*A (ubi1)	O	Charity *et al.* 2002
radiata	Cot (MZE)	*npt*II (nos), *uid*A (35S)		Grant *et al.* 2004
radiata	EM (IZE)	*npt*II (ubi1), *uid*A, *bar* (35S)	SE	Charity *et al.* 2005
rigida x taeda	EM (IZE)	*npt*II (nos), *uid*A (35S)	SE	Connett-Porceddu *et al.* 2007
strobus	EM (IZE)	*npt*II (nos), *uid*A (2x35S, PAL2), *uid*A, *gfp* (35S)	SE	Levée *et al.* 1999
strobus	MZE (SL)	*npt*II (*nos*), *gfp (35S)*	O	Tang *et al.* 2007
taeda	MZE (SL)	*npt*II, *hpt* (nos), *uid*A (35S)	O	Tang 2001, Tang *et al.* 2001
taeda	MS (SL, AS)	*npt*II (nos), *uid*A (35S, RbcS)	O	Gould *et al.* 2002
taeda	EM (IZE)	*npt*II, *uid*A (35S)	SE	Connett-Porceddu and Gulledge 2005; Connett-Porceddu *et al.* 2007
taeda	MZE (SL)	*uid*A, *hpt* (35S)	O	Tang 2003; Tang *et al.* 2004
taeda	MZE (SL)	*npt*II (nos), *Mt*1d, *Gut*D (35S)	O	Tang *et al.* 2005d
virginiana	MS (SL, AS)	*npt*II (nos), *uid*A (35S, RbcS)	O	Gould *et al.* 2002
virginiana	ESC, Callus (MZE)	*hpt*, *GVG* (35S), *gfp* (4UAS)	O	Tang and Newton 2004b; Tang *et al.* 2005c
virginiana	MZE (SL)	*npt*II (nos), *gfp* (35S)	O	Tang and Newton 2005
virginiana	Callus (MZE)	*npt*II (pl), *Ca*PF1 (35S)	O	Tang *et al.* 2005a, 2006a

[a] AS: adventitious shoot; Cot: cotyledon; ESC: embryogenic suspension culture; EM: embryonal mass; IZE: immature zygotic embryo; MD: meristematic dome; MS: micropropagated shoots; MZE: mature zygotic embryo; Pol: pollen; PC: pollen cone; SL: seedling.

[b] Genes; *bar*: phosphinothricin acetyl transferase; *Ca*PF1: ERF/AP2 pepper transcription factor; *cad*: cinnamyl alcohol dehydrogenase; *cry*1Ac: *Bacillus thuringiensis* (Bt) toxin; *gfp*: green fluorescent protein; *Gut*D: glucitol-6-phosphate dehydrogenase; *GVG*: Gal4 binding/VP16 activation, glucocorticoid receptor; *hpt*: hygromycin phosphotransferase; *Mt*1D: mannitol-1-phosphate dehydrogenase; *npt*II: neomycin phosphotransferase; *PRGer*1*: Pinus radiata* germin*; RNAi:* RNA interference*;* s/a*:* sense/antisense*; uid*A: β-glucuronidase.

[c] Promoters; 35S: cauliflower mosaic virus (CaMV) promoter; 2x35S: double 35S gene promoter; 4UAS: promoter containing four copies of the Gal4 UAS and the -46 to +1 region of 35S; Emp: abscisic acid inducible promoter of the wheat EM gene; Emu: artificial promoter containing ocs enhancer elements; nos: nopaline synthase promoter; PAL2: phenylalanine ammonia-lyase promoter; pl: promoter less gene; RbcS: ribulose-1,5-bisphosphate carboxylase promoter; ubB1/ubi1: sunflower/maize polyubiquitin promoter.

[d] Plant regeneration method; O: organogenesis; SE: somatic embryogenesis; ZE: zygotic embryogenesis

Table 3 Stable PB transformation and plant regeneration in *Pinus* spp.

Species	Explant (source)[a]	Gene (promoter) transferred[a]	RM[a]	Reference
patula	EM (IZE)	*uid*A, *bar* (ubi1)	SE	Nigro *et al.* 2004
pinaster	EM (IZE)	*uid*A, *hpt* (35S)	SE	Trontin *et al.* 2002
radiata	EM (IZE)	*npt*II (35S), *uid*A (2x35S, Emu)	SE	Walter *et al.* 1998
radiata	EM (IZE)	*npt*II (35S), *uid*A (2x35S), *PRGer*1 s/a, *bar* (ubi1)	SE	Bishop-Hurley *et al.* 2001
radiata	EM (IZE)	*npt*II (ubi1, Emu), *uid*A (2x35S), *cry*1Ac (ubi1)	SE	Grace *et al.* 2005
radiata	EM (IZE)	*cad* s/a/RNAi (ubi1)	SE	Wagner *et al.* 2005
rigida x *taeda*	EM (IZE)	*npt*II (nos), *uid*A (35S)	SE	Connett-Porceddu *et al.* 2003
roxburghii	MZE (SL), Cot (MZE)	*uid*A, *bar* (ubi1)	O	Parasharami *et al.* 2006
sylvestris	Pol (PC)	*npt*II (35S, 2x35S), *uid*A (35S, 2x35S, ubB1, EmP)	ZE	Aronen *et al.* 2003
taeda	EM (IZE)	*npt*II, *uid*A (35S)	SE	Connett-Porceddu *et al.* 2003
taeda	MZE (SL)	*npt*II (nos), *cry*1Ac (2x35S)	O	Tang and Tian 2003

[a] See **Table 2**.

the genome by a variety of recombination events. In the AT method, the gene(s) of interest are inserted into the transfer-DNA (T-DNA) region of tumor-inducing plasmid (pTi) from a disarmed *A. tumefaciens* strain. After contact and attachment of bacteria to plant cells, the T-DNA is transferred to the nucleus and then spliced into plant DNA (see Tang and Newton 2003, for a description of the T-DNA transfer process). The target materials are embryonal mass (EM) or other explants, such as mature zygotic embryos, cotyledons, shoot apices of seedlings, and pollen. When non-embryogenic explants are used, the transgenic plants are regenerated by means of shoot organogenesis from the transgenic callus (Charity *et al*. 2002; Tang and Tian 2003; Grant *et al*. 2004; Tang *et al*. 2007) or by applying transformed pollen in controlled crosses (Aronen *et al*. 2003). The results of this work have generated a wealth of knowledge about the stability of gene expression, sources of transgene silencing, and efficacy of various gene promoters, among others (see the species sections of this review). However, SE cultures coupled with cryopreservation have obvious advantages over shoot organogenesis for large-scale production of transgenic pines (Walter *et al*. 2005). First, EM can be rapidly proliferated on either semi-solid or liquid media, providing a source of actively dividing cells that are recognized as the most competent cells for genetic transformation. Moreover, these cells are easily handled in suspension culture and/or on various supports at very low cell densities that favor efficient selection of transformed cells. Second, occurrence of chimeras is rare because a somatic embryo is usually derived from a single cell. Third, it takes considerably less time to recover a transgenic plant from EM. In radiata pine, for instance, 8-10 months was sufficient to regenerate transgenic somatic plants, whereas 18-24 months was needed to recover transgenic shoots from cotyledons or embryo apical domes (Charity *et al*. 2005). The final main advantage of SE over other regeneration methods is that transformed EM can be easily cryopreserved without any detectable effect on re-growth and transgene expression (Tereso *et al*. 2006b). This is critical because embryogenic cultures can progressively lose their somatic embryo maturation ability as a consequence of aging (Breton *et al*. 2006). The juvenility of transgenic EM can thus be preserved while transformed plants are tested for long-term gene expression. Moreover, high transformation efficiency is also a prerequisite for easy, cost-effective, large-scale production of transgenic material needed for the high-throughput gene testing or extensive screening and final selection of lines that will eventually be authorized for commercial deployment in MVF. For the above reasons, in this review we focused on the protocols developed specifically

for transformation of EM.

Transformation efficiency of pines is affected by a number of factors related to the plant material, e.g., species, family, genotype, explant type and source, as well as the developmental and physiological state of target tissue. A number of methodological issues are also critical for successful transformation and regeneration of transformed plants, namely the gene delivery method (AT or PB), the selection procedure of transformed cells, and the practices to minimize physical (e.g., membrane disruption) and physiological (e.g. metabolic pathways poisoned by toxic compounds) damage to cells during the whole, long process of transformation and selection.

The media for transformation and selection of transformed cells in pines are usually the same as those currently used for EM proliferation (DCR, EM, EDM, EMM, MSG, P6-based medium, mLV; see Klimaszewska *et al*. 2007 and references therein) with appropriate modifications or additives. Once transclones are selected and stabilized, cryopreservation of EM, maturation and germination of somatic embryos, as well as conversion to transformed plants invariably follows the specific protocols developed for each pine species (reviewed by Klimaszewska *et al*. 2007).

Transformation using particle bombardment

Radiata pine was the first transgenic pine produced, after PB transformation of EM, geneticin selection of transformed cells, and subsequent plant regeneration by SE (Walter *et al*. 1998). More than 150 transgenic plants derived from two lines (13 transclones) were established in the greenhouse. Since this key report, protocols have been developed to produce transgenic trees in seven other *Pinus* spp. (**Table 3**). As a physical method of gene transfer, PB optimization mainly relies on modification of bombardment parameters such as microcarrier particle size and speed, distance to target (Walter *et al*. 1998), handling of EM to facilitate the microcarrier penetration into the cells (e.g., use of filter paper as a support to anchor the cells), evaporation of excess water (Walter *et al*. 1994, 2005), and pre-culture of cells on preparation media to preserve cell integrity (Connett-Porceddu *et al*. 2003, see *P. taeda*). This direct, practical, and rapid physical method to deliver foreign DNA into the cells is considered to be independent of genotype, especially in transient gene expression experiments. Moreover, a gene construct could be delivered into virtually any organ, tissue, cell, or even cell compartment. Thus, this method appears best suited for large-scale studies of transient gene expression such as promoter testing. It is also currently the most efficient method to stably transform some pine species (see *P. radiata*).

Transformation using *A. tumefaciens*

The first demonstration of AT in a conifer was published by Levée *et al*. (1997). Transgenic plants were regenerated from EM of hybrid larch. Since then, AT has increasingly become favored over PB for stable gene expression and plant regeneration in pines, with a growing number of published reports (20, **Table 2** vs. 10, **Table 3**, for AT and PB, respectively). The AT method usually results in more predictable transgene integration patterns with a higher percentage (e.g., 100% vs. less than 40% using PB in barley, Travella *et al.* 2005) of low-copy (1-3) and single-locus insertion events, and with less foreign DNA fragmentation and rearrangements (Levée *et al.* 1999; Connett *et al.* 2003; Grace *et al.* 2005; Tang *et al.* 2005d). Such simple insertion events tend to result in stable gene expression and Mendelian inheritance. Integration of multiple copies of a transgene may lead to both transcriptional (TGS) and post-transcriptional gene silencing (PTGS). These regulatory mechanisms, especially PTGS, result in specific RNA degradation and are thought to be important for protecting plants against infections by foreign DNA sequences (e.g. viral DNA) or in down-regulating genes during plant development (Hamilton and Baulcombe 1999; Meyer 2000; Tang *et al.* 2005d). Interestingly, recent results in radiata pine suggest that silencing only affects the very early stages of tree development (see *P. radiata*).

Improvement of AT methodology requires that conditions for interaction of bacteria with plant cells, strains of *A. tumefaciens*, access to plant cells (wounding, vacuum infiltration), and co-cultivation parameters (plant cell and bacteria preparation, density ratios, duration, temperature, bacterial virulence inducers) be optimized. Furthermore, after transformation is completed, the agrobacteria must be eradicated from the cultures before the subsequent *in vitro* steps of selection and regeneration of transformed plant material. It is also an important objective for field release of transgenic trees (see Deployment of Transgenic Pines and Biosafety). The main concern is that the laboratory strain of *A. tumefaciens* could be released into the soil from the roots of the host transgenic plant and subsequently infect other plants or transfer the genes to other microorganisms by horizontal gene transfer (Droege *et al*. 1999; Stewart *et al*. 2000). The persistence of *A. tumefaciens* in genetically engineered trees is of significant interest, because they are likely to remain in the environment for decades. Unfortunately, there are disproportionately fewer publications investigating the survival of residual *A. tumefaciens* in plant tissues after transformation, relative to those on transformation itself. However, a few studies have reported that *A. tumefaciens* persisted in the tissues of other transgenic plant species (herbaceous and trees) for up to 3 months after transformation (Mogilner *et al*. 1993; Cubero and López 2005). One study has shown that *A. tumefaciens* persisted in transgenic tobacco plants that had been in soil for 3–6 months (Matzk *et al.* 1996). Recent studies on residual agrobacteria after transformation of EM of several conifer species showed it persisted for up to 12 months despite the lack of visible bacterial growth on the culture medium (Charity and Klimaszewska 2005). However, no agrobacteria were detected in mature somatic embryos or in needles, branches, stems, or roots of transformed plants grown in a greenhouse for up to 4 years following transformation. One of the critical issues that became apparent in that study was the limitations of the polymerase-chain reaction (PCR) in detecting bacterial DNA in a background of pine needle DNA, which was approximately 10^9 to 10^{10} cells g^{-1} fresh mass (fm) of tissue. Thus, rather than using the PCR method as the sole detection technique when determining if *A. tumefaciens* persists in plant tissues, it is recommended that an enrichment culture of bacterial cells released from macerated plant tissues on bacterial growth medium, followed by PCR of bacterial colonies, be used. Similarly, Cubero and López (2005) also concluded that enrichment techniques followed by PCR should be used for detecting *A. tumefaciens* that may be present, but not actively growing in transformed tissues.

Various strains have been tested in pine transformation, such as succinamopine strains with C58 chromosomal background (EHA105, AGL1), octopine TiAch5 (LBA4404) or C58 (GV2260) strains, and various nopaline C58 strains such as EHA101, GV3101, C58pMP90 (= GV3101pMP90), and GV3850 (Hellens *et al*. 2000). EHA105 harboring the super-virulent pTiBo542 plasmid (Hood *et al*. 1993) was the most efficient strain in various pine species such as loblolly pine (EHA105 > LBA4404 > GV3101; Wenck *et al*. 1999), *P. pinea*, and *P. nigra* (EHA105 > LBA4404 > GV3850; Humara *et al*. 1999a; Lopez *et al*. 2000b). This strain was also successfully used in other species such as *P. eldarica* (Gould *et al*. 2002), *P. radiata* (Charity *et al*. 2005), *P. rigida* × *taeda* (Connett-Porceddu *et al*. 2007), and *P. virginiana* (Tang *et al*. 2005a, 2005c). The C58pMP90 strain was found effective in eastern white pine (Levée *et al*. 1999) and maritime pine (Trontin *et al*. 2002; Tereso *et al*. 2006b). Additional copies of the *virB*, *virC*, and *virG* genes from pTiBo542 were shown to further increase transient reporter gene expression in loblolly pine following infection of EM with EHA105 (Wenck *et al*.

1999). Tang (2003) confirmed these findings in transformation experiments of mature zygotic embryos. As the *virB* gene has been implicated in T-DNA transfer, these results suggest that gene transfer and not just virulence induction may be a limiting factor in AT of pines (Wenck *et al*. 1999).

Selection and reporter genes used in pine transformation

As in most other higher plants, the success of transformation in pine species primarily depends on the efficient delivery and expression of selectable marker genes into the genome, allowing selection of the transformed cells. Only negative selection with antibiotic- or herbicide-resistant genes has been reported in pine. Three selection genes have been involved in stable transformation studies (**Tables 2** and **3**): (1) the commonly used *npt*II gene from *Escherichia coli* encoding neomycin phosphotransferase, which confers resistance to the antibiotics kanamycin or geneticin; (2) the *hpt* (or *aph*IV) gene from *E. coli* encoding hygromycin phosphotransferase, an enzyme capable of detoxifying hygromycin B, a strong inhibitor of protein synthesis; and (3) the *bar* gene isolated from *Streptomyces* spp., encoding the phosphinothricin acetyl transferase responsible for resistance to phosphinothricin, bialaphos and commercial herbicide formulations containing the ammonium salt of phosphinothricin (ammonium glufosinate) such as Basta™ or Buster™. The active ingredient phosphinothricin is an irreversible inhibitor of glutamine synthase. This molecule affects both protein synthesis and the mitochondrial respiratory chain. In each case, the optimal concentration of the selective compound has to be precisely determined. These concentrations are usually specific for each pine species and type of target explant in order to allow selection of transformed cells while preserving their regeneration potential. Inefficient application (type, concentration, and timing of selection after transformation) of the selective agent may result in conditions that are too stringent and detrimental to both transformed and non-transformed cells, or that are insufficiently stringent, resulting in high frequency of non-transformed cells escaping the selection process ("escapes"). Reporter genes facilitate visualization and quantification of gene expression by means of bioassays. The most frequently used genes (**Tables 2** and **3**) are *uid*A (encoding β-glucuronidase) and, more recently, *gfp* (encoding green fluorescent protein). Non-toxic, visual marker genes such as *gfp* may be useful in developing effective selection strategies for transformed cells at the early stages without the need for antibiotic or other selective agent application (see *P. strobus*). The *luc* (luciferase) gene was also found useful in transient expression experiments in radiata pine (Campbell *et al*. 1992).

Chimera

In both the PB and AT techniques, the potential to create chimeric cell lines and plants composed of both transformed and non-transformed cells or, alternatively, of transformed cells from different integration events, may be problematic. No extensive research has been carried out on this topic. In one study, the molecular analysis of plant DNA/T-DNA junctions in different parts of loblolly pine transgenic plants regenerated from adventitious buds from transformed callus demonstrated a single cell origin (Tang *et al*. 2001). Similarly, in radiata pine, the analysis of several adventitious shoots regenerated from five independently transformed callus pieces did not reveal chimera (Grant *et al*. 2004), nor were they detected in transgenic EM lines (Bishop-Hurley *et al*. 2001; Wagner *et al*. 2005). Recently, however, it was shown that three maritime pine somatic plantlets obtained from three different transgenic EM lines (confirmed by molecular analysis) were regenerated from non-transformed cells (Tereso *et al*. 2006b). This result suggests that transformed EM were chimeras made of both transformed and non-transformed cells. Clearly, more attention needs to be given to this aspect of transgenic plant production.

Promoters, gene expression and silencing

Several constitutive promoters such as 35S (*Cauliflower mosaic virus*, CaMV), nos (nopaline synthase), ubi1 (maize ubiquitin), artificial Emu, and rice actin have been used to control gene expression (**Tables 2** and **3**). However, inducible promoters were proposed to offer more flexibility, as they allow the regulation of gene expression. Functional studies would benefit from the availability of a system to induce gene expression at defined developmental stages or under particular conditions. These promoters are quiescent in the absence of inducers, and therefore, will not interfere with physiological activities and plant regeneration while the promoter is inactive. A few of these promoters were studied and found effective in pines for tightly controlling gene expression, e.g., the light-inducible PAL2 (phenylalanine ammonia-lyase) promoter from bean (see *P. strobus*) and a chimeric transcriptional activator GVG using triamcinolone (*P. virginiana*, Tang and Newton 2004b; Tang *et al*. 2005c) or dexamethasone as chemical inducers (Tang and Newton 2004a, see *P. taeda*).

Reverse genetics is increasingly applied in plant species to study the function of genes involved in various metabolic pathways. In long-lived plants such as pines, being able to control the expression of selected genes (i.e., overexpression or gene silencing) in a reduced time frame will be a significant advance. Various gene repression strategies have recently been successfully tested in pines (*P. elliottii*, *P. radiata*, *P. virginiana*), e.g., direct delivery of small interfering RNAs (siRNAs) into cells using PB (Tang *et al*. 2005e) or nanosecond pulsed laser-induced stress wave (LISW, Tang *et al*. 2006b), stable transformation and expression in the cell genome of sense/antisense genes or large RNA interference constructs (RNAi) in the form of an inverted repeat (Wagner *et al*. 2005; Möller *et al*. 2005; see *P. radiata*). Interestingly, in *P. virginiana* cell cultures, the dexamethasone-inducible system (GVG, see above) was applied to stringently control antisense-mediated PTGS of the *gfp* reporter gene (Tang *et al*. 2005b). The degree of *gfp* silencing could be regulated by the concentration of inducer (1-10 mg l^{-1}) and the time of treatment (1-21 days). This result was achieved by using the 4UAS promoter to drive the antisense *gfp* sequence, which is sensitive to the inducible transcription factor GVG.

Field tests and application of transgenic pines

Although transgenic pines from different species (radiata pine, Scots pine, loblolly pine) have apparently been established in more than 50 field tests (FAO 2004) since at least 1996 (OECD database of field trials of genetically modified organisms), published data on field growth performance and transgene expression are scarce. Evaluation of 3- to 8-year-old transgenic radiata pine (Walter *et al*. 2005, see *P. radiata*) and loblolly pine (Connett *et al*. 2003, see *P. taeda*) indicated normal growth in the field and continued stable transgene expression.

To date, only a few attempts to create new transgenic pine genotypes with novel characteristics have been published, including herbicide tolerance (e.g., sulfonylurea, *cfs-r* gene, Walter and Smith 1999) or glufosinate ammonium (*bar* gene, Bishop-Hurley *et al*. 2001, see *P. radiata*; see also Parasharami *et al*. 2006), resistance to insects (*cry1Ac* gene, see *P. radiata* and *P. taeda*), reduced or modified lignin (*cad* gene; see *P. radiata*), tolerance to salt (*Mt1D*/*GutD* genes; see *P. taeda*), heavy metal, heat, and resistance to bacterial pathogens (*CaPF1* gene; Tang *et al*. 2005a, 2006a). In the latter case (*P. virginiana*), the level of antioxidant enzymes and other plant compounds that are considered to play a role in protecting cells from the oxidative damage caused by biotic and abiotic stresses was increased in transgenic plants. Interestingly, enhanced organ growth caused by increasing cell size and cell number was conco-

mitantly observed.

PINUS PINASTER

Research programs were launched in France (FCBA, INRA) and Portugal (IBET) to establish basic protocols needed for efficient delivery of foreign genes into the maritime pine genome using EM as target material. Stable transformation of EM and regeneration of transgenic plants grown in the greenhouse were first reported by Trontin *et al*. (2002) with molecular evidence (PCR and Southern hybridization). Protocols for both PB and AT were adapted from the methods of Bercetche *et al*. (1992) and Levée *et al*. (1999), respectively, and applied to different genotypes selected from the French breeding program. Subsequently, stable AT of EM was also achieved with Portuguese maritime pine genotypes (Tereso *et al*. 2003, 2006b), but with very low regeneration rate and production of non-transformed plantlets.

Recent developments have aimed at improving transformation efficiency and rapid selection of transformed cells using AT. The PB method is no longer used in maritime pine for stable transformation because of the complex patterns of transgene integration, i.e., multiple loci and amplification of tandem repeats (Trontin *et al*. unpublished). Comparatively, less than three T-DNA copies were generally detected after AT (Tereso *et al*. 2006b; Trontin *et al*. unpublished). In addition to the strong genotype effect (Trontin *et al*. 2002; Tereso *et al*. 2006b), recent progress in AT revealed that transformation efficiency and subsequent regeneration of plants is significantly affected by the EM physiology (age since SE initiation), the *A. tumefaciens* strain, the co-cultivation method, the control of bacterial growth with eradicants, and the selection procedure.

In the studies presented below, the disarmed *A. tumefaciens* strain C58pMP90 (Koncz and Schell 1986) was used to carry binary vectors harboring *hpt* and *uid*A genes under the control of the 35S promoter (pCambia1301 – Hajdukiewicz *et al*. 1994; Trontin *et al*. 2002; pPCV6NFGUS – Mathur *et al*. 1998; Tereso *et al*. 2006b) or 2 *npt*II copies controlled by the double 35S (2x35S) or 19S promoters (pMRKE70Km – Leplé *et al*. 1992; Lelu-Walter, unpublished).

Transformed cultures could be recovered from 4-day-old cell suspensions, but not from 7-day-old suspensions (Tereso *et al*. 2006b). As in other pine species, this result suggests that transformation efficiency is correlated with cell division rate, which was probably higher at the 4th day of culture. In a survey of transformation efficiency of 12 embryogenic lines (10 genotypes), it was found that transformation efficiency was correlated with the age of cultures computed as the total number of subcultures since SE initiation (Trontin *et al*., unpublished). Young lines subcultured for less than 50 weeks were apparently easier to transform (five out of seven genotypes) than older lines propagated for more than 100 weeks (one out of five genotypes). Both analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) tests for means comparison (six transformation experiments) concluded that the juvenile PN519.j line grown from the cryopreserved stock produced significantly higher yields ($P < 0.05$) in both hygromycin-resistant (H+) lines and H+ lines expressing *uid*A (GUS+) than the old PN519.o counterpart (**Fig. 1**). In this study, the significant effect of genotype on transformation efficiency was also confirmed, and using a juvenile version of a recalcitrant genotype, produced negative results. The juvenility of EM is, therefore, an important factor for transformation of receptive genotypes in maritime pine, but not a sufficient criterion to recover transclones from recalcitrant genotypes. Cryopreservation of transclones is thus required in order to preserve their juvenility. The cryopreservation process did not affect the regrowth and stability of transgenic cells in maritime pine (Tereso *et al*. 2006b).

In another set of experiments (Trontin *et al*. unpublished), three disarmed *A. tumefaciens* strains (C58pMP90, LBA4404, AGL1; see Hellens *et al*. 2000) with highly contrasted chromosomal backgrounds and Ti plasmids were tested in transformation experiments involving line PN519 and the binary vector pCambia1301. The nopaline strain C58pMP90 was confirmed as a superior strain (ANOVA, $P < 0.05$) for transforming maritime pine at a high frequency compared with the succinamopine strain LBA4404 and the octopine strain AGL1 (**Fig. 1**). This latter strain yielded only meager results. Interaction with the subculture number was also strongly apparent in these experiments for both the C58pMP90 and LBA4404 strains, as indicated above.

Compared with a direct co-cultivation method, where the bacterial suspension is mixed with EM and immediately collected on a filter paper disk, a 6 h pre-culture method (i.e., flooding the EM pieces with bacterial suspension) did not improve transformation efficiency in maritime pine (13.3 ± 4.8 vs. 9.2 ± 2.9 H+ lines g^{-1} fm; Lelu-Walter, unpublished). Mixing of bacterial suspension with suspended EM and plating on filter papers for co-cultivation is now routinely used for maritime pine (Trontin *et al*. 2002; Tereso *et al*. 2006b).

For genetic transformation of *P. pinaster*, acetosyringone is used as *A. tumefaciens* virulence inducer and usu-

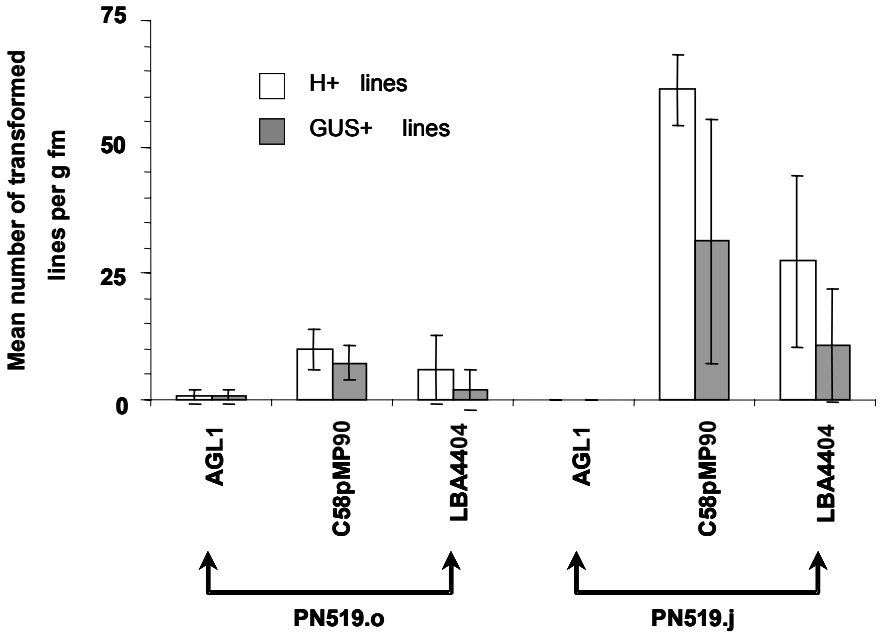

Fig. 1 Transformation efficiency after 12 weeks selection of *P. pinaster* embryogenic line PN519 co-cultivated with *A. tumefaciens* strains AGL1, C58pMP90 and LBA4404 carrying the binary vector pCambia1301 (Trontin *et al*. unpublished). Line PN519.j is a juvenile version of PN519 thawed from the cryopreserved stock (46 subcultures since initiation at the time of experiment). PN519.o is an older counterpart subcultured for more than 100 weeks. H+ lines: hygromycin-resistant lines; GUS+: hygromycin-resistant lines expressing the *uid*A gene; vertical bars: 5% confidence intervals (three independent experiments); fm: fresh mass.

ally added to the co-culture medium at 100 µM (Trontin *et al*. 2002; Tereso *et al*. 2006b). Other virulence inducers such as coniferin or coniferyl alcohol (100 µM) were also compared with acetosyringone (Lelu-Walter *et al*. unpublished). The results showed that acetosyringone had the most significant effect. Transformation rate increased from 22 ± 7 (no acetosyringone) to 40 ± 9 H+ lines g^{-1} fm. An effect was also observed with coniferin (29 ± 31 H+ lines g^{-1} fm), but with highly variable response compared with acetosyringone. No change was detected with coniferyl alcohol.

Agrobacteria growth can usually be controlled on a medium supplemented with 300 mg l^{-1} Augmentin™ (a mixture of amoxicillin and clavulanic acid) for at least 4-5 weeks and up to 12 weeks (Trontin *et al*. 2002). However, agrobacteria growth was invariably observed in the case of one genotype using up to 600 mg l^{-1} Augmentin™, suggesting that a genotype-related protection of agrobacteria during antibiotic treatment can occur. Similarly, ticarcillin combined with clavulanic acid (Timentin™) has been effective for *A. tumefaciens* elimination from nine embryogenic lines (Tereso *et al*. 2006a) using a concentration of 400 mg l^{-1} for at least 12 weeks. In that case, complete removal of casein hydrolysate from the DCR-based medium used for transformation was concomitantly found to be crucial to recover embryogenic tissue growth after co-cultivation (Tereso *et al*. 2006b). Continuous presence of antibiotics or bacteriostatics in the culture medium did not adversely affect the viability of EM.

Both *npt*II and *hpt* genes were tested as selection genes in maritime pine transformation. Kanamycin was reported to inhibit growth of embryogenic cells (Tereso *et al*. 2006a) plated on filter paper within 2 weeks at 20 mg l^{-1}, or EM clumps within 4 weeks at 5 mg l^{-1}. Thus, the hygromycin gene is currently preferred for selection of transformed maritime pine cells. Ten mg l^{-1} hygromycin was found effective by Tereso *et al*. (2006b) to achieve selection of transformed lines from Portuguese genotypes, but 20 mg l^{-1} was proposed by Trontin *et al*. (2002) as the optimal level for most genotypes. Recently, herbicide selection was also tested in maritime pine (Trontin *et al*. unpublished). A gene cassette extracted from pAHC20 (Christensen and Quail 1996) comprising the ubi1 promoter with first intron, the coding sequence of *bar* gene and nos terminator was ligated into pCambia1301 between the *hpt* and *uid*A gene cassettes. The resulting new vector (pCambia1301/pUbi-*bar*-Tnos) could then be used to compare the transformation efficiency of line PN519 following 12 weeks of selection with hygromycin (20 mg l^{-1}) or phosphinothricin (1 mg l^{-1}). Both casein hydrolysate and glutamine should be completely removed from the culture medium to allow efficient phosphinothricin selection within 12 weeks. More phosphinothricin-resistant lines (P+) were recovered compared with H+ lines after two independent experiments (means of 136 P+ vs. 82 H+ lines g^{-1} fm).

Selection was applied when EM growth was noticeable, i.e., after about 7 to 10 days (Trontin *et al*. 2002; Tereso *et al*. 2006b). The continuous use of a selective agent (hygromycin vs. phosphinothricin) in the EM culture medium during the 12-week selection period, however, influenced the success of post-transformation steps, especially the somatic embryo maturation yields (Trontin *et al*. unpublished). A total of 1080 mature somatic embryos were regenerated from transformed and control lines (**Fig. 2**): 387 from H+ lines (mean of 21.5 embryos g^{-1} fm per line), 539 from P+ lines (mean of 29.9 embryos g^{-1} fm per line), and 154 from the non-transformed PN519 line (51.3 embryos g^{-1} fm). Maturation yields ranged from 15 (AC6.1) to 33 (AC3.3) embryos g^{-1} fm in the case of H+ lines, and from 4 (AB7.10) to 42 (AB5.1) embryos g^{-1} fm considering P+ lines. Pairwise SNK tests for means comparison (**Fig. 2**) revealed that transformation yield was significantly reduced for five out of six H+ lines (AC6.1, AC7.1, AB4.1, AB5.3, AB6.8), but only for two out of six P+ lines (AB5.3, AB7.10). A global ANOVA confirmed that maturation yield was diminished following selection using hygromycin compared with phosphinothricin ($P < 0.05$).

Protocols for AT of EM in maritime pine are now sufficiently refined to allow production of transgenic plantlets of the receptive genotypes. One recommended procedure is to co-cultivate juvenile and actively growing EM lines with *A. tumefaciens* strain C58pMP90 in the presence of 100 µM acetosyringone for 2 days in the dark at 25°C. The continuous use of 300 mg l^{-1} Augmentin™ or 400 mg l^{-1} Timentin™ (without casein hydrolysate in the medium) is effective to control agrobacteria growth during the whole duration of post-transformation (up to 12 weeks). When cell growth resumes (after 7-10 days), selection pressure with 10 to 20 mg l^{-1} hygromycin allows the recovery of resistant lines within 12 weeks. Selection using 1 mg l^{-1} phosphinothricin (without casein hydrolysate and glutamine in the medium) can also be used with similar or higher transformation efficiency and improved regeneration ability of transclones compared with hygromycin.

More research is now needed to further refine AT protocols in order to broaden the range of transformable genotypes, including the "recalcitrant" genotypes. Indeed, the implementation of genetic transformation as a new tool for genetic breeding of maritime pine would ultimately require that transformed lines could be produced from any selected, elite clones.

When applied to receptive embryogenic lines, the current protocols can be useful for functional analysis of genes and promoters involved in wood-related and other biosynthetic pathways. Recently, the expression of an heterologous promoter of coniferyl coenzyme A orthomethyl transferase (pCCoaOMT) from poplar (*Populus* sp.), which is known to be preferentially expressed in lignifying xylem cells in the stem has been studied in maritime pine using three embryogenic lines and derived somatic embryos (Lelu-Walter *et al*. unpublished). A construct involving the heterologous pCCoaOMT promoter linked to *uid*A was

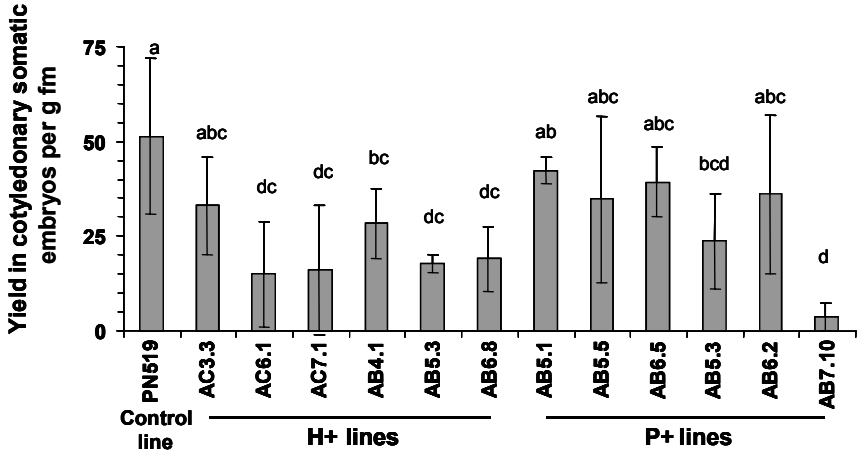

Fig. 2 Yields in *P. pinaster* cotyledonary somatic embryos obtained from hygromycin- (H+) and phosphinothricin-resistant (P+) lines cultured for 12 weeks on selection medium (20 mg l^{-1} hygromycin or 1 mg l^{-1} phosphinothricin) and 4 more weeks on maintenance medium before maturation (Trontin *et al*. unpublished). Both maintenance and maturation media were free of antibiotic. Lines labeled "AC" were transformed with pCambia1301 carrying the *hpt* gene. Lines labeled "AB" were transformed with pCambia1301/Pubi-*bar*-Tnos carrying both *hpt* and *bar* genes. Difference between means (three independent experiments) sharing the same letter is not significant (Student-Newman-Keuls test, α = 0.05); Vertical bars: 5% confidence intervals; fm: fresh mass.

used. Except for one line, the transformation rates were consistent with those previously obtained with the established protocol. Approximately, 40 H+ lines g^{-1} fm could be selected. Validation of the transformation events was realized by transgene expression analysis using the histochemical detection of GUS and RT-PCR. A few transclones were matured in order to regenerate somatic embryos and plants for histochemical and molecular analyses. The expression of *uid*A under control of this promoter was effectively detected in transgenic maritime pine material (EM, somatic embryos).

Similarly, reverse genetic studies of candidate genes for MAS can now be undertaken in maritime pine using AT of model lines. Functional studies of wood quality genes are currently being jointly developed in France by FCBA and INRA.

PINUS RADIATA

Radiata pine EM has been transformed for the first time with *A. tumefaciens* (strain LBA4404) carrying *npt*II and *uid*A genes under the nos and 35S promoters, respectively, by Cerda *et al*. (2002). Transclones were recovered on medium with geneticin (15 mg l^{-1}) and bacteria growth was controlled by carbenicillin (400 mg l^{-1}). Tissue expressing the *uid*A gene was confirmed by PCR. However, no transgenic plants were regenerated and no Southern hybridization results were provided to confirm integration pattern of T-DNA. Subsequently, Charity *et al*. (2005) transformed EM with the *Agrobacterium* strain EHA105, followed by the regeneration of transgenic plants. Transgenic tissue was selected on medium containing geneticin (5-10 mg l^{-1}), and transgenic tissue and embryos showed the expression of the introduced *uid*A gene. The latter work also included transfer of the *bar* gene, and regenerated transgenic plants were resistant against operational applications of the herbicide Buster™. Molecular analysis confirmed the presence of introduced genes, and most transclones had only a few copies of the transgene, confirming a relatively simpler integration pattern than that of PB transformation. This was also reflected in transformation experiments with *A. tumefaciens* of radiata pine cotyledons (Grant *et al*. 2004), where multiple copy integrations and truncated integrations were reported. However, the complexity of gene integration was still much lower compared with PB methods (Walter et *al*. 1998).

Much work in radiata pine transformation was done using PB. This method was used first to transfer *uid*A into EM and to estimate transient *uid*A expression (Walter *et al*. 1994). Various factors, such as the promoter controlling *uid*A expression, physical bombardment conditions, and media composition were tested to optimize the gene transfer protocol, but stable transformation could not be achieved. However, this work showed that a high level of gene transfer into embryogenic cells was achievable and that promoters such as the artificial pEmu led to significantly higher *uid*A expression levels compared with 35S. Wagner *et al*. (1997) showed that the *aph*IV gene (hygromycin resistance) could also be used to select transgenic EM of radiata pine. Other studies on transient expression of *uid*A concluded the superiority of ubi promoter (from a polyubiquitin gene) over 35S and rice actin promoters (McElroy *et al*. 1990; Binet *et al*. 1991).

The first stably transformed transgenic radiata pine trees were reported in 1998 (Walter *et al*. 1998). The EM was bombarded with the *npt*II and *uid*A genes, and transgenic material was selected using geneticin (15 mg l^{-1}). Molecular analysis of transgenic plants confirmed the integration and expression of new genes. The gene integration pattern was complex and tandem repeats, multiple copies, and fragmented integrations were detected. The application of PB resulted in stably transformed radiata pine trees with novel traits of interest for plantation forestry, such as herbicide tolerance (glufosinate ammonium, *bar* gene expression; Bishop-Hurley *et al*. 2001) and insect resistance (painted apple moth, *cry1*Ac gene expression; Grace *et al*. 2005). In these cases, the expression of the transgenes and the expected traits were confirmed using operational spray tests and insect feeding assays.

Recent work has focused on improving transformation frequencies of EM using a particle inflow gun and increasing the number of genotypes that can routinely be transformed (Devillard *et al*. 2007). Embryogenic lines that are readily undergoing maturation and form high numbers of viable somatic embryos usually show very low genetic transformation frequencies when standard PB techniques (Biorad PDS 1000 He) are used, involving dried DNA-coated gold particles on a macrocarrier (Walter *et al*. 1998). A different PB that delivers DNA-coated gold particles in liquid (Particle Inflow Gun; Finer *et al*. 1992), has been used to increase transformation frequencies to up to eight transclones per bombarded plate (Devillard *et al*. 2007). This allowed the production of 80–3200 transclones per day, depending on the genotype. As 20-30 transgenic events are usually sufficient for a scientific experiment, the modified technique offers the potential to include a range of genotypes that have poor plant regeneration ability.

The induced silencing of genes in radiata pine has been studied using sense, antisense, and RNAi technologies in combination with the *cad* gene (cinnamyl alcohol dehydrogenase) (Wagner *et al*. 2005). The data indicated that constitutive expression of RNAi constructs (ubi1 promoter) based on inverted repeats was most efficient in downregulating *cad* in biolistically transformed EM and regenerated plants. Antisense constructs silenced *cad* to a much lesser extent, but sense constructs in some cases led to silencing as well. Using a similar RNAi construct driven by the highly expressing *cad* endogenous promoter, reduced *cad* activity was similarly obtained in most transclones generated from radiata pine xylem-derived callus culture (Möller *et al*. 2005). These data confirmed for the first time that RNAi can efficiently be used in conifers to silence endogenous genes. Quantitative measurements of CAD revealed reduced enzymatic activity in almost all transclones. Furthermore, the accumulation of metabolites such as dihydroconiferyl-alcohol, which also accumulates in a loblolly pine *cad* mutant line, was observed.

Field trials of genetically modified radiata pine plants started in 1996. An enzyme linked immuno sorbent assay (ELISA) was used in a field trial to study the continued expression of the *npt*II gene in up to 8-year-old trees from different transformation events. Interestingly, although gene silencing after PB was observed in immature somatic embryos and very young (0-3 months old) *in vitro* somatic seedlings, such an effect was no longer detectable in trees under non-selective conditions in the field. The transgene expressed continuously during the assessment with trees tested from their 5th to 8th year of age. Although some variation in gene expression was observed, each transclone and every individual plant from all transclones tested continued to express *npt*II (Walter, in preparation). The field tests were also used to study impacts of genetically modified pines on soil microorganisms and native insects. Horizontal gene transfer into other soil microorganisms could not be detected. Furthermore, no negative effects on soil microorganism populations were found, and the plantation site is characterized by high biodiversity for soil microbes and insects (Burgess, unpublished; Lottmann, unpublished).

Genetic engineering of trees for commercial use in plantation forestry is severely limited by the time it takes to genetically transform tissue, regenerate whole trees, and conduct molecular and biochemical analyses. Time and space restrictions by and large limit the testing of genes at high throughput and within an acceptable timeframe. In order to significantly reduce time and to functionally study genes associated with secondary cell wall development in radiata pine, Möller *et al*. (2003) developed an *in vitro* cell culture system for gene testing. Callus cultures were genetically transformable and could be initiated from xylem strips on young radiata pine trees. Following transformation

using PB (Biorad PDS 1000 He), genetically transformed cells were selected on geneticin medium and induced to form secondary walls. Studies using cell wall biochemistry-related genes demonstrated the usefulness of this technology for functional gene analysis in a timeframe of less than 6 months, compared to years required for transgenic tree regeneration and testing of whole transgenic trees. This technique now allows the testing of genes related to primary or secondary cell wall composition and architecture, at acceptable throughput and limited timeframes. Recently, *in vivo* transformation of cambial tissues was similarly reported as an efficient and rapid means for induced somatic sector analysis (van Beveren *et al.* 2006). In this study, cambial zones of plants of eucalyptus, poplar and pine were genetically transformed using *A. tumefaciens*, and the expression of foreign genes was confirmed up to two years following the transformation event. Initial experiments were performed with the *uid*A reporter gene; however the authors predict that this technique can now be used to study cell fate and gene function during secondary growth in stems of forest tree species.

High throughput PB technologies are now available for radiata pine, and a variety of genotypes can be used for transformation and production of transgenic plants. Field studies have shown no negative effects of genetically modified radiata pine trees on the environment, and gene expression appears to remain stable after several years in the field and in the absence of selection for the introduced gene. This makes radiata pine an ideal candidate for deployment of genetically modified material in plantation forestry (MVF). Benefits expected from cell wall modifications include improved pulpability, higher wood quality, and in particular, environmental benefits such as less toxic pulping processes and higher carbon sequestration on a per hectare plantation basis.

PINUS STROBUS

There are only three published reports on stable genetic transformation of EM in eastern white pine using the AT method (Levée *et al*. 1999; Levée and Seguin 2001; Tang *et al*. 2007). In the first study, one EM line was used in co-cultivation with *A. tumefaciens* disarmed strain C58pMP90 containing either of the following binary vectors: pGIN, pBIV, pBIVSAR or pBINm*gfp*5ER. All the plasmids carried the *npt*II selectable marker gene controlled by the nos promoter. The pGIN carried the *uid*A-intron gene controlled by the 35S promoter. The pBIV and pBIVSAR are BINplus derivatives. The pBIV carried the 2x35S-AMV*uid*A*::npt*II fusion. The pBIVSAR was pBIV with scaffold attachment regions (SARs) to reduce the risk of gene silencing.

The transformation involved first mixing the *A. tumefaciens* suspension with the EM suspension (200 mg fm in one ml of mLV medium) in 1:1 ratio, collection of cells on a filter paper disk, and co-culture on a semi-solid mLV medium with PGR and acetosyringone in the dark at 25ºC for 2 days. Acetosyringone was also earlier added to the cell suspension. After co-cultivation, the bacterial growth was controlled by washing the cells in a solution of tetracycline (25 mg ml^{-1}), followed by culture on a medium with 250 mg l^{-1} cefotaxime. The selection of transformed cells was carried out 1 week later, when EM displayed growth, on a medium with 25 mg l^{-1} kanamycin (concentrations up to 75 mg l^{-1} were tested). Kanamycin-resistant cell colonies (K+ lines) were distinguished as small white clumps of growing cells 5-6 weeks after infection with *A. tumefaciens*.

The mean transformation frequency over five experiments was four K+ lines g^{-1} fm of co-cultivated EM. The transformation frequency was higher if acetosyringone was present in the medium for at least three of the four constructs. The PCR analysis of 50 K+ lines confirmed the transgenic nature of the lines and showed no escapes. The Southern hybridization analysis of eight K+ lines revealed incorporation of mostly two to many copies of T-DNA. There was a large variation in the *uid*A expression among the lines and constructs, as determined by the fluorometric GUS analysis. The study of the variability of the relative GUS activity over a 5-month period showed that the lines transformed with BIVSAR fluctuated little compared with the lines transformed with BIV. This would suggest that SAR elements might stabilize the expression of a gene, which otherwise might be affected by environmental or physiological factors.

The transclones producing GFP were also obtained, and some cells emitting fluorescence were detected under UV light after the selection process was completed. However, during the selection, no cells could be identified, possibly because there was insufficient accumulation of the protein needed for direct visualization under UV light. In another study involving transformation of eastern white pine zygotic embryo-derived callus with *gfp*, it was determined that integrating more than one T-DNA copy into the same chromosome caused gene silencing (PTGS), and thus a lack of fluorescence (Tang *et al*. 2007). However, there was no difference in shoot differentiation and development among transclones with single vs. multiple T-DNA inserts.

Another study has shown that the inducible PAL2 promoter from bean is functional in transgenic EM lines (Levée and Seguin 2001). The *A. tumefaciens* strain used for co-cultivation was C58pMP90 carrying the binary vector pBIN-PAL2-*uid*A. All 15 selected lines expressed *uid*A following exposure to UV. Compounds such as jasmonic acid and salicylic acid added to the medium activated the *uid*A expression to low levels and in a few lines only; however, they enhanced the expression if followed by UV irradiation. An opposite effect was observed if the cell lines were treated with okadaic acid, which inhibits phosphatase activity, suggesting that the induction of the heterologous PAL2 promoter is consistent with induction of PAL in angiosperms.

Stable genetic transformation using the AT method is achievable for eastern white pine, and may serve as a tool for research as well as for future creation of trees with novel traits once transgenic trees are tested for the long-term gene expression and persistence of a specific trait.

PINUS TAEDA

Although SE of loblolly pine has been improved significantly over the last 20 years and is commercially available, very little has been published on genetic transformation of EM and regeneration of transgenic plants. Recent progress is mostly reported in issued US patents or in published applications (Sederoff *et al*. 1989; Connett-Porceddu *et al*. 2003; Connett-Porceddu and Gulledge 2005; Connett-Porceddu *et al*. 2007).

Stable transformation of EM and routine regeneration of transgenic plants was obtained in *P. taeda* and one hybrid (*P. rigida* × *P. taeda*) through the application of patented PB (Connett-Porceddu *et al*. 2003) and AT methods (Connett-Porceddu *et al*. 2007). Among PB parameters that were optimized, identification of the appropriate developmental stage of the early somatic embryos and use of special media formulations (preparation media) to condition and protect the cells from injury before, during, and after bombardment were critical factors for successful transgene delivery. The success of transformation using the AT method depends on the culture system applied to eradicate or control growth of *A. tumefaciens* after co-cultivation. Moreover, the selection strategy of transformed cells after both PB and AT appeared crucial for regeneration of transformed lines and plants from a wide range of genetic backgrounds. The use of special types of membrane support to handle the cells (Connett-Porceddu *et al*. 2007), as well as ABA in the medium (Connett-Porceddu and Gulledge 2005), did significantly improve production of transgenic plants.

When using the PB transformation method, the key factor was to determine precisely the most amenable embryo stage for the DNA-coated particle delivery. As in other pines, loblolly pine embryogenic cultures can be separated

into different cell fractions. Although all fractions were able to differentiate mature somatic embryos and plants, only the fractions containing stage II to pre-stage III immature embryos (torpedo-shaped embryos) produced transgenic cultures and plants (Connett *et al*. 2003; Connett-Porceddu *et al*. 2003). Another factor was decreased water potential of the medium on which the EM was bombarded. Transformation efficiency is significantly enhanced when cells are bombarded on embryo maturation medium, which typically contains osmotically active molecules such as sucrose, maltose, inositol, PEG, glycerol, and sorbitol, as opposed to maintenance medium. It is plausible that the slightly plasmolyzing conditions prevented possible leakage from the cells during or after bombardment. The development of harvestable embryos subsequent to exposure to different "bombardment" media was variable among four EM lines. Depending on the line, media containing combination of maltose (6%) and PEG (7%), sucrose (6%), sucrose (3%) and glycerol (4.6%) or sorbitol (4.6-18.2%) were optimal for regeneration of transgenic plants.

When using the AT method, the critical factor for recovery of transclones was the mode of eradicating *A. tumefaciens* from the cultures. If the eradicants were incorporated in the gelled media throughout most of the culture period following transformation, it was often detrimental to the growth and regeneration ability of embryogenic cells (Connett-Porceddu *et al*. 2007). Therefore, an alternative technique was developed using a double layer or biphasic culture system that abolishes the need for continuous use of eradicants. In this method, cells were cultured on a bilayer culture medium composed of the gelled maintenance medium overlaid with a thin, gelled or preferably liquid film directly pipetted or saturated into a filter paper and containing antibiotics. Only a few milliliters of medium overlay were necessary to restrict growth of *A. tumefaciens* (strains GV2260, EHA105), and the cost associated with antibiotics could thus be significantly reduced. Following this method, the use of eradicants such as Timentin^{TM} at 800 mg l^{-1} and Augmentin^{TM} at 500 mg l^{-1} was not significantly detrimental, and in some cases, even appeared to be beneficial to the growth of embryogenic cells. Embryo formation and germination were also not significantly different whether or not these eradicants were incorporated into the bilayer system. In addition, the combined use of specific membranes (see below) as supports of embryogenic cells over a biphasic culture medium was found particularly important to facilitate rapid and complete subculture and minimize carryover of both agrobacteria and compounds released by necrotic cells. Such supports did not create a barrier to antibiotic diffusion into the cells.

The use of support membranes (Connett-Porceddu *et al*. 2007) for plating embryogenic cells before and after AT or PB was identified as a critical point for successful recovery of transformed lines and plants. Membranes made of non-swelling fibers, such as polyester or fluoropolymer (pore size ranging from a few microns and up to about half the size of the cells) were found to greatly facilitate the transfer of cell material among different media and culture phases with minimal physical damage, while increasing the likelihood that most cells plated in a thin layer will be exposed to the selective agents. As well, media components might penetrate more readily through the polyester membranes compared with commonly used filter papers or nylon membranes. In addition, polyester membranes limited the development of detrimental conditions at the interfaces, such as anaerobicity or accumulation of exudates from necrotic cells. As a result, the use of polyester or fluoropolymer membranes considerably speeded up the selection process, and transformation efficiency was 5-6 times greater compared with other supports. In experiments with mixtures of transformed and non-transformed cells, it was found that both the nylon membranes and filter papers were unable to capture all transformation events, and allowed the growth of non-transformed cells that would ultimately regenerate escapes. Using geneticin as selective agent (15 mg l^{-1}), incidence of escapes was reduced to less than 1% with polyester membranes, whereas an escape rate of 11% was reported with previous selection schemes (Connett *et al*. 2003; Connett-Porceddu *et al*. 2003).

With improved selection procedures of transformed cell lines based on the combined use of ABA and polyester support membranes, transformation efficiency of embryogenic lines from 14 widely diverse seed families was reported to be as high as 60-80%, including elite families producing "recalcitrant" genotypes (80%) that were never previously transformed (Connett *et al*. 2003; Connett-Porceddu and Gulledge 2005).

The first field tests of transgenic loblolly pine plants were apparently established around 2000 (Connett *et al*. 2003; Connett-Porceddu *et al*. 2003, 2007). Preliminary results, after 3 years since planting, indicated that growth and phenotype of transgenic plants (expressing *uid*A) were similar to non-transformed plants with high survival rate. In a sample of PB-transformed plants carrying one to more than five transgene copies, constitutive expression appeared stable through multiple years including after clonal propagation of transgenic tree stock. No clear correlation between copy number and stable expression of the transgene could be detected (Connett *et al*. 2003).

Inducible gene expression systems based on chemical-responsive transcription factors (TF) could be a powerful tool in functional studies of genes and promoters to induce gene expression at different developmental stages or under particular conditions. The dexamethasone-inducible GVG system (Gal4 binding/VP16 activation, GR glucocorticoid receptor; Moore *et al*. 2006) was tested in loblolly pine cell suspension cultures established from zygotic embryo-derived callus clones (Tang and Newton 2004a). After AT transformation, three transgenic cell lines carrying a single copy of the m*gfp*5ER gene targeting the recombinant GFP to endoplasmic reticulum were selected for inducible gene expression experiments. Maximum *gfp* fluorescence and transcript levels were detected after 48-72 h with 5 mg l^{-1} inducer, which was not toxic to the cells. Thus, the dexamethasone-inducible GVG system appears to tightly and exclusively control m*gfp*5ER expression. No interference between the 4UAS recognition sequence and loblolly pine endogenous transcriptional activators was observed.

Dendrolimus punctatus and *Crypyothelea formosicolla* are major pests threatening the wood production of loblolly pine. The *cry1Ac* gene encoding a *Bacillus thuringiensis* (Bt) insecticidal protein was introduced and constitutively expressed (under 2x 35S promoter) in the loblolly pine genome after PB of mature zygotic embryos (three seed families; Tang and Tian 2003). The *cry1Ac* mRNAs were detected in total RNA extracted from four transgenic plants. In 42 out of 48 plants tested (87%), a polyclonal antibody detected the CRY1Ac protein in needle extracts. The immunosignal was not detected in six plants carrying more than one copy of the c*ry1Ac* gene. This result could be explained by low expression level of the protein (below the detection threshold), possible PTGS or recombination events within T-DNA that altered *cry1A*. These plants were not resistant to larvae of *D. punctatus* and *C. formosicolla*. In contrast, larvae placed on transgenic calli or plants producing CRY1Ac ceased feeding and became stunted. After 7 days, larval mortality of both species was as high as 65-76%, but only 7-10% in the case of control assays with non-transformed plants. However, the level of resistance and development stage of the larvae could be related in some cases, suggesting that the use of genes with different resistance mechanisms would ideally be required to maintain a Bt strategy.

Transgenic loblolly pine plants regenerated from mature zygotic embryos were recently reported to have considerably increased salt tolerance (Tang *et al*. 2005d). This was achieved by the co-integration and constitutive expression (35S) of mannitol-1-phosphate dehydrogenase (*Mt1D*) and glucitol-6-phosphate dehydrogenase (*GutD*) genes. Eighteen transgenic plants were selected for analysis of

mannitol and glucitol contents and for salt tolerance assays. Although loblolly pine does not normally produce mannitol, both mannitol and glucitol were detected in needles of transgenic plants in quite similar amounts, but with some differences in expression levels among different lines (3.1-6.5 µmol g^{-1} fm for glucitol and 3.6-6.7 µmol g^{-1} fm for mannitol). Transcripts of *Mt1D* were also detected in plants derived from the three investigated seed families. When watered over 9 weeks with solutions containing 85 or 120 mM NaCl (5-7 g l^{-1}), transgenic plants from all three seed families had survival rate from 52% to 78%, and non-transformed plants survived at rate from 2% to 7%. The mechanisms by which sugar alcohols such as mannitol and glucitol increased salt tolerance included adjustment of cytosolic osmotic potential, protection of membranes and proteins, and stimulation of metabolic pathways normally involved in stress tolerance.

Much progress has been made in genetic transformation of loblolly pine in developing robust PB and AT protocols that are applicable to most genetic backgrounds. The expertise level needed to engineer a novel tree with high efficiency has apparently been reached. Both organogenic (especially adventitious budding from mature zygotic embryos) and embryogenic regeneration systems have been developed (**Table 2** and **Table 3**) and are efficient enough for application. Transformation methods targeting organogenic explants appeared to fulfill the requirements for flexible gene delivery to multiple genotypes with moderate clonal replication, such as in functional studies of candidate genes (Nehra *et al*. 2005). Using EM as the target material further opens the door to high throughput gene testing and clonal replication of selected transclones for scaling up at the commercial level. Transgenic loblolly pines are now routinely produced to establish pre-commercial field tests in the USA (Connett *et al*. 2003). The main objective in loblolly pine is to significantly improve growth rate and wood formation in order to reduce the duration of rotation cycles and associated cost for industry.

DEPLOYMENT OF TRANSGENIC PINES AND BIOSAFETY

The reasons for using genetic transformation technology on forest trees include the creation, with improved efficiency, of new genetic traits that are not available from the "natural" (i.e., classical) mode of breeding to express foreign genes or difficult-to-breed indigenous genes with low heritability. New transgenic phenotypes resulted from the regulation of gene expression by various ways such as constitutive, tissue-specific, or inducible gene silencing (TGS, PTGS) and over expression (see examples in the *Pinus* spp. Sections above). Potential commercial and environmental benefits of transgenic trees in forestry are numerous, and include: (1) increased ecological competence by engineering transgenes to confer resistance to diseases, insect attacks, and abiotic factors such as drought, cold stress, salt, and herbicides; (2) restriction of gene flow by engineering sterility; (3) improved wood product quality and productivity by modifying expression of cell wall-, growth- and flowering-related genes; (4) increased environmental competitiveness of forest resources (renewable energy source, carbon sequestration, phytoremediation, less toxic pulping process, reduced soil erosion problems, etc.); and (5) derivation of new products such as bio-fuel and chemicals (Mullin and Bertrand 1998; Strauss *et al*. 2001; Fenning and Gershenzon 2002; Sedjo 2006).

Potential high benefits and recent advances in genetic transformation technology have led to intense interest in using the technology in commercial forestry, such as MVF, especially in pines (Klimaszewska *et al*. 2007). It is envisaged that genetic transformation is likely to be applied to already improved tree varieties as a value-added component of MVF. As the introduction of value-added transgenic varieties is mainly to be within the context of MVF, it is important to develop an ecologically sound MVF strategy before deploying transgenic trees. Therefore, all aspects of the general requirements for implementing MVF are equally applicable for deploying transgenic trees. The MVF strategy based on somatic embryogenesis as described in Klimaszewska *et al*. (2007) is based on careful exploitation of indigenous genetic variability that exists in nature within species or among closely related, compatible species. In most breeding programs, it is likely that genetic engineering will further contribute to the optimal use of such indigenous variability in variety design. As it will also be possible to take advantage of foreign gene resources, the deployment of genetically engineered trees may be associated with common (non-specific) or additional (specific) risks compared with classical varieties, depending on the gene introduced. Therefore, studies on the biosafety of transgenic varieties in their respective environment should be considered to evaluate and manage potential benefits and both specific and non-specific risks before transgenic trees are deployed.

Using the vast experience gained from genetically modified crop plants in agriculture, several biosafety issues related to environmental concerns have been identified that should be considered when evaluating risks associated with transgenic deployment in long-lived trees (Mullin and Bertrand 1998; Burdon 1999). These are all highly interdisciplinary and complex issues (Conner *et al*. 2003). Most of these risks are not specific to transgenic trees but are relevant to plantation forestry in general (Strauss *et al*. 2001): (1) spread of new genes to wild populations; (2) increased weediness and invasiveness; (3) impact on non-target organisms; (4) ecosystem interactions; (5) species integrity and biodiversity; and (6) adaptability of newly bred genotypes and varieties. Specific risks recognized as intrinsic to transgenic plants included (7) modification of genome structural integrity through the process of randomly introducing genes that will affect plant development and fertility, resistance to biotic and abiotic stresses, etc.; (8) transfer of antibiotic resistance genes to microorganisms or other selection genes to weeds; and (9) the stability of transgenic traits over the long term, especially in the case of long-lived trees.

Intense research programs are currently being developed in plants to find more precise and stable methods to introduce genes in the DNA (e.g., site-specific recombination, Kumar *et al*. 2006) and to find alternative ways to the use of selection genes, including positive, phenotype-based selection, and marker-free methods (Penna *et al*. 2002; de Vetten *et al*. 2003; Sreekala *et al*. 2005). Among non-specific risks, the major concern has been the possible spread of transgenes from transformed varieties to natural populations or related organisms. In such a case, the obvious mode of gene flow is sexual reproduction through wind-borne pollen, which is difficult to control. Isolation barriers have been suggested, but these are likely to be only partially effective, as observed for crop plants. Genetic engineering of flowering sterility (Strauss *et al*. 1995) is an attractive option to reduce the extent of spread, and recent results – including in forest trees – are very promising (Lännenpää *et al*. 2005). A male cone-specific promoter from *P. radiata* was recently used to express a stilbene synthase gene (STS) in anthers of transgenic *N. tabacum* plants, resulting in complete male sterility in 70% of transformed plants (Höfig *et al.* 2006). Such a promoter-STS construct may be useful for the ablation of pollen formation in coniferous gymnosperm. The promoter-STS construct is currently been tested in transgenic radiata pine (Walter pers. comm.). Pollen from some conifer species (for example *Cryptomeria japonica*) can lead to strong allergenic responses in people and a sterility approach could potentially be used to reduce the amount of pollen shed, thereby reducing the incidence of allergenic reactions. Using a different approach the suppression of expression of allergenic proteins located in the outer pollen wall, may reduce pollen allergenicity. However, pollen of the most important plantation forest tree species, *Pinus radiata* and *Pinus taeda*, shows very low allergenicity. Nevertheless, it appears extremely difficult to ensure the perfect stability of the sterility, especially in view of long

rotations of forest trees, but partial flowering prevention would help to control the extent of spread of new varieties. In the case of persistence of *A. tumefaciens* in plant tissue transformed by AT methods, one additional risk of transgene dispersal is the release of engineered agrobacteria into the soil, where horizontal gene transfer to other microorganisms and plants, especially weeds, can occur. Adapted molecular detection methods of *A. tumefaciens* should be used to minimize the risk (see Genetic Transformation of Pines: General Considerations). Another concern about the escaped genes is the change in invasiveness caused by the putative selective advantage conferred by the transgene (Dale 1992; Raybould and Gray 1994). In cases such as pest resistance, increased invasiveness of transgenic varieties is likely to be ecologically and evolutionary unstable and far lower than some exotic species known to be invasive (Strauss *et al*. 2001). Even if the transgene does not escape, it still may have an adverse impact on other organisms because of possible toxicological effects when consumed or through nutrient recycling (Mullin and Bertrand 1998). Such an effect is already present among species, varieties, families, and clones under conventional plantation practices (Strauss *et al*. 2001). Field testing of transgenic varieties should help to choose the least harmful genes for non-target organisms. The introduction of novel traits by genetic transformation may also lead to changes in forest management that could have either negative or positive impacts on ecosystem processes. For example, conferring herbicide tolerance could lead to increased use of chemical inputs that may have a potentially adverse impact on the ecosystem (Goldburg 1992). However, herbicide-resistant plants may result in the use of more environmentally benign herbicide with reduced impact on soil erosion and carbon emission (Strauss *et al*. 2001). When compared with "natural" forests, plantation forestry is commonly accused of reducing biodiversity. The extensive use of value-added transgenic trees may reduce diversity even further in plantations, leading eventually to the deployment of only a few clones. Clearly, the genetic diversity is progressively reduced through the process of domesticating trees to obtain higher genetic gain per breeding generation. Obviously, MVF is a strategy capable of balancing genetic gain and genetic diversity. In this process, an appropriate strategy to mitigate against the risk of failure in transgenic forestry would be to progressively deploy multiple transgenic clones from multiple transformation events. Additionally, there could be unexpected side effects that can arise from the total process of genetic transformation (Burdon 1999). There is currently no clear evidence of such an effect from the large-scale use of transgenic plants in agriculture, but this risk should be addressed in long-lived plants such as forest trees. Testing of transgenic trees, before deployment – with both complete containment in the laboratory and partial containment in the field – is crucial, but may not detect all the side effects as the expression of genes may be significantly delayed. Continued and strict evaluation of transgenic varieties in pre-commercial and initial commercial release (**Fig. 3**) would be required for long-term and progressive assessment of environmental risk and complete validation of the benefits-to-risks ratio of such new varieties (Strauss *et al*. 2001).

Even when a great deal is known about a transclone, an absolute guarantee of safety is not possible. Risk assessment in relation to the deployment of transgenic trees is needed to evaluate the risk management strategies intended to minimize risk (Mullin and Bertrand 1998). Such risks trigger the regulation of environmental release in many jurisdictions. Although there is considerable controversy over its meaning, scope, and application (Conner *et al*. 2003), the precautionary principle is widely recognized as an approach to manage risk when there is scientific uncer-

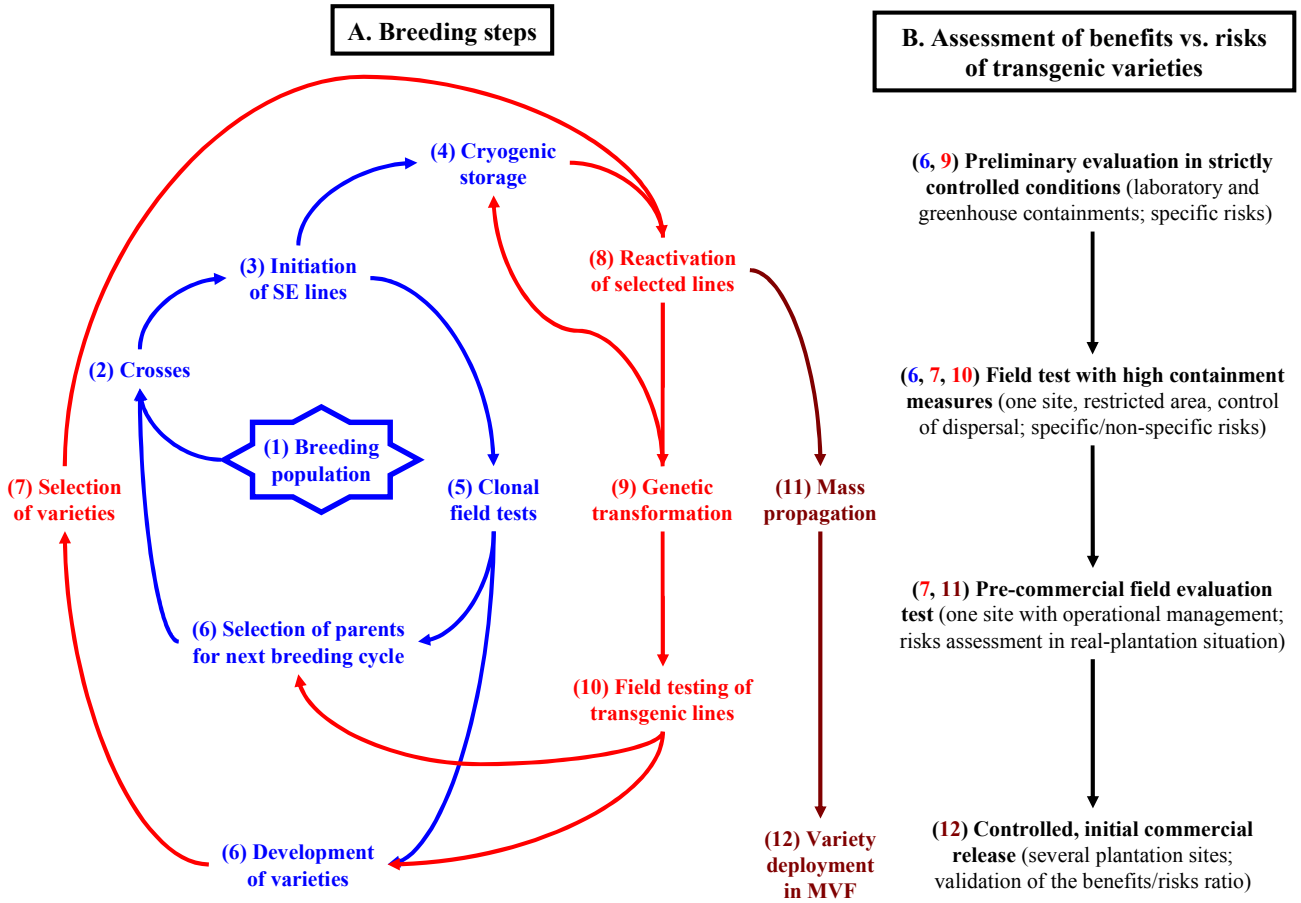

Fig. 3 A current view of technical (**A**) and biosafety (**B**) implementation of genetically modified trees in pine breeding programs as a value-added component of multi-varietal forestry (MVF, see Klimaszewska *et al*. 2007). Breeding steps 1–6 (in blue): long-term management of breeding populations; steps 7–10 (in red): variety design; steps 11–12 (in brown): variety deployment. The breeding steps related with risk assessment are indicated. SE: somatic embryogenesis.

tainty. In Canada, for example, the Canadian Food Inspection Agency (CFIA) exercises precaution in its decision to permit environmental release of trees with novel traits (including transgenic trees) within the science-based management. In regard to novel plants, an example of the precautionary principle is the insect resistance or herbicide tolerance management plan that CFIA requires as a condition of environmental release. In cases where a risk is present following environmental release, the precautionary principle requires that action must be taken to mitigate the risk even when the scientific base is inconclusive. Another approach is "substantial equivalence," which is a concept applied within the precautionary principle. It demands comparison of novel plants with existing and familiar plants. Although this determination does not mean that transgenic trees are identical to comparison trees, it means that, through testing and analysis, novel trees are to be equally safe for their respective use in the environment. The regulatory framework of environmental release of trees with novel traits in Canada has been reviewed by Finstad *et al*. (2007). In Europe, the European Community (EC) imposes strict rules on genetically modified organisms, and transgenic trees may only be introduced into the environment in conformity with Directive 2001/18/EC. This directive and supplemented decisions provide detailed guidance on the objective, elements, general principles (including the precautionary principle), and methodology of full environmental risk assessment. Explicit reference has been made to possible delayed effects of transgenic trees on health and environment because of their potential longevity. The directive imposes a step-by-step evaluation of genetically modified plants with progressive reduction of containment measures and increase of field test area (**Fig. 3**). Provisions for risk assessment are founded on comprehensive scientific evaluation by each member state within the EC. In France, for example, the scientific information pack is examined by the Biomolecular Engineering Commission (CGB), which provides case-by-case recommendations and prescription for field release of transgenic plants. In the United States, transgenic trees are regulated by the Animal and Plant Health Inspection Service (APHIS). The developers of transgenic trees must obtain authorization from APHIS before importing, transporting across state lines, or field testing these plants. Field testing is a precondition of deregulation, which is necessary for the transgenic trees to be commercialized (Sedjo 2004). In New Zealand, the development, field testing, and commercial deployment of genetically modified organisms falls under the Hazardous Substances and New Organisms Act. Applications can be made to the Environmental Risk Management Authority of New Zealand, which evaluates genetically engineered plants case by case, and with particular focus on the risks and benefits of the proposed activity. Field tests and commercial release require public consultation and a public hearing where any perceived risk can be raised and discussed. The Authority must make a decision based on scientific evidence, also taking into account cultural issues. There is no commercial release of genetically engineered organisms in New Zealand, however, there are field tests of genetically modified agricultural plants and trees.

Transgenic trees are the product of a powerful technology that promises many benefits. It is important to capture those benefits while ensuring public safety and maintaining environmental integrity. The deployment of transgenic trees is also a social issue and, despite scientific endeavors, there are knowledge gaps in developing an effective assessment and management of biosafety. This is an appropriate time to open dialog among scientists, the public, and regulators for careful integration of this powerful technology in forestry.

CONCLUDING REMARKS

Technical requirements to genetically transform pine have been largely defined in the past decade. To date, stable expression of selected foreign genes was reported in 17 pine species, with transgenic plant regeneration achieved in ten species. Both PB and AT methods were developed and in some cases fully optimized, i.e., with sufficient refinements to be applicable to a wide range (up to 80%) of genetic backgrounds (radiata pine, loblolly pine). Embryonal masses and mature zygotic embryos are currently the most frequently used target explants from which transgenic plants can be regenerated by SE or adventitious budding, respectively. Production of reasonable amounts of transgenic plant material for molecular and physiological studies of mutant phenotypes no longer appears to be a challenging task. Similarly, it is likely that pine functional genomics research will largely benefit in the short term from ongoing (overexpression and silencing, inducible expression) and forthcoming technological developments (tissue-specific promoters, site-specific recombination, etc.) for regulated gene expression in regenerated plants (Busov *et al*. 2005). Compared with association studies, such reverse genetic approaches are expected to more readily contribute to the demonstration of links between gene and phenotype at higher throughput and within a reduced timeframe, especially when specific, transformable *in vitro* cell culture systems are available (e.g., study of xylogenesis-related genes in callus cultures).

Considering that technological developments are still in the early stages in pines, recent achievements to create new genotypes with quite "rough" transformation systems are very promising (biotic and abiotic stress tolerance, enhanced growth, see *P. radiata* and *P. taeda* sections above). Based preferably on the powerful SE technology for plant regeneration, genetic transformation can be viewed as an impressive "generator" of innovative commercial products with very exciting novel traits. By introducing genetically modified trees in pine breeding programs (**Fig. 3**), new varieties with added value, that yet maintain the gene combination in already improved varieties, could be delivered after each breeding cycle. As a probable component of MVF, there will be specific and non-specific environmental concerns to be soundly assessed before deployment of such transgenic varieties. An absolute guarantee of safety cannot be ascertained for any new tree variety obtained through genetic engineering or breeding for the long term. However, risks should be considerably minimized through progressive and continued case-by-case evaluation of new products from the laboratory to the field that ensures a high degree of environmental safety, including strictly controlled precommercial and initial commercial releases in real plantation situations (**Fig. 3**).

ACKNOWLEDGEMENTS

We thank Mrs. Caroline Simpson (Canadian Forest Service – Atlantic Forestry Centre) for English editing. *Pinus pinaster* work was partly funded by EU (GEMINI, QLK5-CT-1999-00942).

REFERENCES

Aronen TS, Nikkanen TO, Häggman HM (2003) The production of transgenic Scots pine (*Pinus sylvestris* L.) via the application of transformed pollen in controlled crossings. *Transgenic Research* **12**, 375-378

Bercetche J, Dinant M, Coosemans N, Pâques M, Matagne RF (1992) Particle gun: a new transformation possibility in *Picea abies*. *Annals of AFOCEL* **1991**, 29-48

Binet MN, Lepetit M, Weil JH, Tessier LH (1991) Analysis of a sunflower polyubiquitin promoter by transient expression. *Plant Science* **79**, 87-94

Bishop-Hurley SL, Zabkievicz RJ, Grace LJ, Gardner RC, Wagner A, Walter C (2001) Conifer genetic engineering: Transgenic *Pinus radiata* (D. Don) and *Picea abies* (Karst) plants are resistant to the herbicide Buster. *Plant Cell Reports* **20**, 235-243

Breton D, Harvengt L, Trontin JF, Bouvet A, Favre JM (2006) Long-term subculture randomly affects morphology and subsequent maturation of early somatic embryos in maritime pine. *Plant Cell, Tissue and Organ Culture* **87**, 95-108

Burdon RD (1999) Risk management issues for genetically engineered forest trees. *New Zealand Journal of Forest Science* **29**, 375-390

Busov VB, Brunner AM, Meilan R, Filichkin S, Ganio L, Gandhi S, Strauss S (2005) Genetic transformation: a powerful tool for dissection of adaptative

traits in trees. *New Phytologist* **167**, 9-18
Campbell M, Kinlaw C, Neale D (1992) Expression of luciferase and β-glucuronidase in *Pinus radiata* suspension cells using electroporation and particle bombardment. *Canadian Journal of Forest Research* **22**, 2014-2018
Cerda F, Aquea F, Gebauer M, Medina C, Arce-Johnson P (2002) Stable transformation of *Pinus radiata* embryogenic tissue by *Agrobacterium tumefaciens*. *Plant Cell, Tissue and Organ Culture* **70**, 251-257
Charity JA, Klimaszewska K (2005) Persistence of *Agrobacterium tumefaciens* in transformed conifers. *Environmental and Biosafety Research* **4**, 167-177
Charity JA, Holland L, Donaldson SS, Grace L, Walter C (2002) *Agrobacterium*-mediated transformation of *Pinus radiata* organogenic tissue using vacuum-infiltration. *Plant Cell, Tissue and Organ Culture* **70**, 51-60
Charity JA, Holland L, Grace LJ, Walter C (2005) Consistent and stable expression of the *npt*II, *uid*A and *bar* genes in transgenic *Pinus radiata* after *Agrobacterium tumefaciens*-mediated transformation using nurse cultures. *Plant Cell Reports* **23**, 606-616
Choi WY, Lee BS, Lee KJ, Chung HK, Lee SK, Shim SY (1988) Gene transfer into *Pinus densiflora* and *Quercus acutissima* by *Agrobacterium tumefaciens*. *Research Reports of the Institute of Forest Genetics of Korea* **24**, 121-126
Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* **5**, 213-218
Conner AJ, Glare TR, Nap JP (2003) The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. *The Plant Journal* **33**, 19-46
Connett-Porceddu MB, Gulledge JE (2005) Enhanced selection of genetically modified pine embryogenic tissue. Patent N° 6,964,870 B2, USA, filed: 10, 2001
Connett MB, Becwar MR, Gulledge JE, Gladfelter HJ, Kothera R, Roberts SB, Schwuchow SG, Nehra NS, Kodrzycki RJ (2003) Transgenic loblolly pine from diverse elite families. In: Espinel S, Barredo Y, Ritter E (Eds) *Sustainable Forestry, Wood Products and Biotechnology*, AFA Press, Vitoria-Gasteiz, Spain, pp 227-231
Connett-Porceddu MB, Becwar MR, Kodrzycki RJ, Schwuchow SG (2003) Particle-mediated conifer transformation. Patent N° 6,518,485 B1, USA, filed: May 25, 1999
Connett-Porceddu MB, Gladfelter HJ, Gulledge JE, McCormack RR (2007) Enhanced transformation and regeneration of transformed embryogenic pine tissue. Patent N° 7,157,620 B2, USA, filed: Oct. 10, 2001
Cubero J, López M (2005) *Agrobacterium tumefaciens* persistence in plant tissues after transformation. In: Peña L (Ed) *Methods in Molecular Biology* (Vol 286), *Transgenic Plants Methods and Protocols*, Humana Press, Inc, Totowa, NJ
Dale PJ (1992) Spread of engineered genes to wild relatives. *Plant Physiology* **100**, 13-15
de Vetten N, Wolters AM, Raemakers K, van der Meer I, ter Stege R, Heeres E, Heeres P, Visser R (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nature Biotechnology* **21**, 439-442
Devillard C, Geddes B, Holland L (2007) Influence of tissue culture on transformation of *Pinus radiata* embryogenic tissue using a particle inflow gun and a novel approach for maturation of somatic embryos. Poster presentation at the 17th meeting of the NZ branch of the IAPTC&B, 25-28 February, Rotorua, New Zealand
Diner AM (1999) Genetic transformation in *Pinus palustris* (longleaf pine). In: Bajaj YPS (Eds) *Transgenic Trees*, Springer-Verlag, Berlin, pp 185-191
Droege M, Puehler A, Selbitschka W (1999) Horizontal gene transfer among bacteria in terrestrial and aquatic habitats as assessed by microcosm and field studies. *Biology and Fertility of Soils* **29**, 221-245
FAO (2004) Preliminary review of biotechnology in forestry, including genetic modification. Forest genetic resources working paper FGR/59E, Forest Resources Development Service, Forest Resources Division, Rome, Italy, 116 pp
Fenning TM, Gershenzon J (2002) Where will the wood come from? Plantations forests and the role of biotechnology. *Trends in Biotechnology* **20**, 291-296
Fernando D, Owens J, Misra S (2000) Transient gene expression in pine pollen tubes following particle bombardment. *Plant Cell Reports* **19**, 224-228
Finer JJ, Vain P, Jones MW, McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports* **11**, 323-328
Finstad K, Bonfils AC, Shearer W, MacDonald P (2007) Trees with novel traits in Canada: regulations and related scientific issues. *Tree Genetics and Genomics* **3**, 135-139
Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. *Trees* **18**, 115-135
Goldburg RJ (1992) Environmental concerns with the development of herbicide tolerant plants. *Weed Technology* **6**, 647-652
Gomez-Maldonado J, Crespillo R, Avila C, Canovas FM (2001) Efficient preparation of maritime pine (*Pinus pinaster*) protoplasts suitable for transgene expression analysis. *Plant Molecular Biology Reporter* **19**, 361-366
Gould JH, Zhou Y, Padmanabhan V, Magallenes-Cedeno M, Newton RJ (2002) Transformation and regeneration of loblolly pine: shoot apex inoculation with *Agrobacterium*. *Molecular Breeding* **10**, 131-141
Grace LJ, Charity JA, Gresham B, Kay N, Walter C (2005) Insect-resistant transgenic *Pinus radiata*. *Plant Cell Reports* **24**, 103-111
Grant JE, Cooper PA, Dale TM (2004) Transgenic *Pinus radiata* from *Agrobacterium tumefaciens*-mediated transformation of cotyledons. *Plant Cell Reports* **22**, 894-902
Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Molecular Biology* **25**, 989-994
Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science* **286**, 950-952
Hellens R, Mullineaux P, Klee H (2000) A guide to *Agrobacterium* binary Ti vectors. *Trends in Plant Science* **5**, 446-451
Höfig KP, Möller R, Donaldson L, Putterill J, Walter C (2006) Towards male sterility in *Pinus radiata* – a stilbene synthase approach to genetically engineer nuclear male sterility. *Plant Biotechnology Journal* **4**, 333-343
Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* **2**, 208-218
Huang Y, Tauer CG (1994) Integrative tranformation of *Pinus taeda* L. and *P. echinata* Mill. by *Agrobacterium tumefaciens*. *Forest Genetics* **1**, 23-31
Humara JM, Lopez M, Ordas RJ (1999a) *Agrobacterium tumefaciens*-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of *uid*A gene transfer. *Plant Cell Reports* **19**, 51-58
Humara JM, Lopez M, Ordas RJ (1999b) Transient expression of the *uid*A gene in *Pinus pinea* cotyledons: a study of heterologous promoter sequences. *Plant Cell, Tissue and Organ Culture* **56**, 69-78
Klimaszewska K, Trontin JF, Becwar M, Devillard C, Park YS, Lelu-Walter MA (2007) Recent progress on somatic embryogenesis of four *Pinus* spp. *Tree and Forestry Science and Biotechnology* **1**, 11-25
Koncz C, Schell G (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Molecular and General Genetics* **204**, 383-396
Kumar S, Allen GC, Thompson WF (2006) Gene targeting in plants: fingers on the move. *Trends in Plant Science* **11**, 159-161
Lännenpää M, Hassinen M, Ranki A, Hölttä-Vuori M, Lemmetyinen J, Keinonen K, Sopanen T (2005) Prevention of flower development in birch and other plants using a BpFULL1::BARNASE construct. *Plant Cell Reports* **24**, 69-78
Leplé JC, Brasileiro ACM, Michel MF, Delmotte F, Jouanin L (1992) Transgenic poplars: expression of chimeric genes using four different constructs. *Plant Cell Reports* **11**, 137-141
Levée V, Seguin A (2001) Inducible expression of the heterologous PAL2 promoter from bean in white pine (*Pinus strobus*) transgenic cells. *Tree Physiology* **21**, 665-672
Levée V, Garin E, Klimaszewska K, Séguin A (1999) Stable genetic transformation of white pine (*Pinus strobus* L.) after co-cultivation of embryogenic tissues with *Agrobacterium tumefaciens*. *Molecular Breeding* **5**, 429-440
Levée V, Lelu MA, Jouanin L, Cornu D, Pilate G (1997) *Agrobacterium tumefaciens*-mediated transformation of hybrid larch (*Larix kaempferi* × *L. decidua*) and transgenic plant regeneration. *Plant Cell Reports* **16**, 680-685
Lindroth AM, Grönroos R, Clapham D, Svensson J, von Arnold S (1999) Ubiquitous and tissue-specific *gus* expression in transgenic roots conferred by six different promoters in one coniferous and three angiosperm species. *Plant Cell Reports* **18**, 820-828
Loopstra CA, Stomp AM, Sederoff RR (1990) *Agrobacterium*-mediated DNA transfer in sugar pine. *Plant Molecular Biology* **15**, 1-9
Lopez M, Humara JM, Rodriguez R, Ordas RJ (2000a) Transient *uid*A gene expression in electroporated cotyledonary protoplasts of *Pinus nigra* ssp. *salzmannii* and in bombarded cotyledons. *Canadian Journal of Forest Research* **30**, 448-455
Lopez M, Humara JM, Rodriguez R, Ordas RJ (2000b) Factors involved in *Agrobacterium tumefaciens*-mediated gene transfer into *Pinus nigra* Arn. ssp *salzmannii* (Dunal) Franco. *Euphytica* **114**, 195-203
Magnussen D, Clapham D, Gronroos R, von Arnold S (1994) Induction of hairy and normal roots on *Picea abies*, *Pinus sylvestris* and *Pinus cortorta* by *Agrobacterium rhizogenes*. *Scandinavian Journal of Forest Research* **9**, 46-51
Matzk A, Mantell S, Schiemann J (1996) Localization of persisting *Agrobacterium tumefaciens* in transgenic tobacco plants. *Molecular Plant-Microbe Interactions* **9**, 373-381
Mathur J, Szabados L, Schaefer S, Grunenberg B, Lossow A, Jonas-Straube E, Schell J, Koncz C, Koncz-Kalman Z (1998) Gene identification with sequenced T-DNA tags generated by transformation of *Arabidopsis* cell suspension. *The Plant Journal* **13**, 707-713
McAfee BJ, White EE, Pelcher LE, Lapp MS (1993) Root induction in pine (*Pinus*) and larch (*Larix*) spp. using *Agrobacterium rhizogenes*. *Plant Cell, Tissue and Organ Culture* **34**, 53-62
McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. *The Plant Cell* **2**, 163-171
Meyer P (2000) Transcriptional transgene silencing and chromatin component.

Plant Molecular Biology **43**, 221-234
Mihaljevic S, Stipkovic S, Jelaska S (1996) Increase of root induction in *Pinus nigra* explants using agrobacteria. *Plant Cell Reports* **15**, 610-614
Mogilner N, Zutra D, Gafny R, Bar-Joseph M (1993) The persistence of engineered *Agrobacterium tumefaciens* in agroinfected plants. *Molecular Plant-Microbe Interactions* **6**, 673-675
Möller R, McDonald AG, Walter C, Harris PJ (2003) Cell differentiation, secondary cell-wall formation and transformation of callus tissue of *Pinus radiata* D. Don. *Planta* **217**, 736-747
Möller R, Steward D, Phillips L, Flint H, Wagner A (2005) Gene silencing of cinnamyl alcohol dehydrogenase in *Pinus radiata* callus cultures. *Plant Physiology and Biochemistry* **43**, 1061-1066
Moore I, Samalova M, Kurup S (2006) Transactivated and chemically inducible gene expression in plants. *Plant Journal* **45**, 651-683
Morris JW, Castle LA, Morris RO (1989) Efficacy of different *Agrobacterium tumefaciens* strains in transformation of pinaceous gymnosperms. *Physiological and Molecular Plant Pathology* **34**, 451-461
Mullin TJ, Bertrand S (1998) Environmental release of transgenic trees in Canada – potential benefits and assessment of biosafety. *The Forestry Chronicle* **74**, 203-219
Nehra NS, Becwar MR, Rottmann WH, Pearson L, Chowdhury K, Chang SJ, Wilde HD, Kodrzycki RJ, Zhang CS, Gause KC, Parks DW, Hinchee MA (2005) Forest biotechnology: innovative methods, emerging opportunities. *In Vitro Cellular and Developmental Biology – Plant* **41**, 701-717
Newton RJ, Dong N, Marek-Swize K, Cairney J (1993) Transformation of slash pine. In: Proceedings of the 22nd Southern Forest Tree Improvement Conference, Atlanta, Georgia, NTIS Springfield, pp 390-402
Nigro SA, Makunga NP, Jones NB, van Staden J (2004) A biolistic approach towards producing transgenic *Pinus patula* embryonal suspensor masses. *Journal of Plant Growth Regulation* **44**, 187-197
OECD database on field trials of genetically modified organisms. Available online: http://webdomino1.oecd.org/ehs/biotrack.nsf
Parasharami VA, Naik VB, vonArnold S, Nadgauda RS, Clapham DH (2006) Stable transformation of mature zygotic embryos and regeneration of transgenic plants of chir pine (*Pinus roxburghii* Sarg.). *Plant Cell Reports* **24**, 708-714
Penna S, Sagi L, Swennen R (2002) Positive selectable marker genes for routine plant transformation. *In Vitro Cellular and Developmental Biology – Plant* **38**, 125-128
Raybould, AF, Gray AJ (1994) Will hybrid of genetically engineered crop invade natural communities? *Trends in Ecology and Evolution* **9**, 85-89
Saborio F, Moloney MM, Tung P, Thorpe TA (1999) Root induction in *Pinus ayacahuite* by co-culture with *Agrobacterium tumefaciens* strains. *Tree Physiology* **19**, 383-389
Salaj T, Moravcikova J, GrecNiquet L, Salaj J (2005) Stable transformation of embryogenic tissues of *Pinus nigra* Arn. using a biolistic method. *Biotechnology Letters* **27**, 899-903
Sederoff R, Stomp AM, Chilton WS, Moore LW (1986) Gene transfer into loblolly pine by *Agrobacterium tumefaciens*. *Bio/Technology* **4**, 647-649
Sederoff R, Stomp AM, Moore LW, Chilton SW (1989) Method for transforming pines. Patent N°4,886,937, USA
Sedjo RA (2001) Biotechnology in forestry: considering the costs and benefits. *Resources* **145**, 10-12
Sedjo RA (2004) Regulation and international trade: Issues for transgenic trees. In: McCord S, Kellison R (Eds) *Conference Proceedings on New Century, New Trees*. *Biotechnology as a Tool for Forestry in North America*, Research Triangle Park, NC. Nov. 16-17, 2004, pp 77-85
Sedjo RA (2006) Towards commercialization of genetically engineered forests: economic and social considerations. *Resources for the Future Report*, RFF press, Washington, DC. Available online: www.rff.org/Documents/RFF-Rpt-CommercializationGEForests.pdf, 45 pp
Sreekala C, Wu L, Gu K, Wang D, Tian D, Yin Z (2005) Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/loxP system. *Plant Cell Reports* **24**, 86-94
Stewart C, Richards H, Halthill M (2000) Trangenic plants and biosafety: science, misconceptions and public perception. *BioTechniques* **29**, 832-843
Stomp AM, Loopstra CA, Chilton WS, Sederoff RR, Moore LW (1990) Extended host range of *Agrobacterium tumefaciens* in the genus *Pinus*. *Plant Physiology* **92**, 1226-1232
Strauss SH, Coventry P, Campbell MM, Pryor SN, Burley J (2001) Certification of genetically modified forest plantations. *International Forestry Review* **3**, 85-102
Strauss SH, Rottmann WH, Brunner AM, Sheppard LA (1995) Genetic engineering of reproductive sterility in forest trees. *Molecular Breeding* **1**, 5-26
Tang W (2001) *Agrobacterium*-mediated transformation and assessment of factors influencing transgene expression in loblolly pine (*Pinus taeda* L.). *Cell Research* **11**, 237-243
Tang W (2003) Additional virulence genes and sonication enhance *Agrobacterium tumefaciens*-mediated loblolly pine transformation. *Plant Cell Reports* **21**, 555-562
Tang W, Newton RJ (2003) Genetic transformation of conifers and its application in forest biotechnology. *Plant Cell Reports* **22**, 1-15
Tang W, Newton RJ (2004a) Glucocorticoid-inducible transgene expression in loblolly pine (*Pinus taeda* L.) cell suspension cultures. *Plant Science* **166**, 1351-1358
Tang W, Newton RJ (2004b) Regulated gene expression by glucocorticoids in cultured Virginia pine (*Pinus virginiana* Mill.) cells. *Journal of Experimental Botany* **55**, 1499-1508
Tang W, Newton RJ (2005) Transgenic Christmas trees regenerated from *Agrobacterium tumefaciens*-mediated transformation of zygotic embryos using the green fluorescent protein as a reporter. *Molecular Breeding* **16**, 235-246
Tang W, Tian YC (2003) Transgenic loblolly pine (*Pinus taeda* L.) plants expressing a modified delta-endotoxin gene of *Bacillus thuringiensis* with enhanced resistance to *Dendrolimus punctatus* Walker and *Crypyothelea formosicola* Staud. *Journal of Experimental Botany* **54**, 835-844
Tang W, Charles TM, Newton RJ (2005a) Overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) confers multiple stress tolerance and enhances organ growth. *Plant Molecular Biology* **59**, 603-617
Tang W, Kinken K, Newton RJ (2005b) Inducible antisense-mediated post-transcriptional gene silencing in transgenic pine cells using green fluorescent protein as a visual marker. *Plant Cell Physiology* **46**, 1255-1263
Tang W, Luo HS, Newton RJ (2004) Effects of antibiotics on the elimination of *Agrobacterium tumefaciens* from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. *Plant Cell, Tissue and Organ Culture* **79**, 71-81
Tang W, Newton RJ, Charles TM (2005c) High efficiency inducible gene expression system based on activation of a chimeric transcription factor in transgenic pine. *Plant Cell Reports* **24**, 619-628
Tang W, Newton RJ, Lin J, Charles TM (2006a) Expression of a transcription factor from *Capsicum annuum* in pine calli counteracts the inhibitory effects of salt stress on adventitious shoot formation. *Molecular Genetics and Genomics* **276**, 242-253
Tang W, Newton RJ, Weidner DA (2007) Genetic transformation and gene silencing mediated by multiple copies of a transgene in eastern white pine. *Journal of Experimental Botany* **58**, 545-554
Tang W, Peng XX, Newton RJ (2005d) Enhanced tolerance to salt stress in transgenic loblolly pine simultaneously expressing two genes encoding mannitol-1-phosphate dehydrogenase and glucitol-6-phosphate dehydrogenase. *Plant Physiology and Biochemistry* **43**, 139-146
Tang W, Sederoff R, Whetten R (2001) Regeneration of transgenic loblolly pine (*Pinus taeda* L.) from zygotic embryos transformed with *Agrobacterium tumefaciens*. *Planta* **213**, 981-989
Tang W, Weidner DA, Hu BY, Newton RJ, Hu XH (2006b) Efficient delivery of small interfering RNA to plant cells by a nanosecond pulsed laser-induced stress wave for post-transcriptional gene silencing. *Plant Science* **171**, 375-381
Tang W, Weidner D, Newton RJ (2005e) Quantitative analysis of siRNA-mediated GFP silencing in transgenic pine cells. *Plant Science* **168**, 741-746
Taniguchi T, Ohmiya Y, Kurita M, Kondo T (2004) Transient expression of the green fluorescent protein gene in *Cryptomeria japonica*, *Chamaecyparis obtusa*, *Pinus densiflora*, *Pinus thunbergii*, *Larix kaempferi* and *Acanthopanax sciadophylloides* following particle bombardment. *Bulletin of the Forest Tree Breeder Center* **20**, 1-8
Tautorus T, Bekkaoui F, Pilon M, Datla R, Crosby W, Fowke L, Dunstan D (1989) Factors affecting transient gene expression in electroporated black spruce (*Picea mariana*) and jack pine (*Pinus banksiana)* protoplasts. *Theoretical and Applied Genetics* **78**, 531-536
Tereso S, Miguel C, Maroco J, Oliveira MM (2006a) Susceptibility of embryogenic and organogenic tissues of maritime pine (*Pinus pinaster*) to antibiotics used in *Agrobacterium*-mediated genetic transformation. *Plant Cell, Tissue and Organ Culture* **87**, 33-40
Tereso S, Miguel C, Zoglauer K, Valle-Piquera C, Oliveira MM (2006b) Stable *Agrobacterium*-mediated transformation of embryogenic tissues from *Pinus pinaster* Portuguese genotypes. *Journal of Plant Growth Regulation* **50**, 57-68
Tereso S, Zoglauer K, Miguel C, Oliveira MM (2003) Establishing a genetic transformation system in *Pinus pinaster*. In: Espinel S, Barreto Y, Ritter E (Eds) *Sustainable Forestry, Wood Products and Biotechnology*, DFA-AFA Press, Vitoria-Gasteiz, Spain, pp 195-204
Tian LN, Séguin A, Charest PJ (1997) Expression of the green fluorescent protein gene in conifer tissues. *Plant Cell Reports* **16**, 267-271
Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. *Plant Cell Reports* **23**, 780-789
Trontin JF, Harvengt L, Garin E, Vernaza ML, Arancio L, Hoebeke J, Canlet F, Paques M (2002) Towards genetic engineering of maritime pine (*Pinus pinaster* Ait.). *Annals of Forest Science* **59**, 687-697
Tzfira T, Yarnitzky O, Vainstein A, Altman A (1996) *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis* Mill. *Plant Cell Reports* **16**, 26-31
van Beveren KS, Spokevicius AV, Tibbits J, Wang Q, Bossinger G (2006) Transformation of cambial tissue *in vivo* provides an efficient means for induced somatic sector analysis and gene testing in stems of woody plant species. *Functional Plant Biology* **33**, 629-638
Victor DG, Ausubel JH (2000) Restoring the forests. *Foreign Affairs* **79**, 127-

144

Villalobos-Amador E, Rodriguez-Hernandez G, Perez-Molphe-Balch E (2002) Organogenesis and *Agrobacterium rhizogenes*-induced rooting in *Pinus maximartinezii* Rzedowsky and *P. pinceana* Gordon. *Plant Cell Reports* **20**, 779-785

Wagner A, Moody J, Grace LJ, Walter C (1997) Transformation of *Pinus radiata* based on selection with hygromycin B. *New Zealand Journal of Forestry Science* **27**, 280-288

Wagner A, Phillips L, Narayan RD, Moody JM, Geddes B (2005) Gene silencing studies in the gymnosperm species *Pinus radiata*. *Plant Cell Reports* **24**, 95-102

Walter C, Find JI, Grace L (2005) Somatic embryogenesis and genetic transformation in *Pinus radiata*. In: Jain SM, Gupta PK (Eds) *Protocol for Somatic Embryogenesis in Woody Plants*, Springer, Dordrecht, The Netherlands, 77, pp 11-24

Walter C, Smith DR (1999) Genetic transformation of *Pinus radiata*. In: Bajaj YPS (Ed) *Transgenic Trees*, Springer-Verlag, Berlin, pp 193-211

Walter C, Grace LJ, Wagner A, White DWR, Walden AR, Donaldson SS, Hinton H, Gardner RC, Smith DR (1998) Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. *Plant Cell Reports* **17**, 460-468

Walter C, Smith DR, Connett MB, Grace LJ, White DWR (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

Wenck AR, Quinn M, Whetten RW, Pullman G, Sederoff R (1999) High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Molecular Biology* **39**, 407-416