Transgenic Plant Journal ©2007 Global Science Books

Genetic Transformation of Conifers: Applications in and Impacts on Commercial Forestry

Ravindra B. Malabadi[1,2*] • K. Nataraja[1]

[1] Division of Plant Biotechnology, Department of Botany, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka state, India
[2] Present address: Forest biotechnology lab (6.06), Instituto de Tecnologia Quimica e Biologica (ITQB), Universidade Nova de Lisboa, Av. da República, Apartado 127, 2781-901, Oeiras, Portugal

Corresponding author: * **MLBD712@rediffmail.com**

ABSTRACT

This review highlights methods of gene transfer technology in trees, particularly conifers, and its possible applications to commercial forestry. Two methodologies have mainly been used on conifers: biolistic and *Agrobacterium*-mediated genetic transformation. DNA transfer using the latter method makes the use of physical processes only to achieve transformation. Since there is no dependence on living organisms, i.e. bacteria, the limitations inherent in organisms such as *Agrobacterium tumefaciens* do not apply. Information of mechanisms for T-DNA transfer to plant cells by *A. tumefaciens* is provided, focusing on the role played by the different components of the virulence system. There are many differences between both methods of gene transfer technology, and these are highlighted in conifer transgenic systems. The first transgenic trees produced by biolistics following the cloning of mature Indian pines, *Pinus roxburghii*, and success in the *Agrobacterium*-mediated transformation of Himalayan blue pine (*P. wallichiana*) are some of the major breakthroughs in forest biotechnology that might help in solving current problems of tree breeding. There are many problems in traditional breeding programs such as a slow production due to long maturation times and the slow growth rate of trees; however, biotechnological approaches have the potential to provide significant improvement in tree growth and quality. Despite the large number of marker genes that exist for plants, only a few have been used for most research and improvement studies in conifers. As the production of transgenic plants is labor-intensive, expensive and difficult for most recalcitrant conifers, practical issues govern the choice of selectable marker genes that are used. This review will also focus some of the important applications of this technology, including the deployment of transgenic plants under field conditions, and its impacts on the environment.

Keywords: *Agrobacterium*, bacterial artificial chromosome, biolistic, lignin, environment, marker genes, somatic embryos, transient transgene expression, yeast artificial chromosome, wood

CONTENTS

INTRODUCTION 289
SELECTOR AND MARKER GENES 290
BIOLISTIC GENE TRANSFER 292
  Strategies of biolistics 293
  Influencing factors 294
  Non-requirement of vectors 295
  Multiple gene transformation 296
STABLE EXPRESSION AND REGENERATION 296
  Molecular characterization 297
  Limitations 299
AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION 300
  Transient and stable expression 300
  Regeneration of transgenic plants 304
TRANSFORMATION USING ELECTROPORATION 304
APPLICATIONS OF GENE TRANSFER IN COMMERCIAL FORESTRY 305
  Bioremediation 305
  Wood modification 305
  Insect and herbicide resistance 306
  Gene expression 306
  Flowering control 306
  Molecular improvement 306
STEPS TOWARDS THE ADVANCEMENT OF TECHNOLOGY 307
LIMITATIONS OF TRANSGENE TECHNOLOGY 307
PROBLEMS OF PUBLIC ACCEPTANCE 308
CONCLUSIONS 309
REFERENCES 309

INTRODUCTION

A very powerful tool in forest biotechnology is genetic transformation in order to transfer relevant genes from bacteria, fungi, animals or plants into conifers. One important aim of genetic manipulation of conifers is the increase of resistance against various fungal pathogens. In the last 15 years many techniques have been developed such as tissue

Received: 15 October, 2007. Accepted: 30 October, 2007.

Invited Review

culture, genetic transformation, and genome analysis of various conifer species (Mathews and Campbell 2000; Sutton 2002). A range of targets are of interest for genetic engineering in conifers e.g. modification of lignin and/or cellulose ratio/content, pest resistance, improving growth rate, wood properties and quality, herbicide resistance and tolerance to abiotic stresses (Lelu and Pilate 2000; Newton *et al*. 2001; van Raemdonck *et al*. 2001). The sufficient production of wood without disturbing primary forest will be one of the most important issues for the near future. Global demand for wood and supply are greatly influenced by the fact that indigenous tree populations are becoming scarce and their exploitation is increasingly viewed as socially and environmentally unacceptable (Charity *et al*. 2005). Their solutions will also provide the means of increased overall competitivity of the industry while optimizing the use of wood products in an expanding range of applications. Considering the increase of world demand for wood, improving wood quality to better fit industrial requirements becomes a major objective for tree breeders. Hitherto, breeding for wood traits has been hampered by the cost of traditional assays, the need to wait until trees are nearly mature to be evaluated, high heterozygosity and autoincompatibility (Birch 1997; Cervera *et al*. 1998, 2000; Tang and Newton 2003, 2005; Tang *et al*. 2006) The recent development of molecular tools for genomic analysis of woody species makes it possible to transfer or identify genes controlling wood traits.

Genetic engineering techniques are available for various conifer species of interest in plantation forestry, and now is an important field in plant biotechnology (Tang and Newton 2003). Fillatti *et al*. (1987) was the first to report transgenic *Populus* trees followed by many forest tree species were used to transfer many economically important genes conferring traits such as virus, insect and herbicide resistance (Birch 1997). However, transgenic conifers were only reported in the last decade (Huang *et al*. 1991), followed by results reported in several conifers viz., *Pinus kesiya* (Malabadi and Nataraja 2007b), *Pinus roxburghii* (Malabadi and Nataraja 2007a), *Pinus wallichiana* (Malabadi and Nataraja 2007c, 2007i). *Larix kaemperi* × *L. deciduas* (Levee *et al*. 1997), *Pinus strobus* (Levee *et al*. 1999; Tang *et al*. 2007), *Picea glauca*, *Picea mariana*, *Picea abies* (Klimaszewska *et al*. 2001), loblolly pine (*Pinus taeda*) (Tang *et al*. 2001a), *Pinus radiata* (Cerda *et al*. 2002; Charity *et al*. 2002; Grant *et al*. 2004; Charity *et al*. 2005; Grace *et al*. 2005), *Picea glauca* (Le *et al*. 2001), Norway spruce (*Picea abies*), *Pinus taeda* (Wenck *et al*. 1999), *Pinus nigra* (Lopez *et al*. 2000), *Pinus pinaster* (Trontin *et al*. 2002; Tereso *et al*. 2006), Douglas fir (*Pseudotsuga menziesii*) (Dandekar *et al*. 1987), *Picea abies* (Walter *et al*. 1999), *Pinus radiata* (Walter *et al*. 1998), *Pinus patula* (Nigro *et al*. 2004), *P. banksiana* (Mc Afee *et al*. 1993), *P. pinea* (Sul and Korban 1998), *P. sylvestris* (Aronen *et al*. 1996), *P. taeda* (Tang *et al*. 2001a) and *P. roxburghii* (Parasharami *et al*. 2006).

Somatic embryogenesis in many conifers throughout the world using vegetative shoot apices or secondary needles (Bonga and Pond 1991; Ruaud *et al*. 1992; Bonga and von Aderkas 1993; Ruaud 1993; Westcott 1994; Bonga 1996; Smith 1997; Malabadi and van Staden 2003; Bonga 2004; Malabadi *et al*. 2004; Malabadi and van Staden 2005a, 2005b, 2005c, 2006; Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b; Aronen *et al*. 2007; Malabadi and Nataraja 2007f, 2007h) provides the best opportunities to produce transgenic plants in a number of species that will lead to their application in commercial forestry (Malabadi and Nataraja 2007a). The first transgenic trees produced by using embryogenic tissue derived from the vegetative shoot apices of mature trees were reported in an Indian pine, *Pinus roxburghii* (Malabadi and Nataraja 2007a). This is the major breakthrough in forest biotechnology, and certainly solves the current problems of tree breeding. With the various gene transfer methods currently available, simple placement or transfer of DNA into a plant cell is no longer a limiting factor (Birch 1997; Tang and Newton 2003; Malabadi and Nataraja 2007a). However, both the mechanisms for DNA transfer to a plant cell and targeting of the DNA to a complex tissue or organ competent for regeneration is another major issue to be considered for effective and successful transformation. Now-a-days there are many genes available for use in conifer transformation experiments. However, most of those have been used as reporter genes for establishing a model transformation system, and very few have been used for novel phenotypes or for tolerance to various stresses (Merkle and Dean 2000; Pena and Seguin 2001; Herschbach and Kopriva 2002; Walter 2002). A model transformation system is very much needed before transfer of an economical trait gene into conifer tree species can be accomplished. However, many cultivars of those transgenic tree lines are now in field trials. This review paper gives an overview of genetic transformation via particle bombardment, electroporation, and *Agrobacterium* in conifers, and its applications in commercial forestry.

SELECTOR AND MARKER GENES

Selectable marker genes have been pivotal to the development of plant transformation technologies because the marker genes allow scientists to identify or isolate the cells that are expressing the cloned DNA, and to select for the transformed progeny (Bevan *et al*. 1983; Bower *et al*. 1996; Miki and McHugh 2004). As only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Since the selectable marker gene is usually co-transformed with a gene of interest. It is usually constructed as a chimeric gene using regulatory sequences that ensure constitutive expression throughout the plant (Tian *et al*. 2000; Miki and McHugh 2004). Once the transgenic plant is generated and characterized, the selectable marker gene generally no longer serves an essential purpose. If the selectable markers are to remain expressed within the transgenic plant, it is important for both scientific and economic reasons that the selectable marker gene does not have broad pleiotropic effects (van den Elzen *et al*. 1985; Miki and McHugh 2004).

At presently 50 or more marker genes used for transgenic and transplastomic plant research have been assessed for efficiency, biosafety, scientific applications and commercialization (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). However, only few marker genes have been used in conifers due to their ease of selection, expression and integration in pine genomes. Selectable marker genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or non-conditional on the presence of external substrates (Joersbo and Okkels 1996; Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). Positive selection marker genes are defined as those that promote the growth of transformed tissue whereas negative selectable marker genes result in the death of the transformed tissue (Barrell *et al*. 2002; Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). A conditional-positive selection system consists of a gene coding for a protein, usually an enzyme, that confers resistance to a specific substrate that is toxic to untransformed plant cells or that encourages growth and/or differentiation of the transformed cells (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). The positive selectable marker genes that are conditional on the use of toxic agents, such as antibiotics, herbicides or drugs were the first to be developed and exploited. In each case the gene codes for an enzyme with specificity to a substrate to encourage the selective growth and proliferation of the transformed cells. The substrate may be toxic or non-toxic to the untransformed cells. The *npt*II gene, which confers kanamycin resistance by inhibiting protein synthesis, is the classical example of a system that is toxic to untransformed cells (Miki and McHugh 2004). Newer strategies include positive selectable marker genes which are not conditional on external substrates but which alter the physiological processes that govern plant development (Twyman *et al*. 2002;

Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006).

A valuable companion to the selectable marker genes are the reporter genes, which do not provide a cell with a selective advantage, but which can be used to monitor transgenic events and manually separate transgenic material from non-transformed material. Some reporter genes can be adapted to function as selectable marker genes through the development of novel substrates (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). In case of conifers, a variety of selection systems are essential as no single selectable marker gene was found to be sufficient for all circumstances (Tian *et al*. 2000). Further, it was concluded from the number of studies in plants that no adverse biosafety effects have been reported for the marker genes that have been adopted for widespread use; biosafety concerns should help direct which markers will be chosen for future wood development (Flavell *et al*. 1992). Common sense dictates that marker genes conferring resistance to significant therapeutic antibiotics should not be used (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). The development of strategies for eliminating selectable marker genes to generate marker-free plants has been well reported in recent years (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). Among the several technologies described, two have emerged with significant potential (Matsunaga *et al*. 2002). The simplest is the co-transformation of genes of interest with selectable marker genes followed by the segregation of the separate genes through conventional genetics (Ebinuma *et al*. 1997, 2001). The more complicated strategy is the use of site-specific recombinases, under the control of inducible promoters, to excise the marker genes and excision machinery from the transgenic plant after selection has been achieved (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). As no single selection system is adequate for all purposes, there is need for several systems. In case of conifers, there are at least two selection systems are very common and this is based on the 90% of the scientific publications. These were selection on antibiotic kanamycin or hygromycin and the herbicide BASTA, and phosphinothricin (Brukhin *et al*. 2000; Tian *et al*. 2000; Charity *et al*. 2005). Transgenic plants showed that selectable markers that confer resistance to kanamycin or BASTA or phosphinothricin were the most common (Brukhin *et al*. 2000; Tang and Newton 2003). As herbicide resistance provides a natural selectable marker system, herbicide resistant lines and varieties can usually be produced without the need for other selectable marker genes. The popularity of these selection systems reflects the efficiency and general applicability of their use across a wide range of conifer species and regenerable tissue culture systems (Tian *et al*. 2000; Brukhin *et al*. 2000; Barrell *et al*. 2002).

The gene coding for the green fluorescent protein (GFP) from jellyfish (*Aequorea victoria*) was introduced into conifer tissues by microprojectile bombardement and its transient expression was detected in black spruce (*Picea mariana*), white spruce (*Picea glauca)* and white pine (*Pinus strobus*) embryonal masses, suspension culture, somatic embryos, and pollen (Tian *et al*. 1997). The successful expression of GFP gene in various tissues suggests that it will be a useful reporter/marker gene for conifers. GFP transgene was stable over multiple subcultures (Tian *et al*. 1999). The GFP gene and the gene conferring resistance to kanamycin (*npt*II) were introduced in black spruce (*Picea mariana*), white spruce (*Picea glauca*) and white pine (*Pinus strobus*) by biolistic or *Agrobacterium* method technology (Tian *et al*. 1999). GFP has become a powerful reporter gene to complement selectable marker genes and can be used to select for transformed material alone. The great advantage of GFP as a non-conditional reporter is the direct visualization of GFP in living tissues in real time without invasive procedures such as the application or penetration of cells with substrate and products that may diffuse within or among cells. Both considerations provide a significant improvement over GUS and LUC as reporter genes (Tian *et al*. 1997, 1999).

The bacterial enzyme *β*-glucuronidase, which is coded by the *E. coli uidA* (*gus*A) gene is the most widely used reporter gene in many plant species including conifers (Huang *et al*. 1991; Aronen *et al*. 1996; Birch 1997; Walter *et al*. 1999; Wenck *et al*. 1999; Klimaszewska *et al*. 2001; Tang *et al*. 2001a; Trontin *et al*. 2002; Tang and Newton 2003; Grant *et al*. 2004; Miki and McHugh 2004; Charity *et al*. 2005; Grace *et al*. 2005; Tang and Newton 2005; Tereso *et al*. 2006; Malabadi and Nataraja 2007a, 2007b, 2007c, 2007i; Tang *et al*. 2007). This enzyme utilizes the external substrate 4-methyl umbelliferyl glucuronide for measurements of specific activity and 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histochemical localization (Jefferson 1987). It is therefore, a conditional non-selectable-marker gene. GUS activity is found widely in microorganisms but there is very little background activity in plants (Miki and McHugh 2004). The GUS enzyme is very stable within plants and is non-toxic when expressed at high levels. The major drawback with the use of GUS as a reporter is that the assays are destructive to the plant cells. A useful feature of GUS as a reporter is that it can be fused with other proteins (Jefferson *et al*. 1987). For example GUS fusion with selectable marker genes such as *npt*II allows the visualization of transformation in addition to selection (Cerda *et al*. 2002; Charity *et al*. 2005; Malabadi and Nataraja 2007i). The capacity to generate fusions with other proteins has extended the usefulness of GUS for gene tagging experiments and has resulted in the discovery of novel genomic elements such as cryptic gene regulatory elements (Miki and McHugh 2004). GUS genes have frequently been co-transformed with selectable marker genes, for example, the *bar* selectable marker gene, to facilitate the selection of transformed conifer tissues (Newton *et al*. 2001; Nigro *et al*. 2004; Charity *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). GUS expression was used as a reporter to help detect transformation events in tissue culture during the production of a number of plant lines approved for commercialization (Miki and McHugh 2004).

Luciferase as a reporter, offers several advantages including the capability of monitoring gene expression patterns non-destructively in real time with great sensitivity. Electroporation of *P. radiata* protoplasts with a plasmid containing a firefly luciferase reporter gene driven by a 35S promoter resulted in expression levels that were 2-3.5 times that of background (Campbell *et al*. 1992). Extracts from *P. radiata* suspension culture cells containing 800 μg soluble protein inhibited the β-glucuronidase activity of *E. coli* extracts by 50%. Aliquots of *P. radiata* extracts containing less than 100 μg of total soluble protein did not exhibit any inhibitory effect (Campbell *et al*. 1992). The firefly (*Photinus pyralis*) luciferase catalyzes the ATP-dependent oxidative decarboxylation of luciferin. After the reaction occurs luciferase is inactive until the oxyluciferin is released from the enzyme complex (Miki and McHugh 2004). Luciferase is often used with other marker genes as an internal control and is also used as a visual marker of transformation for the manual selection of transgenic material undergoing selection (Campbell *et al*. 1992; Miki and McHugh 2004).

Bacterial aminoglycoside 3-phosphotransferase II, also known as neomycin phosphotransferase II (*npt*II), was shown to be effective as a selectable marker tested in many plant species including conifers (Miki and McHugh 2004). The *npt*II gene is the most frequently used selectable marker gene for generating transgenic plants in conifers for research purposes (Le *et al*. 2001; Klimaszewska *et al*. 2001; Cerda *et al*. 2002; Klimaszewska *et al*. 2003; Grant *et al*. 2004; Charity *et al*. 2005; Grace *et al*. 2005; Malabadi and Nataraja 2007i). Selection on kanamycin was used to recover insertions into expressed genes or gene regulatory elements to probe the plant genome for new and novel genes and regulatory elements that are not accessible through conventional cloning strategies (Miki and McHugh 2004). The amplification of the inserted *npt*II gene has generally achieved 10,000 copies per cell and can accumulate up to 1% of total protein (Miki and McHugh 2004). Further a

1993 WHO workshop concluded that the use of the *npt*II marker gene in genetically modified plants posed no risks to human health (WHO 1993; Nap *et al*. 2003; Miki and McHugh 2004).

Hygromycin B is an aminocyclitol antibiotic inhibitor of protein synthesis and in plants, the antibiotic is very toxic (Miki and McHugh 2004). Hygromycin B phosphotransferase confers resistance on bacteria, fungi, animal cells and plant cells. Chimeric genes have been shown to be effective in selection with diverse plant species, including conifers. This enzyme, when used as a selectable marker with *npt*II was not found to be effective (Twyman *et al*. 2002). Hygromycin B is the second most frequently used antibiotic for selection after kanamycin in conifers (Tian *et al*. 2000; Tereso *et al*. 2006; Tang *et al*. 2007). It was also reported that hygromycin resistance was an effective selectable marker for biolistic transformation of black spruce (*Picea mariana*) (Tian *et al*. 2000). Hygromycin was also successfully used to select transgenic radiata pine tissue after transformation with a vector containing the *aph*IV gene (Wagner *et al*. 1997). Tissue resistant to this antibiotic proliferated and became visible 4-6 weeks after transformation of radiata pine (Wagner *et al*. 1997). In *P. pinaster* genetic transformation, from 52 hygromycin-resistant lines obtained, 47 showed stable *uid*A gene expression (Tereso *et al*. 2006).

Bialophos resistance gene or the L-isomer of phosphinothricin (PPT; glufosinate ammonium) is the active ingredient of several commercial broad spectrum herbicide formulations for example, Basta™. An analogue of L-glutamic acid, PPT is a competitive inhibitor of glutamine synthetase ultimately results in the accumulation of toxic ammonium levels resulting in plant cell death (OECD 1999; Miki and McHugh 2004). Bacterial acetyltransferases that confer resistance to bialophos (consisting of two L-alanine residues and PPT) have been used in plants to achieve resistance to herbicides that contain PPT (Miki and McHugh 2004). The *bar* (bialophos resistance) gene from *Streptomyces hygroscopicus*, driven by plant promoters was shown to be an effective selectable marker gene in many plants, including conifers (Nigro *et al*. 2004; Charity *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). Transgenic *P. pinaster* (Maritime pine) have been achieved by co-culture of embryonal suspensor masses with *A. tumefaciens* carrying two constructs with a *bar* gene (phosphinothricin acetyltransferase, Basta tolerance) under the control of constitutive promoters P35S or *Pubi* (from maize polyubiquitin gene) (Trontin *et al*. 2007). Transgenic plants were recovered, and transgenes were detected in needles after 18 months with 95% of the *bar* gene integration. These transgenic plants also showed significant Basta tolerance occurred in about half of tested plants (48%) compared to non-transformed controls. Bar selection is currently used to generate transgenic maritime pine expressing genes with direct, practical interest and in functional genomic research studies (Trontin *et al*. 2007).

Chloramphenicol acetyl transferase from *E. coli* Tn 9 has been used for the selection of many plant transformants including conifers with the *cat* gene driven by the *nos* promoter (Wilson *et al*. 1989; Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006). GUS and CAT were used as reporter proteins in protoplasts from embryogenic suspension cultures of *Picea glauca* (Moench) Voss (white spruce) (Wilson *et al*. 1989). Plasmid DNA enclosing chimeric GUS and CAT constructs, using the CaMV 35S promoter, was introduced into *P. glauca* protoplasts using polyethylene glycol (PEG). Transient expression was detected 12 to 40 h after PEG-mediated DNA delivery. CAT expression was enhanced if PEG-mediated delivery was performed on ice rather than at room temperature (Wilson *et al*. 1989). The highest level of expression for CAT, and the lowest signal-to-noise ratio, was found 24 h after PEG-mediated DNA transfer. Both GUS and CAT provided results that were quantifiable and can therefore, be used as reporter genes in *P. glauca* (Wilson *et al*. 1989). Chloramphenicol was much less efficient than selection on kanamycin conferred by the *npt*II gene. The inefficiency has limited the use of the *cat* gene as a selectable marker, however, the sensitivity assay for enzyme activity enhanced its use as a reporter gene for transformation events in early studies. This enzyme is no longer widely used as a reporter gene (Miki and McHugh 2004; Tian 2006; Tian *et al*. 2006).

BIOLISTIC GENE TRANSFER

Biolistic or particle bombardment is used as a direct gene transfer method for plant transformation, and relies entirely on physical or chemical principles to deliver foreign DNA into the plant cells. In this method there is no dependence on bacteria, so the limitations inherent in organisms such as *A. tumefaciens* do no apply (Tang and Newton 2003; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). There are several different direct DNA transfer methods that have been described, including particle bombardment (Stomp *et al*. 1991; Christou *et al*. 1992; Pena and Seguin 2001; Nigro *et al*. 2004), microinjection (Crossway *et al*. 1986), transformation of protoplasts mediated by polyethylene glycol or calcium phosphate (Datta *et al*. 1990), electroporation (Fromm *et al*. 1986), and transformation using silicon carbide whiskers (Frame *et al*. 1994). Among these methods, biolistic gene transfer method has been used the most widely for generating transgenic conifer trees, and the delivery of transgene into embryogenic tissues by particle bombardment remains the principle direct DNA transfer technique in plant biotechnology (James 2003: Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). Further the ability to deliver foreign DNA directly into regenerated cells, tissues and organs appears to provide the best method for achieving truly genotype-independent transformation bypassing *Agrobacterium*-host specificity and tissue-culture-related regeneration difficulty (Birch 1997; Tang and Newton 2003; Nigro *et al*. 2004; Altpeter *et al*. 2005). There is no biological limitation to the actual DNA delivery process, so genotypic specificity is not a limiting factor (Birch 1997; Tang and Newton 2003; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). Advances in the transformation of crop plants by particle bombardment have demonstrated that foreign DNA can be inserted virtually into any tissues and that cells are impacted by particle bombardment (Birch and Bower 1994; Newton *et al*. 2001; Taylor and Fauquet 2002; Altpeter *et al*. 2005). There is also a widely-held belief that *Agrobacterium*-mediated transformation is more precise, more controllable, and therefore, cleaner than biolistics, but this axiom does not stand up to close scrutiny. There have been some reports of vector backbone co-transfer by *A. tumefaciens* (Wenck *et al*. 1999; Mathews and Campbell 2000; Popelka and Altpeter 2003): it was clear that biolistic gene transfer allows much more precise control over transgene structure (Walter *et al*. 1998; Popelka and Altpeter 2003; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). The absence of biological constraints, at least until DNA has entered the plant cell, means that particle bombardment is a versatile and effective transformation method, not limited by cell type, species or genotype. There are no intrinsic vector requirements so transgenes of any size and arrangement can be introduced and multiple gene co-transformations are straightforward. There are many parameters which influence successful delivery of foreign DNA into plant cells, and among them the condition of the explants prior to bombardment, transient activity of the marker gene, depth of particle penetration, degree of tissue damage as a function of acceleration force, and timing of selection (Walter *et al*. 1998; Tang and Newton 2003; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). Indeed, a major advantage of biolistic gene transfer method is that the delivered DNA can be manipulated to influence the quality and structure of the resultant transgene loci. This has been demonstrated in recently reported strategies that favor the recovery of transgenic plants containing intact, single-copy integration events, and demonstrating high-level transgene

Table 1 Transformation of conifers by particle bombardment.

Species	Bombarded tissue	Plasmid vectors	Gene expression	References
P. roxburghii	Embryogenic tissue from mature trees	pAHC25	stable expression	Malabadi and Nataraja 2007a
P. kesiya	Embryogenic cells	pAHC25	stable expression	Malabadi and Nataraja 2007b
P. wallichiana	Embryogenic cells	pAHC25	stable expression	Malabadi and Nataraja 2007c
P. radiata	Embryogenic cells	pMYC3425	stable expression	Grace *et al.* 2005
P. patula	Embryogenic cells	pAHC25	transient expression	Nigro *et al.* 2004
P. sylvestris	Pollen grains	pCGUΔ0	transient expression	Aronen *et al.* 1998, 2003
P. abies	Embryogenic tissue	pSeGer1, pAsGer1, pCW122	stable expression	Bishop-Hurley *et al.* 2001
P. abies	Embryogenic tissue	pUBi. Spi2.bar	stable expression	Elfstrand *et al.* 2001
P. abies	Embryogenic tissue	pAHC25	stable expression	Clapham *et al* 2000
P. aristata	Pollen tubes	pBi122	transient expression	Fernando *et al.* 2000
P. griffithii	Pollen tubes	pBi122	transient expression	Fernando *et al.* 2000
P. monticola	Pollen tubes	pBi122	transient expression	Fernando *et al.* 2000
P. abies	Embryogenic tissue	pCW122	transient expression	Walter *et al.* 1999
P. radiata	Embryogenic tissue	pRC101, pCW122	stable expression	Walter *et al.* 1998
P. glauca	Embryonal masses	P35S-GFP, mGFP	transient expression	Tian *et al.* 1997, 1999
P. glauca	Pollen grains	mGFP4	transient expresion	Tian et al. 1997
P.mariana	Pollen grains	mGFP4	transient expression	Tian et al. 1997
L. laricina	Embryogenic cells	pRT66GUS, pRT55GUS pRT99GUS	stable expression	Klimaszewska *et al.* 1997
P. strobes	Embryogenic cells	P35S-GFP, mGFP	transient expression	Tian *et al.* 1997
P. mariana	Embryogenic cells	PRt99GUS, pBI426	stable expression	Charest *et al.* 1996
P. radiata	Embryogenic cells	pEmuGN, pCW103, p40CSD35SIGN, pCW5, pCW6, pCW122	transient expression	Walter *et al.* 1994
P. glauca	Embryogenic cells	pBI426	stable expression	Bommineni *et al.* 1993
L. decidua	Embryogenic cells	pRT99GUS	transient expression	Duchesne *et al.* 1993
L. leptolepis	Embryogenic cells	pRT99GUS	transient expression	Duchesne *et al.* 1993
L. leptoeuropae	Embryogenic cells	pRT99GUS	transient expression	Duchesne *et al.* 1993
L. (*entrolepis* × *hybrid larch*)	Embryogenic cells	pRT99GUS	transient expression	Duchesne *et al.* 1993
P. glauca	Embryogenic cells	pTUBT41100	stable expression	Ellis *et al.* 1993
P. abies	Somatic embryo cultures	pRT99GUS	stable expression	Robertson *et al.* 1992
P. radiata	Embryogenic cells	pBI122, pCAMVLN	transient expression	Campbell *et al.* 1992
P. mariana	Embryogenic callus	pRT99GUS, pMB113kp	transient expression	Duchesne and Charest 1991
P. glauca	Embryos , seedlings	pUC19	transient expression	Ellis *et al.* 1991
P. taeda	cotyledons	pBI122	transient expression	Stomp *et al.* 1991
Pseudotsuga menziesii	cotyledons	pTVBTGUS	transient expression	Goldfrab *et al.* 1991

expression (Walter *et al*. 1998; Malabadi and Nataraja 2007a, 2007b, 2007c). In *Pinus*, transient expression of the GFP gene in embryogenic masses of *P. strobus via* particle bombardment was achieved (Tian *et al*. 1997, 1999; **Table 1**). Transient expression of *uid*A in cotyledon cells of *P. taeda* by particle bombardment was reported (Stomp *et al*. 1991), and these results demonstrated that biolistics has the potential for the production of transgenic plants in pine. In *P. radiata*, regenerated pines with transient transgene activity were obtained by bombarding embryogenic tissue (Walter *et al*. 1998; **Table 1**).

In pines, another area of interest is particle-mediated pollen transformation. Pollen is the natural carrier of genetic material, and is therefore, also a good target for foreign gene delivery (Aronen *et al*. 2003). Pollen grains are natural vectors for gene transfer because they are involved in sexual reproduction (Haggman *et al*. 1997; Aronen *et al*. 1998; Fernando *et al*. 2000). Pollen transformation may also be extended to *in vitro* fertilization, a new development that promises a novel approach to conifer breeding. The pollen lots of Scots pine (*P. sylvestris*) were transformed by particle bombardment, resulting in transient transformation frequencies varying from 15 to 49% of the germinated pollen grains, and bombarded pollen was used to pollinate megasporangiate strobili. The morphology and growth of transgenic seedlings was normal (Aronen *et al*. 2003). A biolistic particle delivery system was also used to genetically transform pollen tubes of three species of white pine (*P. aristata*, *P. griffithii* and *P. monticola*) (Fernando *et al*. 2000). Successful gene delivery was demonstrated in three species of white pine by transient GUS expression (Fernando *et al*. 2000). Pollen transformation and its applications in controlled crossings is performed without selection, and screening of progeny can also be performed on the basis of the transgene itself, for instance using PCR. Progeny screening is, however, laborious and the involvement of selection medium or spraying with an herbicide if this is used as a selectable matter might be feasible (Aronen *et al*. 2003). This is another important application of biolistic gene method that can significantly play an important role in commercial forestry, and recalcitrant pines could be easily transformed.

Strategies of biolistics

In plants genetic transformation occurs in two distinct stages: DNA transfer into the cell followed by DNA integration into the genome. Among these two stages, DNA integration is much less efficient than the DNA transfer stage, with the result that only a small proportion of the cells that initially receive DNA actually become stably transformed. On the other hand in the remaining cells the DNA enters the cells and may be expressed for a short duration (transient expression), but it is never integrated and is eventually degraded by nucleases (Herschbach and Kopriva 2002; Altpeter *et al*. 2005). Transient expression occurs almost immediately after gene transfer, it does not require the regeneration of whole plants, and it occurs at a much higher frequency than stable integration. Therefore, transient expression can be used as a rapid assay to evaluate the efficiency of direct DNA transfer and to verify the function of expression constructs. Indeed, transient expression following particle bombardment with a reporter gene such as *uid*A or *gfp* is used routinely to compare different expression constructs and identify those with the most appropriate activity in pines for example, *Larix deciduas* (Duchesne *et al*. 1993), *Larix leptolepis* (Duchesne *et al*. 1993), *Larix leptoeuropae* (Duchesne *et al*. 1993), *Larix eurolepis* × hybrid *larch* (Duchesne *et al*. 1993), *P. glauca* (Ellis *et al*. 1991; Tian *et al*.

1997), *P. mariana* (Duchesne and Charest 1991; Charest *et al*. 1996), *P. aristata* (Fernando *et al*. 2000), *P. griffithii* (Fernando *et al*. 2000), *P. monticola* (Fernando *et al*. 2000), *P. radiata* (Campbell *et al*. 1992; Walter *et al*. 1994), *P. strobus* (Tian *et al*. 1997), *P. taeda* (Stomp *et al*. 1991), and *Pseudotsuga menziesii* (Goldfarb *et al*. 1991; **Table 1**). Transient expression following particle bombardment may also be used to produce small amounts of protein rapidly for testing in many plant species (Twyman *et al*. 2002a, 2002b, 2003; Altpeter *et al*. 2005). Biolistic gene transfer also demonstrates considerable significance and versatility, since in addition to nuclear transformation, it permits the transformation of plastids, a process that can not be achieved with *Agrobacterium* spp. This is because the T-DNA complex is targeted to the nucleus (Altpeter *et al*. 2005). Furthermore, Biolistic gene transfer is the only method that can be used for mitochondrial transformation (Johnston *et al*. 1988), although this has yet to be achieved in higher plants. Biolistics is also useful in transformation strategies involving plant viruses (Altpeter *et al*. 2005). Hoffman *et al*. (2001) used particle bombardment for the mechanical transmission of poleroviruses, and particle bombardment is routinely employed for the inoculation of whole plants and leaf tissues with viruses that are difficult to introduce via conventional mechanical infection. Particle bombardment also has an important role to play in extending virus-induced gene silencing into economically important crop plants (Altpeter *et al*. 2005).

Influencing factors

One of the major advantages of particle bombardment is that it does not rely on the biological limitations of any single group of microorganisms (Ellis *et al*. 1993; Altpeter *et al*. 2005). Further it does not depend on any particular cell type as long as the DNA can be introduced into the cell without killing it (Fernando *et al*. 2000; Altpeter *et al*. 2005). The production of transgenic plants from transformed cells depends only on the ability of such cells to exhibit totipotency under the culture conditions employed (Duchesne *et al*. 1993; Altpeter *et al*. 2005). The lack of cell type dependence also allows particle bombardment to be used in the study of subcellular trafficking and storage protein deposition (Altpeter *et al*. 2005). This is another example how transient expression can be used to provide data rapidly and inexpensively without the need to regenerate transgenic plants. This strategy is feasible in all the target tissues where a sufficient proportion of cells can be transiently transformed by bombardment, and has been demonstrated in species and tissues that are not amenable to *Agrobacterium* (Clapham *et al*. 2000; Altpeter *et al*. 2005). For stable transformation and the recovery of transgenic plants, particle bombardment is restricted only by the requirement to deliver DNA into regenerable cells. By removing almost all the incidental biological constraints that limit other transformation methods, particle bombardment has facilitated the transformation of some of the most recalcitrant plant species (Bommineni *et al*. 1993; Charest *et al*. 1996; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). Therefore, in this context, particle bombardment is superior to other transformation methods because both single cells and organized tissues can be used as transformation targets, and the conditions chosen to target superficial cells or cells residing in deeper layers in organized tissues, allowing transformation of cell types that have traditionally been difficult to reach, such as dividing cells in the apical meristem (Tang and Newton 2003; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a). The ability to transform diverse cell types by particle bombardment facilitates a broad range of applications that are difficult or impossible to achieve by other transformation methods. This is critical when the rapid analysis of large numbers of constructs in a specific tissue or cell type is required. However, the method remains extremely versatile generating transformants with relative ease for a wide range of tree and crop species. A wide variety of cell and tissue explants have been utilized for biolistics, although as a general guide such explants should contain a large proportion of healthy and easily regenerable cells, preferably enriched with cells approaching nuclear division. Examples include apical meristem cells, embryos, seedlings, megagametophytes, xylem, pollen, needles, buds, cell suspension cultures, embryogenic callus, cell aggregate cultures and roots. Transformation efficiency also depends upon the regeneration capacity, and the efficiency of selection, which in turn depends upon how different explants, are handled.

In the case of conifers, two types of explants such as immature zygotic embryos (dissected from immature cones) and mature zygotic embryos (dissected from seeds) from cross-pollinated trees have been used as the starting material for the induction of an embryogenic system. The embryogenic tissue as a result of embryo cloning was used for both biolistic and *Agrobacterium*-mediated genetic transformation in conifers. Another important starting material for biolistics are the pollen grains (Aronen *et al*. 2003). Other than explants, the bombardment conditions such as microcarrier travel distance and target distance are also important influencing factors, affecting the biolistic gene transfer in many plants, including conifers (Heiser 1992; Fernando *et al*. 2000; discussed below).

Successful production of transgenic seedlings of Scots pine (*P. sylvestris*) was achieved by biolistic-gene-transfer through the application of transformed pollen in controlled crossings (Aronen *et al*. 203). This resulted in transient transformation frequencies varying from 15 to 49% of the germinated pollen grains (Aronen *et al*. 2003). Pollen grains were found to be the best explants for biolistics. However, transient expression frequencies in the bombarded pollen were high, but the final frequency of transgenic plants obtained was very low (Aronen *et al*. 2003). There are several reasons for this since pine pollen grains consist of two cells, a larger tube cell and smaller cell. In order to obtain transgenic progeny, the generative nucleus should be transformed. Low transformation frequency among progeny is related to the histochemical screening; the timing of sampling may have been sub-optimal for a part of the seedlings, the majority of which was tested without an acetone pretreatment, which seems to enhance GUS expression in needles covered by a waxy cuticle (Aronen *et al*. 2003). In their method, Aronen *et al*. (1994) tested needle tips from each of the seedlings histochemically for GUS expression with slight modifications, and chlorophyll was removed by washing the samples in ethanol (70%). An acetone pre-treatment was added to the needle protocol: the excised needles were immersed in 95% acetone for 30 min to facilitate substrate penetration (Hawkins *et al*. 1997), and then rinsed three times in 100 mM potassium phosphate buffer (pH 7.0) before incubation in the test solution for GUS analysis (Aronen *et al*. 2003). The acetone treatment has been used earlier to facilitate the penetration of substrate for GUS into stem pieces of deciduous woody plants (Aronen *et al*. 1994; Hawkins *et al*. 1997; Regan *et al* 1999; Tuominen *et al*. 2000; Aronen *et al*. 2003). In another biolistic method of pollen transformation of three species of whit pine (*P. aristata*, *P. griffithii* and *P. monticola*), the frequency of transformation was affected by the bombardment conditions such as microcarrier travel distance and target distances respectively (Fernando *et al*. 2000). In all the three species of white pine (*P. aristata*, *P. griffithii* and *P. monticola*), significant differences were observed between the different distances of target specimens (Fernando *et al*. 2000). Here optimum bombardment conditions were performed with a rupture disc pressure of 900 psi, a gap distance of 1.5 cm, a microcarrier travel distance of 8 mm, and a target distance of 6.9 or 12 cm (Fernando *et al*. 2000). A target distance of 6 cm resulted in a significant number of transformed pollen tubes in all species of white pine (Fernando *et al*. 2000). Further, none of the *P. aristata* pollen tubes were transformed at a target distance of 12 cm, while none of the *P. griffithii* pollen tubes were transformed at target distances

of 9 or 12 cm. In *P. monticola*, the number of pollen tubes transformed at target distances of 6 and 9 cm were not significantly different from each other but were significantly lower than those at a target distance of 12 cm (Fernando *et al*. 2000). Target distance has a major effect on the efficiency of DNA delivery because as it increases, the density of bombardment decreases (Heiser 1992). Since particle-mediated transformation appears as a statistical process, a shorter distance maximizes the probability of hitting specimens. In other pines, a target distance of 9.5 cm was shown to produce the highest percentage of pollen transformation (Haggman *et al*. 1997; Aronen *et al*. 2003). In another biolistic study of *P. patula*, the 70 mm microcarrier travel distance from the stopping plate to the target tissue was found to be optimum, and resulted in the highest transformation efficiencies (40% and 47%) of both GUS and *bar* transgenes, respectively (Nigro *et al*. 2004). They also reported that the pretreatment with sorbitol served to maintain tissue integrity during biolistics, which is another important factor influencing biolistic transformation (Nigro *et al*. 2004). Microscopic analysis after particle bombardment in *P. patula* showed cell-burst in treatments lacking the osmotic treatment, while a marked, positive effect was observed on culture cell integrity by inclusion of an osmoticum in the medium prior to biolistic transfer (Nigro *et al*. 2004). Osmotic enhancement was suggested to have resulted from plasmolysis of the cells that may have reduced cell damage by preventing or making protoplasm extrusion less likely from bombarded cells (Li *et al*. 1994; Nigro *et al*. 2004), and may have improved particle penetration itself (Nigro *et al*. 2004). In *P. patula*, the inclusion of 0.25 M sorbitol had the most beneficial effects on the day 0 of line 1, and on day 14 of line 2, which was the only treatment to produce harvestable embryos suitable for plant regeneration (Nigro *et al*. 2004).

Transgenic radiata pine plants containing a *Bacillus thuringiensis* (*Bt*) toxin gene, *cry*1Ac, were produced by means of biolistic transformation of embryogenic tissue derived from immature zygotic embryos of *P. radiata* (Grace *et al*. 2005). In most of the pines, the embryogenic system for genetic transformation is always obtained from either mature or immature zygotic embryos, and they served as the best starting material for the induction of embryogenic tissue (Klimaszewska *et al*. 2001; Cerda *et al*. 2002; Klimaszewska *et al*. 2003; Grant *et al*. 2004; Nigro *et al*. 2004; Grace *et al*. 2005; Tereso *et al*. 2006; Tang *et al*. 2007). This is simply embryo cloning, which is also an important influencing factor for biolistic transformation. However, use of an embryo as an explant has several disadvantages including heterozygosity as a result of cross-pollination (Malabadi and van Staden 2005a, 2005b, 2005c). The proportion of gene loci of an individual tree lines that are in a heterozygote is called its degree of heterozygosity. Heterozygosity leading to larger genetic variation is a common phenomenon in trees, including conifers (Sharma *et al*. 2007). That is, immature zygotic embryos (actually whole megagametophytes containing multiple zygotic embryos) are induced to undergo what might best be described as continuous cleavage polyembryony following extrusion of the zygotic embryos from the megagametophyte (Becwar *et al*. 1991; Malabadi *et al*. 2002, 2003). Thus, what is actually being carried out is embryo cloning. While the zygotic embryos from which the cultures are initiated may represent superior half-sib or even full-sib families (if they are the product of controlled pollinations), the fact remains that they are unproven genetically. To add to the uncertainty of the genetic value of material propagated via somatic embryogenesis, many workers have observed that embryogenic cultures are usually not initiated from the dominant zygotic embryo in the megagametophyte, but rather from one of the subordinate embryos that would most likely have aborted had the seed been allowed to mature (Becwar *et al*. 1991; Malabadi *et al*. 2002). Furthermore, it was shown that a certain percentage of the embryogenic cultures initiated using this approach may actually be mixtures of genotypes, derived from multiple zygotic embryos that were present in the megagametophyte at the time of extrusion (Becwar *et al*. 1991; Malabadi *et al*. 2002, 2005; Malabadi and Nataraja 2007k, 2007l, 2007m). These drawbacks of the current approach for initiating embryogenic pine cultures from seed embryos could be avoided if a method was available for initiating embryogenic cultures from tissues of mature, proven pine trees. However, mature tree tissues of most pines are known to be highly recalcitrant to vegetative propagation of any kind and the general consensus is that they must be "rejuvenated" to make them amenable to propagation via such approaches as rooted cuttings or tissue culture, including somatic embryogenesis.

At present an embryogenic system derived from vegetative shoot apices or secondary needles of mature pines have been well established in at least a few conifers (Bonga and Pond 1991; Ruaud *et al*. 1992; Bonga and von Aderkas 1993; Ruaud 1993; Westcott 1994; Smith 1997; Bonga 1996, 2004; Malabadi *et al*. 2004; Malabadi and van Staden 2005a, 2005b, 2005c, 2006; Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b; Aronen *et al*. 2007; Malabadi and Nataraja 2007f, 2007h), and an embryogenic system could be used for genetic transformation studies. Another important advantage of using vegetative shoot apices of mature pines as a starting material for genetic transformation is that cells are actively dividing, hence their higher regeneration capacity, and serve as the best starting material for biolistic transformation. These cells are generated by the meiotic division of meristematic tissue, and meristimatic cells possess higher regeneration potential, withstand higher biolistic pressure showing maximum cell integrity compared to cells derived from embryo cloning (Malabadi and Nataraja 2007a). Another reason might be that during cloning of mature trees, the single somatic cell is programmed towards embryogenesis under the stress conditions of cold-pretreatment (Malabadi *et al*. 2004; Malabadi and van Staden 2005a, 2005b, 2005c). Stress induced by cold-pretreatment might make the cells more resistant, and are ready to withstand biolistic pressure resulting in the compact cell integrity of cells (Malabadi and Nataraja 2007a). On the other hand the cells resulting from embryo cloning are much elongated and loosely arranged cells since they are originated not due to any stress conditions but from the embryo only, that resulted in the bursting and loss in cell integrity during biolistic transformation (Nigro *et al*. 2004; Malabadi and Nataraja 2007b, 2007c). This might help in solving the current problems of regeneration of transgenic lines by biolistics. This will also result in the stable transformation of a particular tree line under study, and the transgenic lines could be used for commercial forestry since they have defined genetic characters of superior parents.

Recently transgenic trees produced by using embryogenic tissue derived from cloning mature trees by biolistic-mediated transformation were reported in an Indian pine *Pinus roxburghii* (Malabadi and Nataraja 2007a). The transformation efficiency was higher than our other studies of *P. kesiya* and *P. wallichiana* (Malabadi and Nataraja 2007b, 2007c) by using the embryogenic tissue of mature trees, and also resulted in the stable expression of transgenes (Malabadi and Nataraja 2007a; **Table 1**). In another study, the embryogenic tissue of mature trees of *P. wallichiana* was also successfully used for genetic transformation studies, and resulted in the production of transgenic plants in three lines using *Agrobacterium*-mediated genetic transformation (Malabadi and Nataraja 2007i). Therefore, the transgenic lines are clearly defined with genetically-inherited characteristics of their parents. This is the main advantage of cloning mature trees, and could be used for the biolistic-gene-transformation in the remaining conifers.

Non-requirement of vectors

The most important advantage of biolistics is that during transformation, vectors are not required for transgene expression (Tang and Newton 2003; Nigro *et al*. 2004; Alt-

peter *et al*. 2005). The exogenous DNA used in transformation experiments typically comprises a plant expression cassette inserted in a vector based on a high-copy-number bacterial cloning plasmid (Tang and Newton 2003; Altpeter *et al*. 2005). Neither of these components is required for transgene expression (Birch 1997; Altpeter *et al*. 2005). The vector backbone is therefore, superfluous. The vector backbone typically includes a bacterial origin of replication and selectable marker, allowing the expression cassette to be cloned in *E. coli*. The expression cassette typically consists of a promoter, open reading frame and polyadenylation site that are functional in plant cells. Once this plasmid has been isolated from the bacterial cultures, it is purified and used directly as a substrate for transformation. On the other hand in *Agrobacterium*-mediated genetic transformation, the transgene must be placed between T-DNA repeats, and further sequences such as *overdrive* and *transfer enhancer* may be required for efficient DNA delivery (Malabadi and Nataraja 2007i). There are no such biological constraints in biolistics and no vector DNA sequences are required for the delivery (Altpeter *et al*. 2005). During *Agrobacterium*-mediated transformation, the T-DNA is naturally excised from the vector during the transformation process. This frequently although not always, prevents the integration of vector backbone sequences into the plant genome (Fang *et al*. 2002), necessitating time-consuming sequence analysis of transgene insertion sites following *Agrobacterium*-mediated gene transfer whereas biolistic gene transfer involves no such processing. Cloning vectors are used in biolistic for convenience rather than necessity (Clapham *et al*. 2000; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a).

Multiple gene transformation

Biolistics is the most convenient method for multiple gene transfer to plants since DNA mixtures comprising any number of different transformation constructs can be used, with no need to complex cloning strategies, multiple *Agrobacterium* strains or sequential crossing (Bishop-Hurley *et al*. 2001; Altpeter *et al*. 2005). Therefore, co-transformation is the simultaneous transformation of a plant cell with two or more transgenes. Multiple gene transfer to plants is necessary for sophisticated genetic manipulation strategies such as the stacking of transgenes specifying different economically important traits (Elfstrand *et al*. 2001; Altpeter *et al*. 2005; Grace *et al*. 2005) although this can be achieved by single gene transformation followed by the crossing of plant species carrying different transgene(s). This is much quicker and more straightforward to introduce all the necessary genes simultaneously. But due to heterozygosity of pines, this makes crossing approaches difficult and time consuming (Malabadi and van Staden 2005a). Particle bombardment is the most convenient method for multiple gene transfer to plants since DNA mixtures comprising any number of different transformation constructs can be used, with no need for complex cloning strategies, multiple *Agrobacterium* strains or sequential crossing (Altpeter *et al*. 2005).

STABLE EXPRESSION AND REGENERATION

Stable expression and regeneration of transgenic conifers has been reported by many workers, e.g. *P. kesiya* and *P. wallichiana* (Malabadi and Nataraja 2007b, 2007c), *L. larcicina* (Klimaszewska *et al*. 1997), *P. abies* (Robertson *et al*. 1992; Walter *et al*. 1999; Clapham *et al*. 2000; Bishop-Hurley *et al*. 2001; Elfstrand *et al*. 2001), *P. glauca* (Bommineni *et al*. 1993; Ellis *et al*. 1993), *P. mariana* (Charest *et al*. 1996), *P. radiata* (Walter *et al*. 1998; Bishop-Hurley *et al*. 2001; **Table 1**). Recently for the first time stable expression and transgenic plants were regenerated from embryogenic tissue derived from vegetative shoot apices of mature trees of *P. roxburghii* (Malabadi and Nataraja 2007a). Transgenic radiata pine plants containing a *Bt* toxin gene, *cry*1Ac, were produced by means of biolistic transformation of embryogenic tissue (Grace *et al*. 2005). Using the selectable marker gene *npt*II and corresponding geneticin selection, 20 independent transgenic lines from five genotypes were established (Grace *et al*. 2005). Over 200 plants regenerated from ten transgenic lines were successfully transferred to soil. The integration and expression of the introduced genes in transgenic tissue and/or plants were confirmed by PCR, southern hybridization. Bioassays with larve of the painted apple moth, *Teia anartoides*, demonstrated that transgenic plants displayed variable levels of resistance to insect damage, with one transgenic line being highly resistant to feeding damage (Grace *et al*. 2005).

Biolistics has also been used to produce transgenic plants in conifers for the functional analysis of genes (Bishop-Hurley *et al*. 2000) and promoters (Moyle *et al*. 2002), and for the development of early screening technologies for new introduced traits that promise to be significant advantage to conifer biotechnology (Walter 2002). In *P. radiata*, a particle bombardment system was established by genetically transforming embryogenic tissue (Walter *et al*. 1998). The average number of stable, geneticin-resistant lines recovered was 0.5 per 200 mg fresh wt bombarded tissue. More than 150 transgenic *P. radiata* plants were produced from 20 independent transformation experiments with different clones (Walter *et al*. 1998). Clapham *et al*. (2000) established an efficient production method of transgenic plantlets of *P. abies* from embryogenic suspension cultures using a particle inflow gun. Embryogenic colonies resistant to Basta appeared 2 months after bombardment. Of over 100 independent Basta-resistant sublines tested, 65% expressed the co-transformed reporter gene and over 80% of the sublines retained their embryogenic potential. Over 200 Basta-resistant sublines from four cell lines have been established, of which 138 are confirmed as transformed (Clapham *et al*. 2000). Further stable transformation of *P. mariana* by particle bombardment has been accomplished by Charest *et al*. (1996). An efficient particle bombardment has been developed by stably transforming several *P. abies* embryogenic tissue lines. Stable transformation of *P. abies* tissue was obtained following bombardment of mature somatic embryos with pRt99Gus (Robertson *et al*. 1992). Stable transformation of *P. glauca* by biolistic and transgenic regenerated plantlets were obtained by transforming embryogenic cultures (Ellis *et al*. 1993). Biolistics was also used regenerate whole plants in *P. glauca*, *P. mariana*, *P. radiata*, *P. kesiya*, *P. roxburghii*, and *P. wallichiana* (Ellis *et al*. 1989, 1991; Charest *et al*. 1993; Walter *et al*. 1994; Malabadi and Nataraja 2007a, 2007b, 2007c). The expression of foreign genes in conifers was observed on meristematic cells that have the ability to rapidly divide and have a high rate of metabolic activity (Malabadi and Nataraja 2007g). Cells with high metabolic activity are most likely to be active in endogenous gene expression with all the functions for gene transcription and translation actively expressed (Malabadi and Nataraja 2007g). In *P. kesiya*, the introduction of a *bar*-GUS cassette under the control of the ubiquitin promoter was achieved through biolistic gene transfer (Malabadi and Nataraja 2007b). Expression of positive histochemical GUS activity (41%) in the bombarded embryogenic tissue was observed. PCR analysis of *bar* transgenes (46%) transformation efficiency indicated successful genetic modifications of *P. kesiya* embryogenic tissue by using the pAHC25 plasmid (Malabadi and Nataraja 2007b). Similarly in *P. wallichiana* the expression of positive histochemical GUS activity (39%) in the bombarded embryogenic tissue was observed. PCR analysis of *bar* transgenes (52%) transformation efficiency indicated that 50% of the selected plants showed gene integration and expression (Malabadi and Nataraja 2007c). Further positive histochemical GUS activity (31%) in the bombarded embryogenic tissue was observed during biolistic gene transfer of *P. roxburghii* (Malabadi and Nataraja 2007a). PCR analysis of *bar* transgenes (54%) transformation efficiency indicated stable genetic transformation of *P. roxburghii* embryogenic tissue by using the pAHC25 plasmid. This was achieved using the embryogenic tissue derived from the vegetative shoot api-

ces of mature trees of *P. roxburghii* (Malabadi and Nataraja 2007a). Incorporation of the introduced genes into the genome was confirmed by PCR and Southern blot analysis of embryogenic callus and regenerated transformed plants, as well as spruce budworm (*Choristoneura fumiferana* (Clemens)) feeding trials with transformed tissues (Ellis *et al*. 1993).

Molecular characterization

It is a widely held belief that particle bombardment produces large, multi-copy, and highly complex transgenic loci that are prone to further recombination, instability and silencing (Clapham *et al*. 2000; Altpeter *et al*. 2005; Grace *et al*. 2005). While it may be true that the delivery of whole plasmids by particle bombardment can lead to an increased proportion of complex transformation events compared to *Agrobacterium*-mediated transformation, recent experiments have shown that particle bombardment can be turned to favor the generation of plants with simple transgenic loci containing a small number of intact transgene copies (Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). Multi-copy transgenic plants generated by particle bombardment tend to have all the transgene copies at a single locus, regardless of how many different transformation cassettes have been used (Altpeter *et al*. 2005). In contrast, *Agrobacterium*-mediated transformation tends to be segregated; larger populations of plants are required to achieve homozygosity (Birch 1997; Tang and Newton 2003; Charity *et al*. 2005). When transferring the transgenes into a new genetic background *via* traditional breeding, breeding lines carrying the same gene at multiple loci are more difficult to use than those where all the genes are present at the same locus (Birch 1997; Tang and Newton 2003; Altpeter *et al*. 2005). In this respect, particle bombardment is advantageous over *Agrobacterium*-mediated transformation (Klimaszewska *et al*. 1997; Altpeter *et al*. 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). The requirement for extra copies of *vir*B, which has been implicated in T-DNA transfer, suggests that DNA transfer and not just virulence may be a limiting factor in transformation (Wenck *et al*. 1999). *Agrobacterium*-mediated transformation of *P. abies* and *P. taeda* showed the number of T-DNA insertions and the arrangement of insertions (head to tail), which is in good agreement with other transformation studies of conifers (Wenck *et al*. 1999). Although only six lines of *P. abies* and *P. taeda* were analyzed by Southern analysis, two of these had single-copy-insertions. Sequencing of the right-border junction in four lines indicated a slight truncation from the processing site of the right border in two of them (Wenck *et al*. 1999). Processing of the left border was revealed in the two head-to-tail insertions isolated. Both of these were truncated within 8 bp of the left border sequence. Even though the isolated fragment from these head to tail insertions have the same right border and left border processing points, sequencing and Southern analysis show them to be independent lines (Wenck *et al*. 1999).

On the other hand the Southern analyses of two transgenic lines by *Agrobacterium*-mediated transformation of embryogenic tissue of each of *P. glauca* and *P. abies* revealed a simple integration pattern with one or three copies of the T-DNA inserted in different loci (Klimaszewska *et al*. 2001). In this study of 12 transgenic lines of *P. mariana* analyzed, eight contained one to three copies of T-DNA integrated in different loci, and four lines displayed a more complex integration pattern. These later lines showed more than five T-DNA integration sites, some of which might contain T-DNA repeats (Klimaszewska *et al*. 2001). The method of transformation had no effect on the integration pattern of T-DNAs in *P. mariana* and *P. abies* (Klimaszewska *et al*. 2001); however, the majority of the transgenic lines had a relatively simple T-DNA integration pattern, a case also noted for *P. strobus* (Levee *et al*. 1999) and *P. abies* (Wenck *et al*. 1999). On the other hand 70 transgenic tissue lines (translines) obtained by *Agrobacterium*-mediated transformation of three spruce species (*P. mariana*, *P. glauca* and *P. abies*) were characterized with respect to the integration pattern of the GUS gene, and the level of GUS activity was determined in 81 lines (Klimaszewska *et al*. 2003). These results demonstrated that the majority of the *P. mariana* translines (18/22) integrated multicopies (2-4) of the transgene, whereas mostly single integrations were detected in the other two species. The activity levels of GUS varied widely among the individual translines of *P. mariana*. The average level of GUS activity, in lines that integrated one gene copy, was the highest in white spruce followed by black spruce and Norway spruce (Klimaszewska *et al*. 2003).

In *P. radiata*, a biolistic method for genetic transformation was achieved using embryogenic tissue (Walter *et al*. 1998). The average number of stable, geneticin-resistant lines recovered was 0.5 per 200 mg fresh weight bombarded tissue. Expression of the *uid*A reporter gene was detected histochemically and fluorimetically in transformed embryogenic tissue and in derived mature somatic embryos and regenerated plants. More than 150 transgenic *P. radiata* plants were produced from 20 independent transformation experiments with four different embryogenic clones (Walter *et al*. 1998). Cerda *et al*. (2002) reported embryonal masses from immature zygotic embryos of *P. radiata* were genetically transformed using *A. tumefaciens*. The pBI121 vector containing *uid*A and *npt*II genes was introduced into the embryogenic tissue of *P. radiata*. Molecular characterization showed the insert is often (although not quantified) a single insertion event. Low transgene copy number introduced by *Agrobacterium*-mediated transformation in *P. radiata* reduces undesirable traits such as gene silencing (Cerda *et al*. 2002). To overcome limitations with biolistics such as high gene copy number and gene silencing, *Agrobacterium*-mediated transformation protocols have been developed for *P. radiata* (Walter *et al*. 2002). The gene transfer frequency, as measured by transient expression of the reporter gene *uid*A was improved to 60% and plants were regenerated adventitiously from detached cotyledons or from apical meristematic domes, via the epicotyl shoot in *P. radiata* (Charity *et al*. 2002). However, since the efficiency for regeneration was low and there was some evidence of chimerism, alternative regeneration protocols have recently been evaluated (Walter *et al*. 2002). Chimerism is an extremely rare disorder that mixes the chromosomal population in a single organism (Wegner 2006). In these cases, chimerism may manifest as the presence of two sets of DNA, or organs that do not match the DNA of the rest of the organism. Genotypic variations can be genomic, chromosomic or genic. Genomic mutations affect the number of chromosomes (the ploidy) and can be detected by cytometry or chromosome counting. Unlikecytometry, the time-consuming chromosome counting enables an unfailing detection of all, even the smallest, modifications in chromosome number. Chromosomic mutations like inversions, deletions or translocations and genic mutations can be detected by genetic molecular markers like RFLPs (restriction fragment length polymorphisms), ISSR (Inter simple sequence repeats), RAPDs (random amplified polymorphic DNA), Microsatellite DNAs, AFLPs (Amplified length polymorphisms), SNPs (Single nucleotide polymorphisms), that can detect DNA sequence modifications (Malabadi *et al*. 2006; Malabadi and Nataraja 2006b, 2007j). It is also important to remember that mutations can occur on nuclear as well as on mitochondrial (mt) or chloroplast (cp) DNA (Fourre *et al*. 1997). Southern hybridization analysis of buds from transgenic *P. radiata* derived from a biolistic transformation experiment confirmed the presence of transgenes and their copy number was determined (Walter *et al*. 1998). Radiata pine explants transformed with the *npt*II and the *uid*A genes showed distinct hybridization signals in this analysis, indicating the presence of integrated sequences (Walter *et al*. 2002). These signals were absent in non-transformed material of *P. radiata*. The results further indicated high copy numbers of transgenes and transgene fragments. In some cases several hundred copies of the transgene ap-

peared to be present in the transgenic tissue of *P. radiata* (Walter *et al*. 2002). This integration pattern is typical for transclones that are produced using biolistics, in contrast to *Agrobacterium*-mediated transformation where single or low copy numbers of the transgene are usually reported (Huang *et al*. 1991; Tzfira *et al*. 1996; Wenck *et al*. 1999). Transgenic radiata pine (*P. radiata*) plants containing a *Bt* toxin gene, *cry1Ac*, were produced by means of biolistic transformation of embryogenic tissue (Grace *et al*. 2005). Over 200 plants regenerated from ten transgenic lines were successfully transferred to soil. Bioassays with the larvae of the painted apple moth, *Teia anartoides*, demonstrated that transgenic plants displayed variable levels of resistance to insect damage, with one transgenic line being highly resistant to feeding damage (Grace *et al*. 2005). This investigation has the potential to mitigate a serious risk to forestry, insect damage, by using an environmentally sustainable technology (Grace *et al*. 2005).

High copy numbers and fragmented copies can potentially have negative effects on long-term gene expression in transgenic plants, but conclusive results on gene silencing in transgenic conifers are not yet available (Walter *et al*. 2002). The issue of gene silencing resulting from multiple copies and fragmented copies is of great importance to genetic engineering in trees, particularly since expression must be maintained over a period of 30 years or more. Field trials with transgenic conifers will help to better understand long-term gene expression in conifers, and possibly lead to strategies to avoid silencing and expression-instability (Walter *et al*. 2002). Therefore, it is clear that for large-genome conifers, the detection of single transgene copy integrations by Southern hybridization is difficult when DNA is isolated from green needles. The alignment of PCR and Southern results is not stringent, and the reliance on PCR alone may lead to the false identification of putative transgenics. Charity *et al*. (2002) also showed that while their putative transgenics from cotyledon explants of *P. radiata* gave positive PCR results, they were unable to prove transformation by Southern analysis in any of their lines. Other investigations in pine species have overcome this limitation by analyzing the plant T-DNA junctions. Recent work suggests a number of common features in most cases of successful T-DNA integration. Usually one intact copy is integrated by illegitimate recombination. A single intact copy may be accompanied by a variable number of extra copies, which may be rearranged into head-to-head or head-to-tail orientation, incomplete copies, and/or truncated fragments (Grant *et al*. 2004). The extra T-DNA tends to be integrated into the same position as the first copy – a hotspot – and separated by genomic filler DNA (Kumar and Fldung 2001; Grant *et al*. 2004). In Southern hybridization results of *P. radiata* incomplete transfer leading to truncated copies of the T-DNA was common, and these arrangements and/or truncations are very similar in all cases (Grant *et al*. 2004). Further in *P. radiata*, only 4 of the 26 independently transformed plants by *Agrobacterium* method showed the expected integration of a complete copy of the T-DNA (Grant *et al*. 2004). The remaining 22 transformants appeared to have a truncated or rearranged copy of the T-DNA. It is possible that truncation/rearrangements are due to the CaMV 35S promoter (Grant *et al*. 2004). On the basis of *Agrobacterium* transformation study in *P. radiata*, it was also concluded that rearrangement most commonly occurs in the area of the *nos* terminator and/or the CaMV 35S promoter (Grant *et al*. 2004), and recombination events clustered within the CaMV 35S promoter and recombination occurred at a high frequency (Grant *et al*. 2004). Another study of *Agrobacterium*-mediated transformation of embryogenic cell cultures of *P. radiata* also revealed the expression of the *npt*II, *uid*A and *bar* genes in up to ten plants of each individual line (Charity *et al*. 2005). Molecular analysis revealed the expression of the *npt*II gene varied among the ten lines, while within ten replicates of the same line, *npt*II expression appeared to be consistent, with the exception of one line. Like-wise the level of GUS activity varied among transgenic lines, but was relatively consistent in plants derived from the same tissue (Charity *et al*. 2005). Southern hybridization analysis of embryogenic tissue and green needle tissue from putative transgenic lines of *P. radiata* demonstrated a relatively low number of gene insertions (from one to nine) of both the *bar* and *npt*II genes in the nine transgenic lines tested (Charity *et al*. 2005). Hence the relation between gene copy number and gene expression in most other plant species appears to be quite complex since transgene copy number can be positively (Klimaszewska *et al*. 2003) or negatively (Vaucheret *et al*. 1998) related to the activity of genes. However, as indicated in the literature (Hobbs *et al*. 1993; Dai *et al*. 2001), where the level of transgene expression does not always correlate with transgene copy number, it appears that there may be effects other than the co-ordinated expression of transgenes (Charity *et al*. 2005). The lack of a relationship between gene expression may be partially explained by the position within the genome into which the T-DNA has integrated, commonly called the position effect (Charity *et al*. 2005). Another factor determining expression in *P. radiata* could have been the activity of the promoter driving gene expression. Therefore, without a more thorough and comparative analysis between *P. radiata* plants derived from either biolistic-mediated or *Agrobacterium*-mediated transformation studies, it is very difficult to make more than general conclusions (Charity *et al*. 2005). A stable *Agrobacterium*-mediated transformation of embryogenic tissues from *P. pinaster* Portuguese genotypes also revealed at least one gene copy and at least two copies of the T-DNA inserted in different loci (Tereso *et al*. 2006). However, the needles of the regenerated somatic plantlets were GUS-negative and PCR-negative for genes *uid*A, *hpt* and *vir*BG. These results suggest that transformed embryogenic clones showing ability for plant regeneration were chimeras and plants were regenerated from non-transformed cells in *P. pinaster* (Tereso et al. 2006). Tang *et al*. (2007) reported the influence of okadaic acid and trifluoperazine on *Agrobacterium*-mediated transformation in eastern white pine (*P. strobus*). Stable integration of *uid*A gene in the plant genome of eastern white pine was confirmed by PCR/Southern/Northern analysis, and concluded that zygotic embryos are excellent targeting explants for transformation, provided that the *Agrobacterium* is delivered to their interior (Tang *et al*. 2007). *Agrobacterium*-mediated genetic transformation system was established for the first time using the embryogenic tissue derived from the cloning of mature trees of Himalayan blue pine (Malabadi and Nataraja 2007i). Molecular characterization of transgenic plants indicated the presence of a single copy number of the *npt*II gene in *P. wallichiana* (Himalayan blue pine) (Malabadi and Nataraja 2007i). On the other hand transgenic plants were produced in *P. kesiya* and *P. wallichiana* using mature zygotic embryos as the starting material for the induction of embryogenic tissue by biolistics also indicated the presence of a single copy of the *bar* gene in transgenic plants, and GUS by histochemical analysis (Malabadi and Nataraja 2007b, 2007c). In the case of *P. roxburghii*, transgenic plants were produced for the first time by biolistics using embryogenic tissue derived from cloning of mature trees (Malabadi and Nataraja 2007a). Transgenic plants also indicated the presence of a single copy of the *bar* transgene in *P. roxburghii* (Malabadi and Nataraja 2007a). However, the integration of one copy of a transgene does not ensure the same level of expression in different transgenics due to the different sites of integration into the plant genome, commonly called the position effect (Matzke *et al*. 1994: Klimaszewska *et al*. 2003; Tereso *et al*. 2006). Variability in transgene expression levels between individual transgenic plants or cell lines is a general phenomenon described in many plant transformation studies (Klimaszewska *et al*. 2003). In one of the studies reported by Klimaszewska *et al*. (2003), they were able to establish a strong positive correlation between the *gus* copy number and the level of GUS activity in a subset of translines of black spruce for which the copy number could be deter-

mined (between one and five). However, this correlation was much weaker when all the black spruce translines (including the ones with multiple inserts of unknown copy number) were analyzed (Klimaszewska *et al*. 2003). Therefore, it appears difficult to establish a true relation between transgene copy number and expression level in transformed plant material. Hence every clone should be tested for a transgene copy number before making any general conclusion. However, this appears to be more time-consuming, expensive, and found to be a tedious and laborious work.

Limitations

Until recently, one serious limitation to plant transformation technology was the inability to introduce large intact DNA constructs into the plant genome (Altpeter *et al*. 2005). Such large constructs could incorporate multiple transgenes or could comprise a segment of genomic DNA to facilitate the map-based cloning of plant genes. To facilitate map-based cloning efforts, a new generation of vectors (BIBAC, TAC and BAC) was constructed so that a large genomic fragment could be directly transformed into the plant genome via biolistic or *Agrobacterium*-mediated genetic transformation (Hamilton *et al*. 1996; Qu *et al*. 2003). Both BIBAC (binary bacterial artificial chromosome) and TAC (transformation-competent-artificial-chromosome) vectors can replicate in *E. coli* and *A. tumefaciens* and contain all the features that are theoretically required for transferring large DNA inserts into plant genomes, including the plant transformation markers *npt*II for resistance to kanamycin and *hpt* for resistance to hygromycin (Qu *et al*. 2003).

Cloning of exogenous DNA into bacterial artificial chromosomes (BACs) provides a new approach to the analysis of the genomes of higher organisms. BAC libraries containing large genomic DNA inserts are important tools for positional cloning, physical mapping and genome sequencing (Qu *et al*. 2003). **Table 2** shows a comparison between the two most common large DNA fragment cloning systems, YACs and BACs. A number of plant BAC libraries have been constructed e.g., *Arabidopsis*, rice, and sorghum (Woo *et al*. 1994; Choi *et al*. 1995; Zhang *et al*. 1996). Bacterial artificial chromosome vectors utilize the *E. coli* single-copy fertility plasmid and can maintain genomic DNA fragments up to 350 kb. Very little or no rearrangement of the inserts or chimerism have been observed (Choi *et al*. 1995; Zhang *et al*. 1996). Other systems for the cloning of large DNA fragments have been developed. The development of yeast artificial chromosome vectors (YAC) permits cloning of fragments of greater than 500 kb (Burke *et al*. 1987). However, some disadvantages of the YAC system include a high degree of chimerism and insert rearrangement whose limit is its usefulness (Burke 1990). Systems based on the bacterial F-factor (BAC) and bacteriophage P1 (PAC) have much higher cloning efficiencies, improved fidelity, and greater ease of handling as compared with the YAC system (Pierce *et al*. 1992). Due to BAC clone stability and ease of use, the BAC cloning system has emerged as the system of choice for the construction of large insert genomic DNA libraries (Burke *et al*. 1987; Pierce *et al*. 1992).

In *Agrobacterium*-mediated transformation, this limitation has been addressed by the development of BIBAC and TAC vectors (Shibata and Liu 2000). The transfer of YAC (Yeast artificial chromosome) DNA by particle bombardment was also done, but only one cultivar of tomato yielded YAC transformants. YAC was also found to be hygromycin resistant (Altpeter *et al*. 2005). Particle bombardment is therefore, a relatively efficient procedure for generating high-molecular weight-DNA transformants, although it would be useful to compare data from a large number of species. Biolistic transformations, in contrast to *Agrobacterium* (Malabadi and Nataraja 2003, 2006a; 2007d, 2007e), have resulted in fragmented or multicopy integration events of the transgene (Walter *et al*. 1998; Nigro *et al*. 2004), which may lead to transgene silencing (Kumpatla *et al*. 1997).

In the case of conifers, pine has a genome of about 48 pg/2C (24,000 Mb/1C) that is extremely rich in repetitive sequences. The pine needles are highly waxed and rich in polyphenolic substances. It is of significance to demonstrate the feasibility of isolating megabase DNA from these species and constructing a large-insert DNA BAC library for molecular analysis of pine genomes. DNA digestion experiments and pulsed-field gel electrophoresis indicated that the majority of the DNA was larger than 1 Mb, the DNA was readily digestable, and therefore, was amenable to BAC cloning and genome analysis (Peterson *et al*. 2006). From the megabase DNA, Peterson *et al*. (2006) successfully constructed a partial BAC library for loblolly pine. The insert sizes of the BACs ranged from 45 to 210 kb, with an average insert size of about 120 kb. BAC fingerprint analysis showed that the BACs were stable in host cells for over 150 generations. A high degree of variability in restriction patterns was also observed and indicated that pine.

BACs are amenable to fingerprinting. Further the characterization of the pine genome *via* Cot analysis, sequencing of random genomic and Cot-filtered sequences, and study of the organization of repetitive and low-copy sequences by fluorescence *in situ* hybridization (FISH) and macroarray analysis. Collectively, the data generated in this study will provide considerable insight into the structure of the pine genome which in turn will help guide efficient physical mapping and sequencing of pine. To date, high-throughput archival of BAC clones is well underway, Cot analysis has been completed, and Cot-filtered libraries are under construction (Peterson *et al*. 2006). The 454 sequence data will be compared with genomic and Cot-filtered sequences elucidated using standard capillary sequencing techniques. The >60 Mb of sequence data produced to date and the growing BAC library have set the stage for the FISH- and macroarray-based physical mapping of the pine genome (Peterson *et al*. 2006). Additional information on this project is available at www.mgel.msstate.edu.

Therefore, in the near future these vectors will be used for particle bombardement studies in pines. Currently BAC libraries have become invaluable tools in plant genetic research (Peterson *et al*. 2006). However, it is difficult for new practitioners to create plant BAC libraries *de novo* because published protocols are not particularly detailed, and plant cells possess features that make isolation of clean, high molecular weight DNA troublesome. This is the main disadvantage and therefore, it is very difficult to apply in many conifers. Hence, by reducing the obstacles to BAC cloning in plants, new and accelerated progress in plant genomics will be foster. This approach might be very helpful for inserting economically important genes into pine genome via biolistics. This will also improve the genetic trans-

Table 2 Comparison between YAC and BAC cloning systems.

Features	YAC	BAC
Configuration	Linear	Circular
Host	Yeast	Bacteria
Copy number/cell	1	1-2
Cloning capacity	Unlimited	Up to 350 kb
Transformation	Spheroplast (10^7 T/ug)	Electroporation (10^{10} T/ug)
Chimerism	Up to 40%	None to low
DNA isolation	Pulsed-field-gel-electrophoresis-Gel Isolation	Standard plasmid miniprep
Insert stability	Unstable	Stable

formation studies in conifers, particularly in recalcitrant pine species, which has a significant impact on the commercial forestry.

In a research funded by the U.S. Department of Agriculture (USDA) Plant Genome National Research Initiative, a new binary-BAC vector was constructed at Cornell by a team lead by Dr. Carol Hamilton (Hamilton *et al.* 1996; Hamilton 1997; Hamilton *et al.* 1999). Evaluation of the new vector BIBAC demonstrated that the BIBAC was capable of transferring at least 150 kb of DNA, intact, to the plant nuclear genome. The vector was also designed to be suitable for the construction of high molecular genomic DNA libraries so that additional sub-cloning steps would not be needed. To demonstrate that the BIBAC vector is suitable for constructing large insert genomic DNA libraries, they also set out to make high molecular DNA libraries for two different tomato species, *Lycopersicon esculentum*, domesticated tomato, and *L. pennellii*, a wild species of tomato (Hamilton *et al.* 1999). The average insert sizes for these libraries were 125 and 90 kb respectively. The advent of BIBAC technology has made it possible to consider new approaches to long-standing problems in basic plant biology and will facilitate the development of new elite varieties of agronomic crops (Hamilton *et al.* 1999; Qu *et al.* 2003). BIBAC technology will accelerate the identification of agriculturally important genes and make it possible to introduce valuable traits into plants without dragging along deleterious traits (a common problem for classical plant breeders). The ability to introduce high molecular weight DNA, intact into plant chromosomes will also make it possible to investigate long distance affects on gene expression (Qu *et al.* 2003).

The BIBAC system for transfer of high molecular weight DNA to plants would not have been successful without enhancing the ability of *A. tumefaciens* to effect DNA transfer to the plant chromosomes (Hamilton *et al.* 1996; Qu *et al.* 2003). Therefore, this work included basic research that has affected the plant transformation community. At presently many workers throughout the world are interested to use the virulence helper plasmids that made BIBAC technology a success, not because they needed BIBAC technology *per se*, but because they are interested in improving the transformation efficiency for their plant system of interest. In general this is a more common problem for conifers than for model system plants used for basic research (Qu *et al.* 2003; Peterson *et al.* 2006). The critical elements of this new technology, the bacterial strains and plasmids, are not yet available for the public because of patenting. A US patent has been issued for the BIBAC vector and foreign patents are pending (Peterson *et al.* 2006). The Center for Advanced Technology/Biotechnology at Cornell University, USA supported the construction and maintains a "BIBAC website" in support of BIBAC technology.

AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION

The first evidence indicating that *Agrobacterium* as the causative agent of crown gall goes back to more than 99 years (Smith and Towsend 1907). Plant transformation method by *A. tumefaciens*, soil pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Birch 1997; de Buck et al. 2000; Tzfira and Citovsky 2002; Dodueva *et al.* 2007). *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed causing the crown gall disease (Birch 1997; de Buck *et al.* 1999, 2000; Dodueva *et al.* 2007). Ti-plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti-plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The virulence region (*vir*) is a regulon organized in six operons that are essential for the T-DNA transfer (*vir*A, *vir*B, *vir*D and *vir*E) (Tzfira and Citovsky 2002). The process of gene transfer from *A. tumefaciens* to plant cells implies several essential stpes. 1). Bacterial colonization. 2) Induction of bacterial virulence. 3) Generation of T-DNA transfer-complex. 4) T-DNA transfer and 5) integration of T-DNA into plant genome (Hansen *et al.* 1994; Tinland 1996). The mechanism involved in the T-DNA integration has been fully characterized (Gelvin 2000). It is considered that the integration occurs by illegitimate recombination (Tinland *et al.* 1995; Tinland 1996).

Transient and stable expression

Both reporter and important trait gene have been tested in conifers by *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated gene transfer has been applied to many coniferous species (**Table 3**): *A. nordmanniana* (Clapham and Ekberg 1986), *A. procera* (Morris *et al.* 1989), *L. deciduas* (Huang *et al.* 1991), *L. deciduas* (Shin *et al.* 1994; Diner and Karnosk 1987), *L. kaempferi* × *L. deciduas* (Levee *et al.* 1997), *L. laricina* (McAfee 1993), *L. decurrens* (Stomp *et al.* 1990), *P. abies* (Wenck *et al.* 1999; Klimaszewska *et al.* 2001), *P. engelmanni* (Ellis *et al.* 1989), *P. glauca* (Ellis *et al.* 1989; Le *et al.* 2001; Klimaszewska *et al.* 2001), *P. mariana* (Klimaszewska *et al.* 2001), *P. sitchensis* (Ellis *et al.* 1989), *P. banksiana* (McAfee *et al.* 1993), *P. contorta* (Lindroth *et al.* 1999), *P. eldarica* (Stomp *et al.* 1990), *P. elliottii* (Stomp *et al.* 1990), *P. halepensis* (Tzfira *et al.* 1996), *P. jeffreyi* (Stomp *et al.* 1990), *P. lambertiana* (Loopstra *et al.* 1990; Stomp *et al.* 1990), *P. monticola* (McAfee *et al.* 1993), *P. palustris* (Diner 1999), *P. pinea* (Humara *et al.* 1999), *P. ponderosa* (Morris *et al.* 1989), *P. radiata* (Stomp *et al.* 1990; Bergman and Stomp 1992; Cerda *et al.* 2002; Charity *et al.* 2002), *P. taeda* (Sederoff 1986; Stomp *et al.* 1990; Wenck *et al.* 1999; Tang *et al.* 2001a; Gould *et al.* 2002), *P. virginiana* (Stomp *et al.* 1990), *P. sylvestris* (Stomp *et al.* 1990), *P. menziesii* (Ellis *et al.* 1989), *P. menziesii* (Morris *et al.* 1989; Stomp *et al.* 1990), *P. maximartinezii* and *P. pinceana* (Villalobos-Amador *et al.* 2002), *T. heterophylla* (Stomp *et al.* 1990), *P. pinaster* (Trontin *et al.* 2007; Milhinhos *et al.* 2007), *P. strobus* (Tang *et al.* 2007), and *P. wallichiana* (Himalayan blue pine) (Malabadi and Nataraja 2007i).

A. tumefaciens is more than the causative agent of crown gall disease affecting many plant species including conifers. It is also the natural instance for the introduction of foreign genes in plants allowing its genetic manipulation. Although, the gene transfer mechanisms remain largely unknown, great progress has been obtained in the implementation of transformation protocols for many plant species including conifers. Particularly important is the extension of this single-cell transformation methodology to recalcitrant pines. This advance has biological and practical implications. This confirmation implies that any plant can potentially be transformed by this method if a suitable transformation protocol is established. Genetic transformation of woody plants is a promising tool for plant functional genomics and for their genetic improvement, since their breeding has limitations imposed in general by their heterozygosity, long juvenile periods, and autoincompatibility (Birch 1997; Tang *et al.* 2007). The *Agrobacterium*-mediated transformation protocols in conifers differ from one plant species to other and, within species, from one cultivar to other. As a consequence, the optimization of *Agrobacterium*-mediated transformation methodologies requires the consideration of several factors that can be determined in the successful transformation of one species. Firstly, the optimization of *Agrobacterium*-plant interaction on competent cells from different regenerable tissues. Secondly, the development of a suitable tissue culture method for regeneration from trans-

Table 3 *Agrobacterium*-mediated genetic transformation in conifers.

Species	*Agrobacterium* strain	Plasmid vectors	Gene expression	References
P. wallichiana	(A. t.) EHA105	pBi121	stable expression	Malabadi and Nataraja 2007i
P. pinaster	(A. t.) C58/pMP90	pCambia1301	stable expression	Trontin *et al.* 2007
P. pinaster	(A. t.) C58/pMP90	pPCV6NFGUS	stable expression	Milhinhos *et al.* 2007
P. strobus	(A. t.) GV3101	pCAMBIA1301	Stable expression	Tang *et al*. 2007
P. pinaster	(A. t.) C58/pMP90	pPCV6NFGUS	stable expression	Tereso *et al.* 2006
P. radiata	(A. t.) EHA105	pTOK47	stable expression	Charity *et al.* 2005
P. radiata	(A. t.) AGL1, LBA4404, EHA101	pGA643	stable expression	Grant *et al.* 2004
P. mariana	(A. t.) C58/pMP90	pBIV	stable expression	Klimaszewska *et al.* 2001, 2003
P. glauca	(A. t.) C58/pMP90	pBIV	stable expression	Klimaszewska *et al.* 2001, 2003
	(A.t.) EHA105, LBA4404, GV3101	pBi121	stable expression	Le *et al.* 2001
P. abies	(A. t.) C58/pMP90	pBIV	stable expression	Klimaszewska *et al.* 2003, 2001
P. taeda	(A. t.) EHA101, 105	pGUS3, pSSLa.3	stable expression	Gould *et al.* 2002
P. radiata	(A.t.) LBA4404	pBi121	stable expression	Cerda *et al.* 2002
P. radiata	(A. t.) AGL1	pSKY1	stable expression	Charity *et al.* 2002
P. pinceana	(A. r.) A4	pRiA4	stable expression	Villalobos-Amador *et al.* 2002
P. maximartinezii	(A. r.) A4	pRiA4	stable expression	Villalobos-Amador *et al.* 2002
P. taeda	(A. t.) LBA4404	pBi121	stable expression	Tang 2000
P. taeda	(A. t.) GV3101	pCV6NFHygGUSINT	stable expression	Tang *et al*. 2001
P. taeda	(A. t.) EHA105, LBA4404, GV3101	pWWS006	stable expression	Wenck *et al.* 1999
P.contorta	(A. r) LBA9402	pHRGPnt3-GUS	stable expression	Lindroth *et al.* 1999
P. pinea	(A. t.) EHA105, GV3850, LBA4404, C58	P35SGUSint	transient expression	Humara *et al.* 1999
P. abies	(A. t.) EHA105, LBA4404, GV3101	pWWS006	stable expression	Wenck *et al.* 1999
P. palustris	(A. r.) A4; (A. t) 208	pRiA4, wild Ti	stable expression	Diner 1999
L. kaempferi × *L. deciduas* (hybrid larch)	(A. t.) C58/pMp90/pMrKE70Km	P35S-*npt*II-p19S-*npt*II	stable expression	Levee *et al.* 1997
P. halepensis	(A. r.) LBA9402	P35SGUSINT	stable expression	Tzfira *et al*. 1996
L. decidua	(A. r.) 11325	pCGN1133/pWB139	stable expression	Shin *et al.* 1994
L. laricina	(A. r.) A4/R100	A4/pRiA4b	stable expression	McAfee *et al*. 1993
P. banksiana	(A. r.) A4/R100	A4/pRiA4b	stable expression	McAfee *et al*. 1993
P. monticola	(A. r.) A4/R100	A4/pRiA4b	stable expression	McAfee *et al*. 1993
L. decidua	(A. r.) pRi11325	pRi11325	stable expression	Huang *et al.* 1991
P. radiata	(A. t.) C2/74/542	C2/74/542	Gall formation	Bergman and Stomp 1992
Tsuga heterophylla	(A. t.) A136 (pTiEu6)/K27/B3.73/K41	A136 (pTiEu6)/K27/B3.73/K41	Gall formation	Stomp *et al.* 1990
P. menziesii	(A. t.) A281/542	A281/542	Gall formation	Stomp *et al.* 1990
P. virginiana	(A. t.) 542/M2/73/U3	542/M2/73/U3	Gall formation	Stomp *et al.* 1990
P. sylvestris	(A. t.) 542/M2/73/U3	542/M2/73/U3	Gall formation	Stomp *et al.* 1990
P. radiata	(A. t.) A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp *et al.* 1990
P. taeda	(A. t.) A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp et al. 1990
P. elliotti	(A. t.) A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp *et al.* 1990
P. eldarica	(A. t.) U3	U3	Gall formation	Stomp *et al.* 1990
P. jeffreyi	(A. t.) C58	C58	Gall formation	Stomp *et al.* 1990
P. lambertiana	(A. t.) A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp *et al.* 1990
Libocedrus decurrens	(A. t.) C58/M2/73	C58/M2/73	Gall formation	Stomp *et al.* 1990
P. lambertiana	(A. t.) pTiBo542/pEND4	pTiBo542/pEND4	Stable expression	Loopstra *et al.* 1990
A. procera	(A. t.) pTiEU6/K27/B3.73/K41	A136 pTiEU6/K27/B3.73/K41	Gall formation	Morris *et al.* 1989
P. engelmanni	(A. t.) A281/W2/73	pEND4K/pLUX2	Gall formation	Ellis *et al.* 1989
P. glauca	(A. t.) A281/W2/73	pEND4K/pLUX2	Gall formation	Ellis *et al.* 1989
P. sitchensis	(A. t.) A281/W2/73	pEND4K/pLUX2	Gall formation	Ellis *et al.* 1989
P. ponderosa	(A. t.) A136 (pTiEU6)/K27/B3.73/K41	A136 (pTiEu6)/K27/B3.73/K41	Gall formation	Morris *et al.* 1989
P. menziesii	(A. t.) A281/542	A281/542	Gall formation	Morris *et al.* 1989
Pseudotsuga menziesii	(A. t.) A281/W2/73	pEDD4K/pLUX2	Gall formation	Ellis *et al.* 1989
L.decidua	(A. r.) ATCC11325	ATCC11325	Tumor formation	Diner and Karnosky 1987
P. taeda	(A. t.) M2/73/U3	M2/73/U3	Gall formation	Sederoff *et al.* 1986
Abies nordmanniana	(A. t.) C58/pV3304/pV3851/pV2298	C58/pV3304/pV3851/pV2298	Tumor formation	Clapham and Ekberg 1986

A. t. = *Agrobacterium tumefaciens*; A. r. = *Agrobacterium rhizogenes*

formed cells. It has been also reported that the time of pre-culture, inoculation time and length of co-cultivation, acetosyringone, explant size and *Agrobacterium* strain influence transformation efficiency (Birch 1997; Malabadi and Nataraja 2007i; Tang *et al*. 2007). Another important fact is that the availability of suitable genetic materials (bacterial strains, binary vectors, reporter and marker genes, promoters) and molecular biology techniques available in the laboratory are necessary for selection of the DNA to be introduced. This DNA must be able to be expressed in plants making possible the identification of transformed plants in selection medium and using molecular biology techniques to test and characterize the transformation events (Birch 1997).

Successful stable genetic transformation using *A. tumefaciens* strain GV3101 carrying a binary vector pCAMBIA 1301 with mature zygotic embryos as explants was reported in a recalcitrant Christmas tree species of eastern white pine (*P. strobus* L.) (Tang *et al*. 2007). In this study, factors influencing transient GUS expression and hygromycin-resistant callus in eastern white pine were investigated. A 2.0 to 3.5-fold higher rate of hygromycin-resistant callus was obtained with an addition of 2 µM okadaic acid (protein phosphatase inhibitor) or 150 µM trifluoperazine (kinase inhibitor) or sonicated embryos for 45-50s. Their results suggest

that mature zygotic embryos are excellent targeting explants for transformation, and time of sonication was crucial for the effective transformation in eastern white pine (Tang *et al*. 2007). It is also clear that conditions favoring T-DNA delivery are not necessarily the same as those favoring the recovery of stable transformation events. They also found that sonication influenced the efficient delivery of T-DNA into the embryos during transformation. However, sonicating embryos for more than 45-50 s decreased both transient GUS expression and hygromycin-resistant callus formation in *P. strobus* (Tang *et al*. 2007). A combination of 2 µM okadaic acid or 150 µM trifluoperazine and sonicated embryos for 45 s did not significantly increase transient GUS expression and hygromycin-resistant callus formation in eastern white pine, compared to the single application of each because the combination increased the frequency of embryo death (Tang *et al*. 2007). However, Tang *et al*. (2007) also mentioned that how okadaic acid and trifluoperazine increase transient GUS expression and hygromycin-resistant callus formation in eastern white pine is not clear. They speculate that okadaic acid may increase transformation efficiency by inhibiting the activities of protein phosphatases and trifluoperazine may increase transformation efficiency by inhibiting the calmodulin-dependent stimulation of 3′:5′-cyclic nucleotide phosphodiesterase. Protein phosphatases and 3′:5′-cyclic nucleotide phosphodiesterase may play a role in cell defense against *Agrobacterium* infection. A decrease in their activities may facilitate gene transfer from *Agrobacterium* cells to plant cells (Tang *et al*. 2007).

In the case of Himalayan blue pine (*P. wallichiana*), transgenic plants were produced for the first time using the embryogenic tissue derived from the vegetative shoot apices of mature trees (Malabadi and Nataraja 2007i). Genetic transformation was achieved using *Agrobacterium* strain EHA105 fused with a binary vector pBI121 containing *npt*II as a selectable marker gene and *uid*A as a reporter gene, respectively. During the transformation study, an infection period of 5 h was found to be optimum for the embryogenic lines tested (Malabadi and Nataraja 2007i). This was also observed in other conifers such as *P. glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al*. 2001). The optimization of *A. tumefaciens*-plant interaction is probably the most important aspect to be considered. It includes the integrity of bacterial strain, its correct manipulation and the study of reaction in wounded plant tissue, which may develop a necrotic process in the wounded tissue or affect the interaction and release of inducers or repressors of *Agrobacterium* virulence system. The addition of 100 µM acetosyringone to the bacterial suspensions just before infection increased (57% in PW39 line, 36% in PW120 line and 14% in PW and PW10 lines) the transformation efficiency in *P. wallichiana* (Malabadi and Nataraja 2007i). Lower (25-80 µM) or higher concentrations of acetosyringone (more than 100-150 µM) severely negatively affected the transformation efficiency. Therefore in our study 100 µM acetosyringone was optimum for the transformation and significantly increased the transformation rate when compared to the control (Malabadi and Nataraja 2007i). Further, 470 mgl^{-1} of cefotaxime was crucial for the recovery of embryogenic tissue after co-cultivation, and 35 mgl^{-1} of kanamycin was optimum for the selection for all the lines tested. Among the 3 lines tested, the highest transformation efficiency was recorded in PW39 line (57 lines/g fresh wt) than with the embryogenic lines of PW10 and PW120 in *P. wallichiana* (Malabadi and Nataraja 2007i).

The method of transformation in *P. wallichiana* had no effect on the integration pattern of T-DNAs (Malabadi and Nataraja 2007i). A total number of 9 somatic seedlings recovered per gram fresh wt of a transgenic tissue in a PW10 line. The highest percentage of somatic embryogenesis (16%) was recorded in PW120 line with a total number of 12 somatic seedlings recovered per gram fresh wt of transgenic tissue. The integration of the transgenes was confirmed by PCR/Southern/Northern analyses (Malabadi and Nataraja 2007i). In this protocol the efficiency of transformation was high mainly due to the higher infection rate of *Agrobacterium* strain with actively dividing meristematic cells undergoing cleavage polyembryony. The callus growth was very good and cells have prominent nucleolus and cytoplasm. In another study conducted by Klimaszewska *et al*. 2001, where the source of the cells for the transformation experiments in *P. glauca*, *P. mariana* and *P. abies* was from vigorously growing embryogenic cultures derived from mature zygotic embryos, collected from filter paper, and characterized by the ability to produce mature somatic embryos and plants. Therefore, the viability of cultures, bacterial strains and cocultivation conditions were all important in making the cells accessible to the *Agrobacterium* binding, T-DNA transfer, and integration into the plant cell genome (Wenck *et al*. 1999; Lelu-Walter and Pilate 2000). On the basis of this study, we also concluded that the starting explant material might also play an important role in genetic transformation, and a good tissue culture protocol is very much needed for the successful regeneration of plantlets from transgenic tissue. The establishment of a method for the efficient regeneration of one particular species is crucial for its transformation. It is a general opinion that the growth of transgenic tissue is always affected by the tissue culture protocol, and aging of tissue during the whole process of transformation since the number of regeneration of transgenic plantlets was found to be very low compared with the control (non-transformed lines) in many plant species, including conifers (Klimaszewska *et al*. 2003; Tereso *et al*. 2006; Malabadi and Nataraja 2007i; Tang *et al*. 2007). In our study of *P. wallichina*, the tissue culture protocol used was very much reproducible, and it was tested for several growing seasons before applying the genetic transformation studies (Malabadi and Nataraja 2007i). Therefore, a good, reproducible tissue culture protocol certainly helps in establishing an efficient transformation method for the production of transgenic plants. This is the most important factor for solving the current problems of genetic transformation studies, particularly the regeneration of transgenic plants of woody plant species.

Milhinhos *et al*. (2007) reported the use of *Agrobacterium*-mediated transformation for studying nitrogen metabolism regulation by glutamine synthetase and its relation with carbon metabolism and plant development in *P. pinaster* (Maritime pine). They used cryopreserved embryogenic tissue as the starting explant for transformation studies. In this study, after infection with *Agrobacterium*, tissues were washed and subcultured on medium containing 400 mgl^{-1} timentin, which was critical for the recovery of transgenic tissue of *P. pinaster* (Milhinhos *et al*. 2007). Cerda *et al*. (2002) also noticed that 400 mg/l of carbenicillin was seen to have no effect on the growth of the embryogenic masses during *Agrobacterium* transformation of *P. radiata*. Additionally carbenicillin has no detectable effects on histochemical GUS assay in *P. radiata* (Cerda *et al*. 2002). The *npt*II gene, conferring kanamycin resistance, was used as selection marker. They successfully expressed the genes GS1a and GS1b from *P. sylvestris* in transformed *P. pinaster*, with 2000 mature somatic embryos obtained from the transformed tissues, about only 40% were able to elongate the hypocotyls resulting in plantlets (Milhinhos *et al*. 2007). On the other hand genetic transformation of maritime pine (*P. pinaster*) with the *bar* gene was also reported by using the *Agrobacterium* (Trontin *et al*. 2007). They also noticed the low maturation yields of transgenic tissue related with ageing lines during the whole maturation process in *P. pinaster* (Trontin *et al*. 2007).

In another *Agrobacterium* study of *P. pinaster*, the omission of casein hydrosylate from the culture medium during co-cultivation, and subsequent subculture was crucial to control the growth of *Agrobacterium* (Tereso *et al*. 2006). They used the embryogenic tissue derived from immature zygotic embryos as the starting material for the transformation studies of *P. pinaster*, and applied two different transformation protocols (Tereso *et al*. 2006). In many

conifers, an increase in transformation efficiency of embryogenic tissues by adding acetosyringone was reported, such as in *P. strobus* (100 µM; Leeve *et al.* 1999), *P. abies* and *P. taeda* (25-50 µM; Wenck *et al.* 1999), *P. glauca* (50 µM; Le *et al.* 2001) and *P. wallichiana* (100 µM; Malabadi and Nataraja 2007i). However, there was no effect of acetosyringone in a few conifers such as *L. kaempferi* × *L. deciduas* (Levee *et al.* 1997) and French genotypes of *P. pinaster* (Trontin *et al.* 2002). In one protocol, the bacterial drops were spread over embryogenic clumps whereas in the second protocol a mixture of bacterial and embryogenic cell suspensions was plated on filter paper during genetic transformation study of *P. pinaster* (Tereso *et al.* 2006). They eliminated the *Agrobacterium* by transferring the co-cultivated embryogenic tissue to semi-solid proliferation medium without casein hydrosylate supplemented with 400 mgl^{-1} timentin in *P. pinaster* (Tereso *et al.* 2007). The highest frequency of transformation (22 independent transformed lines/g fresh wt of embryogenic clone) was obtained with the second protocol in *P. pinaster* (Tereso *et al.* 2006). Maturation of 44 transformed lines gave rise to three mature somatic embryos, each one coming from a different transformed line in *P. pinaster* (Tereso *et al.* 2006).

In other studies of conifers, *Agrobacterium* was removed by subculturing the infected tissue on semi solid medium supplemented with higher concentrations (300-470 mgl^{-1}) of different antibiotics used for the particular genotype/or species of conifers (Malabadi and Nataraja 2007i). Wenck *et al.* (1999) reported the removal of *Agrobacterium* from the cultures by allowing suspensions to settle in 50 ml sterile tubes and pouring off medium, following another subculture on fresh medium containing 200 mgl^{-1} timentin in *P. abies* and *P. taeda*, respectively. Then this wash was repeated twice, following 4 ml of cells which were transferred to plates containing 400 mgl^{-1} timentin and 10 mgl^{-1} kanamycin (Wenck *et al.* 1999). In our own study of *Agrobacterium* transformation in *P. wallichiana*, the lower concentrations of cefotaxime (100-400 mgl^{-1} and kanamycin (5-30 mgl^{-1}) were not effective (Malabadi and Nataraja 2007i). Therefore, on the basis of a literature survey, the optimum concentration of cefotaxime (in our own study of *P. wallichina*) or other antibiotics such as chloramphenicol, streptomycin, ampicillin, carbenicillin, neomycin, and tetracyclin (350-470 mgl^{-1}) and kanamycin (25-35 mgl^{-1}) in many studies of conifers played an important role in the recovery of transgenic colonies (Klimaszewska *et al.* 2001; Le *et al.* 2001; Malabadi and Nataraja 2007i). Further these antibiotic concentrations and the type of antibiotic used in the transformation study, vary from species to species or from one genotype to genotype in plants including conifers.

An *A. tumefaciens*-mediated transformation protocol was developed for embryogenic cell cultures of *P. radiata* (Charity *et al.* 2005). In this study transgenic lines were only produced when embryogenic tissue was placed on nurse tissue during the *Agrobacterium* co-cultivation and recovery stages of the procedure in *P. radiata*. Nurse tissue may improved the health and recovery of *P. radiata* cell suspensions, perhaps by increasing aeration of cells by physical separation from the medium, and/or by providing essential nutrients or plant growth regulators for better recovery and subsequent growth (Charity *et al.* 2005). In general, exactly how nurse cultures increased the frequency of transformation is unclear since it may have been due to increased tissue health and recovery, and/or possibly the lack of *A. tumefaciens* overgrowth that subsequently led to successful recovery of transformed cells in *P. radiata* (Charity *et al.* 2005). Another reason might be due to secretion of phenols by nurse cultures that act as virulence inducers, promoting cell division in the recipient plant or even reducing the growth of *A. tumefaciens per se* in *P. radiata* (Charity *et al.* 2005). They also noticed that the inoculation of embryogenic tissue with *A. tumefaciens* usually resulted in some tissue browning and cell death, which led to poor recovery of transgenic tissue in *P. radiata* (Charity *et al.* 2005). However, the level of geneticin (15 mg/l) may have been too stringent to allow a good response and recovery of many transgenic lines in *P. radiata* (Charity *et al.* 2005). Therefore, the growth and recovery of cells prior to their transfer to selective media was found to be an important factor when evaluating the level of selective agent needed to maintain a healthy culture. This observation was also consistent with findings of Levee *et al.* (1999) with *P. strobus* and was also true for selection of biolistically-transformed *P. radiata* (Walter *et al.* 1998), and *P. abies* (Walter *et al.* 1999). As with other transformation protocols for conifers (Levee *et al.* 1997; Klimaszewska *et al.* 2001; Trontin *et al.* 2002), there are likely to be differences in the transformation rates for different *P. radiata* embryogenic cell genotypes, and early indications are that this is likely to be the case for the protocol presented in *P. radiata* (Charity *et al.* 2005). Plantlets were regenerated via somatic embryogenesis from 10 of the 11 transgenic lines tested and at least 20 of each line were planted in *P. radiata* (Charity *et al.* 2005). They also noticed that all the transgenic lines in *P. radiata* did not produce somatic embryos. One explanation is that throughout the entire transformation process, that tissue contact with *A. tumefaciens*, long-term maintenance on medium containing an antibiotic, the integration and expression of foreign genes, may all play a part in interfering with, or altering the plant regeneration protocol (Charity *et al.* 2005).

Another method for *A. tumefaciens*-mediated transformation of *P. radiata* cotyledon explants was developed using commercially available open-pollinated seed (Grant *et al.* 2004). The main drawback of this method was the small number of *P. radiata* genotypes that are amenable to transformation and regeneration. Critical factors for successful transformation were survival of the cotyledons after cocultivation and selection parameters in *P. radiata* (Grant et al. 2004). The low survival of cotyledons after cocultivation with *A. tumefaciens* suggested that the cotyledons might be hypersensitive to *Agrobacterium* or that there may be inhibitory compounds present in the pine explants. Aronen (1997) suggested that the inefficiency of transformation of conifer organogenic explants was due to interference by chemical defense compounds such as terpenoids and phenolic constituents, which inhibited later stages of T-DNA transformation. Similarly for stone pine (*P. pinea*), Humara *et al.* (1999) also experienced difficulty in maintaining the cotyledons after cocultivation. They also observed that infection, as measured by transient GUS expression, was high following cocultivation with *A. tumefaciens* but that subsequent survival of explants was low in *P. radiata* (Grant *et al.* 2004). Survival was low irrespective of whether the *Agrobacterium* strain contained a plasmid or not. Despite this low survival, infection with *A. tumefaciens* was high enough to produce stable transgenic plants in *P. radiata* (Grant *et al.* 2004). Another critical factor that relates to the efficiency of the selection protocol, transformants were obtained on medium containing geneticin (10 mg/l and 15 mg/l) kanamycin (10 mg/l) in *P. radiata* (Grant *et al.* 2004). Other reports for organogenic explants of *P. radiata* was by Charity *et al.* (2002) who recovered one shoot from the transformation of apical meristematic dome from embryos. Tang *et al.* (2001a, 2001b) used mature *P. taeda* embryos as explants to obtain transgenic plants, while Gould *et al.* (2002) used shoot apices from seedlings. Other reports have shown the stable integration of transferred genes but plants, were not recovered for *P. taeda* (Wenck *et al* 1999), *P. radiata* (Holland *et al.* 1997) and *P. pinea* (Humara *et al.* 1999). Le *et al.* (2001) reported that the physiological status of tissue was also considered to be an important factor for successful transformation of white spruce (*P. glauca*). They also noticed that rapidly dividing embryogenic suspension cultures, obtained 5 days after a 7 d subculture, provides suitable material to get a high frequency of transformation in white spruce, additionally, co-cultivation in liquid medium and addition of 50 µM acetosyringone were essential for successful transformation (Le *et al.* 2001).

The design of an adequate artificial environment to

favor the interaction of *A. tumefaciens* with plant tissues is another critical factor for the success of genetic transformation experiments. Oxidative burst, phenolization and the subsequent cell death have been described as frequent phenomena during the interaction of *A. tumefaciens* with plant cells, including those of conifers. It was also reported that coculture of explants with *A. tumefaciens* induces rapid necrosis in tissue (Grant *et al.* 2004). Another factor that influences transformation is the enrichment of the media with sugars and a low pH, However, in our own study of transformation with *P. wallichiana* these factors did not affected the rate of transformation (Malabadi and Nataraja 2007i). Good transformation efficiencies were obtained without infecting the explants in low-pH media. Therefore, we assume that osmotic conditions affect the process of recognition of plant cells by the *Agrobacterium* (Malabadi and Nataraja 2007i). These studies demonstrated that several factors are important for the application of *Agrobacterium*-mediated gene transfer in conifers and other plants. Among them is the rate of infection. Infectivity varies among different *Agrobacterium* strains and vectors (Hoekema *et al.* 1983; Hood *et al.* 1993). Infectivity varies for different conifer genera and genotypes (Loopstra *et al.* 1990; McAfee *et al.* 1993; Tang *et al.* 2001). Further infectivity also dependent upon the selection marker genes and selection agents and promoters used (Ellis *et al.* 1989; Bergmann and Stomp 1992; Wenck *et al.* 1999). Humara *et al.* (1999) reported the transfer and expression of foreign chimeric genes in cotyledons of *P. pinea*. In recent years this concept has changed drastically with more successes in many recalcitrant pine species, leading to the availability of a genetic transformation protocols for many plant species, including conifers.

Regeneration of transgenic plants

The first genetic transformation in conifers was reported by Huang *et al.* (1991), since then *Agrobacterium*-mediated gene transfer has been widely used in many plant species of conifers. Transgenic regenerated plantlets were obtained with *L. decidua*, *P. halepensis* and *P. menziesii* (Dandekar *et al.* 1987; Huang *et al.* 1991; Tzfira *et al.* 1996, 1998), and hybrid larch was transformed by *A. tumefaciens* (Levee *et al.* 1997). However, large-scale use of this *Agrobacterium* in conifer transformation has been limited due to difficult propagation of explant material, selection inefficiencies and low transformation frequency (Wenck *et al.* 1999; Tian *et al.* 2000; Trontin *et al.* 2002). In the case of loblolly pine transient expression was increased 10-fold by utilizing modified *Agrobacterium* strains (Wenck *et al.* 1999). It was also a useful technique for large-scale generation of transgenic Norway spruce. Both family and species types are important for the application of *Agrobacterium*-mediated gene transfer in conifers. Embryos of 24 open-pollinated families of loblolly pine (*P. taeda* L.) were used as explants to conduct *in vitro* regeneration (Tang *et al.* 2001). *A. tumefaciens* was used to transform mature zygotic embryos of seven families of loblolly pine. Ninety transgenic plants were regenerated, and 19 transgenic plantlets were established in soil (Tang 2001; Tang *et al.* 2001). In *Picea*, transgenic plants of three *Picea* species, i.e. *P. glauca*, *P. mariana* and *P. abies* were produced by this method of transformation. Transgenic plants were regenerated for all three *Picea* species (Klimaszewska *et al.* 2001). In *P. radiata*, Charity *et al.* (2002) demonstrated that an *Agrobacterium*-mediated transformation protocol could be developed for detached cotyledons of zygotic embryos. Transient expression of *uid*A was improved when detached cotyledons were pre-cultured on half strength medium containing cytokinin benzyl adenine (BA) (0.5 mg/l) for 7 days, wounded by vortexing and then vacuum-infiltrated in a solution of *A. tumefaciens* (Charity *et al.* 2002).

A method for *A. tumefaciens*-mediated transformation of *P. radiata* cotyledon explants was developed using commercially open-pollinated seed (Grant *et al.* 2004). Since more than 80% of genotypes of radiata pine can be regenerated using cotyledons of mature seed, cotyledon explants were co-cultivated with *A. tumefaciens*. Critical factors for successful transformation were survival of the cotyledons after co-cultivation and selection pressure. Of the 105 putative transformants, 70% were positive for integration of the *npt*II gene when analyzed by PCR. This is the first report of transgenic radiata pine plantlets from isolated cotyledons using *A. tumefaciens*-mediated transformation (Grant *et al.* 2004). An efficient and reproducible procedure for the transformation of white spruce (*P. glauca*) embryogenic tissue was developed using *A. tumefaciens* mediated gene transfer. Transgenic plants were regenerated from transformed tissues within 4 months after co-culture (Le *et al.* 2001). Stable transformation of *P. radiata* embryogenic tissue by *Agrobacterium* was also reported by Cerda *et al.* (2002). Further Charity *et al.* (2005) reported the consistent and stable expression of the *npt*II, *uid*A, and *bar* genes in transgenic *P. radiata* after *A. tumefaciens*-mediated transformation using nurse cultures. Tang *et al.* (2007) recently reported that okadaic acid and trifluoperazine enhance *Agrobacterium*-mediated transformation in eastern white pine (*P. strobus*). These results demonstrated that a stable and enhanced transformation system has been established in eastern white pine and this system would provide and opportunity to transform economically important genes into this Christmas tree species (Tang *et al.* 2007).

TRANSFORMATION USING ELECTROPORATION

Plant protoplasts are the important ideal tools for genetic manipulations such as gene transfer, mutation breeding and somatic hybridization (Malabadi 2003). Establishment of protoplast techniques for conifers is of crucial importance to forest biotechnology. Protoplasts can be used to regenerate whole trees and to study transcriptional regulation in woody plants by promoter analysis (Gomez-Maldonado *et al.* 2001). Protoplasts provide a powerful tool for evaluating marker gene expression in plants. Gene expression systems that allow direct comparision between different promoters in plants under transient conditions can be used for a wide range of applications in plant molecular biology (Gomez-Maldonado *et al.* 2001). This is also another method of direct gene transfer used for the genetic improvement, and for studies of gene structure and function in forest trees. Because of electroporation avoids the host-range limitation of *Agrobacterium*-mediated transfer methods, it has the further advantage of being useful for the rapid evaluation of the functionality of plasmid construction, and for assessing transient gene expression, and stable transformation. Electroporation has been utilized to transfer genes into protoplasts isolated from embryogenic cell cultures of *P. glauca* (Bekkaoui *et al.* 1988; Wilson *et al.* 1989), *P. mariana* (Tautorous *et al.* 1989), *P. taeda* (Gupta *et al.* 1988), and *Larix* × *eurolepis* (Charest *et al.* 1991), and from non-embryogenic cultures of *P. radiata* (Campbell *et al.* 1992), and *P. banksiana* (Tautorus *et al.* 1989). According Tautorus *et al.* (1989), transient expression of the chloramphenicol acetyltransferase gene in electroporated *P. mariana* and *P. banksiana* protoplasts was affected by the cell lines used, by voltage, temperature, and by the plasmid concentration and conformation. The commonly used reporter genes, such as firefly luciferase gene, GUS gene can be used to assess gene activity in conifer protoplasts. Bekkaoui *et al.* (1990) reported that the level of gene activity in electroporated *P. glauca*, *P. mariana* and *P. banksiana* protoplasts is dependent on the promoter transferred, electroporation conditions, as well as on the target cell line under investigation. Gupta *et al.* (1988) reported that protoplast viability was reduced from 90% to 45-55% after electroporation. Gene expression was improved by the addition of PEG to the electroporation mixture. Because of the difficulty in plant regeneration from conifer protoplasts, transformation using electroporation is mainly used to study their transient expression of genes and factors influencing transgene expression (Tang and Newton 2003).

APPLICATIONS OF GENE TRANSFER IN COMMERCIAL FORESTRY

There are many applications of gene transfer technology in commercial forestry programmes which can solve the current problems of breeding, as follows.

Bioremediation

Phytoremediation is a process where interactions between vegetation, associated microorganisms, and the host substrate combine to effectively degrade contaminated soils, sediments, and groundwater (Rugh 2001; Pilon-Smits *et al.* 2002; Suresh and Ravishankar 2004; Eapen and D'Souza 2005; Doty *et al.* 2007). Phytoremediation is a rapidly developing technology that shows promise for the effective and safe cleanup of certain hazardous wastes (Drake *et al.* 2002; Bennett *et al.* 2003; Tong *et al.* 2004; Peuke and Rennenberg 2005; Cherian and Oliveira 2005; Kramer 2005; Aggrawal and Goyal 2007). It has the potential to remediate numerous volatile organic compounds (VOCs). Extensive characterization work has demonstrated that two VOCs, tetra chloroethylene (PCE) and trichloroethylene (TCE) are the major components of the VOC-contaminated groundwater. The PCE and TCE are chlorinated ethane's (CE), and have been detected in soils and ground water adjacent to the ecologically-sensitive area. To determine how native and introduced plants and microorganisms might remove and/or degrade PCE and TCE in the existing groundwater plume, an experimental treatability study was conducted. The analytical results obtained from this phytoremediation project also demonstrated that loblolly pines and hybrid poplars (*Trichocarpa* X *deltoides*) removed up to 90% and 100%, respectively, of total VOCs detected in the source groundwater (Macek *et al.* 2000).

The use of transgenic plants to remove contaminants from soil and water promises to have a positive impact on environmental pollution and, in the long-tern, the preservation of natural forests (Herschbach and Kopriva 2002), termed phytoremediation. Over-expression of the bacterial mercuric reductase gene in yellow poplar (*Populus tremula* × *P. alba*) resulted in transgenic plants that were resistant to toxic levels of mercuric ions and were able to release elemental mercury (Rugh *et al.* 1998). In the case of Loblolly pine transformation with *A. tumefaciens* expression vector harboring the rabbit cytochrome gene for detoxification of soils contaminated with ethylenedibromide (Tang and Newton 2003; M. P. Gordon, pers. comm.). More recently poplars with enhanced GSH synthesis and hence elevated capacity for phytochelatin production are compared with wild type plants for the removal of heavy metals at different levels of contamination and under different climatic conditions (Peuke and Rennenberg 2005). These studies indicated that the expression of the GSH gene (glutamylcysteine synthetase) in poplar leads to 2-to 4-fold enhanced GSH concentrations in leaves. In greenhouse experiments under controlled conditions these transgenic poplars showed a high potential for uptake and detoxification of heavy metals and pesticides. The results of this study will help to assess the biosafety risk of the use of transgenic poplar for phytoremediation of soils (Peuke and Rennenberg 2005).

Small, volatile hydrocarbons, including trichloroethylene, vinyl chloride, carbon tetrachloride, benzene and chloroform, are common environmental pollutants that pose serious health effects. Very recently, Doty *et al.* (2007) developed transgenic poplar (*Populus tremula* × *Populus alba*) plants with greatly increased rates of metabolism and removal of these pollutants through the overexpression of cytochrome P450 2E1, a key enzyme in the metabolism of a variety of halogenated compounds. The transgenic poplar plants exhibited in increased removal rates of these pollutants from hydroponic solution. When the plants were exposed to gaseous trichloroethylene, chloroform, and benzene, they also demonstrated superior removal of the pollutants from the air. In view of their large size and extensive root systems, these transgenic poplars may provide the means to effectively remediate sites contaminated with a variety of pollutants at much faster and at lower costs that can be achieved with current conventional techniques (Doty *et al.* 2007).

Wood modification

Modification of wood by altering its chemical structure is a way of its improving its properties, because wood is renewable energy source and industrial material (Chiang 2002; Doblin *et al.* 2002). Wood properties influence pulp and paper quality. Certainly, overall pulp yields are directly related to the cellulose content, changes in hemicellulose content are associated with changes in pulp cohesiveness. Pulping efficiency is related to lignin content. Despite the importance of wood properties on product quality, little progress has been made in improving such traits because earlier methods of assessing wood and fiber characteristics are time-consuming, expensive, and often imprecise. Modern molecular genetics technologies have created new opportunities for wood modification (Li *et al.* 2003). Lignin is the second most abundant organic compound on earth, and represents about 25% of the global wood biomass (Leple *et al.* 1992). Although lignin is an important compound for wood development, it is an obstacle to efficient pulp and paper production because the lignin must be removed in order to extract the cellulose from the wood (Pilate *et al.* 2002). This process is energy consuming and requires the use of polluting chemicals. It is of great interest to try and engineer trees to have a lower lignin component or a lignin that is easily extracted without reducing tree growth rates or bole form (Pilate *et al.* 2002). Other than genetic modification, another method of removing lignin in pulp and paper industries is the treatment of wood pieces with cellulase-free xylanases before processing (Malabadi *et al.* 2007). Cellulase-free xylanases are being used in the biobleaching of paper, which reduces environmentally and unfriendly chlorine consumption (Malabadi *et al.* 2007). Field and pulping performances of transgenic trees with altered lignification have been reported recently (Pilate *et al.* 2002). Conifers could be engineered with lower lignin content, or more syringyl lignin instead of the guaiacyl lignin, although there is no report in this area right now (Herschbach and Kopriva 2002). An increase in syringyl/guaiacyl ratio also accelerated cell maturation in stem secondary xylem in transgenic aspen (Li *et al.* 2003) and their method of co-transfer system should be broadly useful for plant genetic engineering and functional genomics (Li *et al.* 2003). Research over the last decade has mainly focused on understanding the monolignol biosynthesis pathway, paving the way to engineer wood quality for pulping. These studies have shown that lignin polymer is malleable in its composition and structure, that it incorporates many more than the classical three monolignols, and that it readily incorporates products from incomplete monolignol biosynthesis. This malleability opens perspectives to design entirely novel lignins to improve lignin removal for example conversion of plant cell walls to bioethanol (Boerjan 2007). Molecular insight into these pleiotrophic effects is essential if we are to taylor cell walls for end use applications.

In trees the GUS reporter gene has been commonly used for the optimization of transformation protocols, but also in conjunction with candidate genes for functional characterization. More recently GUS has been used in the development and application of Induced Somatic Sector Analysis (ISSA) protocols for the characterization of gene function during wood formation in poplar (Tibbits *et al.* 2007). Their study also showed that ISSA presented GUS had no effect on some cell wall traits, altered cell wall fluorescence in lignified GUS transgenic cells was observed as compared to adjacent non-transgenic cells indicating a change in cell wall chemistry in poplar tree stems (Tibbits *et al.* 2007). The change in lignin composition may have been caused by differential de-glycosylation of the monolignol 4-*O*-gluco-

sides by the GUS protein in poplar.

Insect and herbicide resistance

Defoliation of or damage to plantation trees by insect pests is a recurring problem in North America, Europe, Canada, Australia, India, South Africa and Chile. For example, the bark beetles and white pine weevil represent a major forest health threat. The current mountain pine beetle (MPB; *Dendroctonus ponderosae*) epidemic in Canada is the largest outbreak of a bark beetle in recorded history affecting more than 14 million hectares of lodgepole pine (*Pinus contorta*) in British Columbia (Kolosova *et al*. 2007). Another bark beetle of substantial economic impact in North America is the spruce beetle (*Dendroctonus rufipennis*). Bark beetles vector fungal pathogens, which are involved in killing of host trees. *Ophiostoma clavigerum* is a blue-stain fungal pathogen associated with MPB, while *Leptographium abietinum* is a tree-killing pathogen vectored by spruce beetles (Kolosova *et al*. 2007). In addition to bark beetles, the white pine weevil (*Pissodes strobi*) is one of the most serious insect pests of Sitka spruce and Norway spruce in North America (Kolosova *et al*. 2007). Europian shoot-tip moth (*Rhyaciona buoliana*), a pest endemic to Chilean forests, is responsible for damage to approximately 30% of radiata pine harvest when left untreated and costs US$ 3 million annually to control through the release of wasps that prey on the larvae. Further the Asian gypsy moth (*Lymantria dispar*), which causes considerable damage to several important tree species, is though to be high (Grace *et al*. 2005). Alternatively a transgenic approach through the expression of *Bt* insect control protein genes in transgenic plants could be used. There have been very few reports of transgenic conifers with enhanced insect resistance for example, *P. glauca* (Ellis *et al*. 1993), Larch (Shin *et al*. 1994), and *P. radiata* (Grace *et al*. 2005), have been genetically engineered with the *Bt* toxin gene *cry*1Ac, and trees have been shown to have improved insecticidal activity in insect bioassays (Grace *et al*. 2005). *Bt* toxin inhibits the insects' digestive pathways. This gene has been successfully transformed into several species with varying degrees of success.

Herbicide resistance has allowed the use of more efficient herbicides without concern for plant health in forestry, especially for higher intensity plantation systems (Shin *et al*. 1994). Bishop-Hurley *et al*. (2001) have established a biolistic transformation method, where embryogenic tissues of *P. radiata* and *P. abies* were co-transformed with two plasmid DNAs that contained the bar gene, specifying resistance to the herbicide glufosinate, and the *npt*II gene and *uid*A reporter gene respectively. Transgenic plants survived and continued to grow with minor or no damage to their needles, whereas non transgenic plants regenerated from the same cell lines died within 8 weeks of spraying (Bishop-Hurley *et al*. 2001).

Gene expression

A putative promoter fragment of a *P. radiata* gene encoding a multi-functional *O*-methyltransferase (AEOMT) was isolated from genomic DNA (Moyle *et al*. 2002). The isolated promoter was fused to the GUS reporter gene, and its expression profile was analyzed in transgenic tobacco and in transient transformation experiments with *P. radiata* embryogenic and xylogenic tissue (Moyle *et al*. 2002). Transgenic roots were induced by infection with *A. rhizogenes* strain LBA9402, harboring a binary plasmid construct that contained one of the following promoters such as; *Ubi*-1 from *Zea mays*, *35*S from CaMV, *cdc*2a and *sam*-1 from *A. thaliana*, *HRGPnt*3 from *N. tabacum*, *RSI*-1 from *L. esculentum* (Lindroth *et al*. 1999). Promoters of broad tissue specificity showed GUS staining in most cell types of all the species. The other three promoters were expressed specifically in lateral root primordial. Elfstrand *et al*. (2001) have studied the effects of an endogenous peroxidase-like gene from *P. abies*, *spi*2, on the development and growth of Norway spruce somatic embryo plants. Overexpression of *spi*2 resulted in increased sensitivity to stress, leading to a reduction in epicotyl formation and in height growth compared to control plants. In addition, adding extra copies of genes involved in *Agrobacteriun* virulence and T-DNA transfer to disarmed strains of *A. tumefaciens* increased transformation efficiencies for embryogenic *P. abies* (Wenck *et al*. 1999).

Flowering control

Trees usually have a long juvenile growth period before they reach their reproductive phase. Nevertheless, homologues of genes involved in flower development in *Arabidopsis* and *Antirrhinum* were identified from several tree species; *LEAFY* and *Floricaula* from *P. radiata* and black spruce (Herschbach and Kopriva 2002). Genetic manipulation of flowering genes are mainly aimed to shorten flowering and generation time. Another problem specific to tree species, compared to conventional agricultural crops, is the necessity for long term stability of the transgene over several vegetative periods (Fladung 1999; Kumar and Fladung 2001). Increase in knowledge about the control of flower development in trees opens up strategies to reduce or prevent the danger of vertical gene transfer to the wild tree species via genetic engineering of sterility (Strauss *et al*. 1995). Therefore, there are several risks from transgenes to the native forest ecosystems (Mathews and Campbell 2000), and gene transfer must be part of field-trial assessment with transgenic trees as discussed by McLean and Charest (2000) and Strauss *et al*. (2001). *Cryptomeria japonica* is one of the most important Japanese conifer species. Kurita *et al*. (2007) reported the isolation and characterization of 6 MADS-box genes from *C. japonica* using PCR and RACE method. These genes play an important roles in both the formation of the flower meristem and the determination of floral organ identity. They also reported that one of these genes, M8p-1 was expressed stronger in the male strobilus and that its transcriptional activity was increased as the male strobilus developed (Kurita *et al*. 2007).

Molecular improvement

Tree improvement by genetic transformation provides a powerful new experimental tool for tree molecular improvement. With this technology, foreign genes of commercially important can be transferred with useful phenotypes, unachievable by conventional tree breeding. Transgenic trees are of great importance in testing the roles of specific enzymes in metabolic processes (Elfstrand *et al*. 2001), the functions of different promoters (Lindroth *et al*. 1999). We can also directly identify specific genes of interest from the available genomic sequences. Tree transformation also improves the commercial value of tree lines and this can be captured by industrial investors, such as expressing foreign genes conferring resistance to viruses (Birch 1997; James *et al*. 1998; Tang and Newton 2003). Further in forest trees, environmental conditions during the reproduction can greatly enhance progeny performance. Asante *et al*. (2007) reported the preparation of four subtracted cDNA libraries, 2 forward and reverse pairs, representing genes predominantly expressed in plants growing from seeds obtained after embryogenesis in a cold environment and warm environment after short-day treatment. Among the candidate genes found, the most interesting ones were transcription factors, signaling, cold and water-stress related genes in Norway spruce (Asante *et al*. 2007). Rab-related small GTP-binding proteins are known to be involved in the regulation of the vascular transport system in eukayotic cells. Gonçalves *et al*. (2007a, 2007b) reported the characterization of full-length cDNA *PpRab*1 from *P. pinaster*. In somatic embryos, *PpRab*1 appears to be expressed abundantly in stage T4 and decreasing towards the maturation of somatic embryos and the subcellular localization of *PpRab*1 protein by green fluorescent technology and to develop genetic

complementation assays to prove the function of *PpRab1* protein has been analyzed in *P. pinaster* (Gonçalves *et al.* 2007a, 2007b). These genes could be used for the tree improvement program in conifers and helps in improving the quality of wood for the commercial needs. Social benefits arise largely from the substantial contributions that forest trees make to both the environment, and to economic growth. Economic benefits are becoming increasingly obvious in particular when tree biotechnology leads to the deployment of highly improved trees with genetic improvement in plantation forestry (Walter *et al.* 2007). The productivity of plantation forests is much greater than that of natural stands, making them an essential and renewable resource for timber and wood products. Further recent discussions around climate change and CO_2 sequestration indicate that trees, and in particular those enhanced using biotechnology, will make a substantial contribution to stabilizing the climate through their ability to sequester carbon. In near future transgenic trees are expected to play a substantial role in replacing fossil fuels for the production of bioenergy and as biomaterial feedstocks for biorefineries (Walter *et al.* 2007).

STEPS TOWARDS THE ADVANCEMENT OF TECHNOLOGY

Tree improvement by genetic transformation is an important tool in the forest biotechnology. Both particle bombardment and *Agrobacterium*-mediated gene transfer were sufficiently developed in conifers; however, many cultivars of those transgenic lines are in a field trial program (Birch 1997; Malabadi and Nataraja 2007a, 2007b, 2007c). But the frequency of transformation in currently established transformation protocols are low and the frequency of undesired genetic change or unpredictable transgene expression are high in some conifers (Walter *et al.* 1998; Wenck *et al.* 1999; Tang *et al.* 2001). Another important goal is the development of transformation methods and constructs tailored for predictable transgene expression, without collateral genetic damage (Birch 1997). Further there is no model transformation system available in conifers, although *Arabidopsis* is a widely used model system in plant biology because of its small genome, small plant size, and rapid generation time. However, this can not be exploited in practical transformation systems for conifers (Birch 1997; Tang and Newton 2003). Further the whole process of gene transfer from bacteria to the nuclei of plant cells is not fully understood yet (Sheng and Citovsky 1996).

Unfortunately, there is no guarantee that a transformation plant cell type will prove regenerable since current tissue protocols at least in a few conifer species used in the transformation studies are very poor and found not to be reliable and reproducible. This is the main drawback and hinders the success of genetic transformation. Tissue culture is an important prerequisite for the successful genetic transformation studies. Success rate of any transformation depends upon the tissue culture protocols, and explants in conifers. So we need to have a well established and reproducible tissue culture protocol before thinking of genetic transformation. The type of the explants used as starting material in the conifer genetic transformation is genotype dependent since all the explants such as pollen grains, cotyledon, mature and immature zygotic embryos, embryogenic tissue derived from vegetative shoot apices or secondary needles will not response in all the conifers. Therefore, choice of explants is mainly dependent on the rate of responsive action of a particular explant in many plant species, including conifers. The best way of genetic transformation can be achieved at least in a few conifers by using vigorously growing embryogenic suspension masses derived either from immature zygotic or mature zygotic or from vegetative shoot apices/ secondary needles as the starting material. An embryogenic system developed from either vegetative shoot apices or secondary needles of mature conifers throughout the world (Bonga and Pond 1991; Ruaud *et al.* 1992; Bonga and von Aderkas 1993; Ruaud 1993; Westcott 1994; Bonga 1996; Smith 1997; Bonga 2004; Malabadi *et al.* 2004; Malabadi and van Staden 2005a, 2005b, 2005c, 2006; Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b; Aronen *et al.* 2007; Malabadi and Nataraja 2007f, 2007h) might open a new window for the successful genetic transformation of conifers (Malabadi and Nataraja, 2007a, 2007i). We also need pay attention to strategies for selection, transgene expression and integrating components of transformation. Finally we should also consider and integrate social, legal, and economical issues as well as technical issues from the earliest stages of the project design (Birch 1997). Recently a number of molecular approaches are being developed to restrict gene flow from genetically modified plants to other crops and wild plant populations. The development of transplastomic plants in which the transgenes are incorporated into the chloroplast genome is a new promising technology being developed to reduce the probability of transgene through pollen dispersal (Miki and McHugh 2004). A unique feature of plastids of most plants is that they are maternally inherited, limiting the potential spread of transgenes through pollen. The transformation of plant chloroplast is challenging and so far stable transplastomics have been identified only in tobacco, tomato and potato. Therefore, studies are very much needed in other plants including conifers before this technology can be widely adopted. Cloning of exogenous DNA into BACs provides a new approach to the analysis of the genomes of higher organisms and plant BAC libraries are constructed in many plant species (Woo *et al.* 1994; Choi *et al.* 1995; Zhang *et al.* 1996; Qui *et al.* 2003). It is of significance to demonstrate the feasibility of isolating megabase DNA from these species and constructing a large-insert DNA BAC library for molecular analysis of pine genomes. This could be another possibility in the near future since loblolly pine BAC libraries are partially constructed (Peterson *et al.* 2006), and may be available for the public for improving the pine transformation. All these essential steps will improve the current transformation studies and helps in solving the problems of traditional tree breeding.

LIMITATIONS OF TRANSGENE TECHNOLOGY

Agrobacterium-mediated gene transfer method of choice for many plant biotechnologies laboratories, however, large scale use of this organism in conifer transformation studies has been limited by difficult propagation of explant material, selection efficiencies and low transformation frequencies (Wenck *et al.* 1999). Tree improvement is a very costly and long-standing process, in terms of time, expenditure and availability of technology. Therefore, in order to achieve this goal, basic infrastructure like a good laboratory facilities, with well developed molecular biology technology are needed for the success of genetic transformation. This could be possible only with a long term funding from the government or from the public sector. At this time still it is very difficult to convince the corporate or government for getting funding for this kind of work particularly genetic transformation of plants including conifers. This is mainly because of awareness of genetically-modified (GM) crops by the public, and in many developing countries lots of local area demonstrations by environmentalists against GM crops. There is a growing fear in the public that there will be an imbalance in the ecological niche, and the arrival of new disease to the living organisms including human beings on this earth. Funding agencies are not any more interested in such this type of projects. Due to the lack of funding for many projects on genetic transformation, the work progress have been suffered and ended up with baseless results. These results can not be utilized for the commercialization of genetic transformation protocol but ended up with basic results for just scientific research. Another most important and significant hurdle for the use of transgenic technology in forestry is the intellectual property right that dominates the biotechnology sector (Lucier *et al.* 2001; Tang and Newton 2003). Even the major companies, which own large

patent estates, are often in court with their competitors, fighting over access to major technologies, and several of these companies have chosen to use their patents as weapons to restrict access to technologies and to extend their sphere of control (Tang and Newton 2003). The tight control of technology and information by a few companies is itself a major driver of the high perception or risk associated with biotechnology (Strauss and Bradshaw 2001; Fenning and Gershenzon 2002). The best solution for this problem is to bring major changes in the government and corporate policy about control of intellectual property; the industries are likely to face a hostile public and a skeptical scientific community (Tang and Newton 2003).The second one is gene silencing (Matzke *et al*. 1994; Kumpatla *et al*. 1997; Matzke and Matzke 1998) and it is becoming clear that many of the same mechanisms that act in silencing transgenes are also important for defense against viruses and invasive DNA elements (Fire *et al*. 1998; Tang and Newton 2003). The extent of somaclonal variation and transgene instability is expected to vary depending on the specific transformation system, tree genotypes and vector/gene constructs employed (Birch 1997). The third limitation is that however, it is unknown to what extent transformation causes less obvious genetic damage, such that transgenic clones might need to be re-evaluated in long-term field trials to verify that their yield and adaptability characteristics remain intact (Tang and Newton 2003). This is because most of the industries wish to see tested, well-known clones transformed and used directly, and this is a critical issue for transgenic trees. The presence of selectable-marker genes in genetically modified conifers including other plant species has seriously raised public concern that they will be transferred to other organisms. In the case of antibiotic resistance markers, there is a fear that the presence of these markers in genetically modified trees could lead to an increase in antibiotic resistant bacterial strains (Warwick *et al*. 1999). In case of herbicide-resistance markers, the concern is that the markers will contribute to the creation of new aggressive weeds (Miki and McHugh 2004). These issues should be addressed before field trials or commercialization of genetically modified trees as a part of the regulatory process (Tang and Newton 2003). Dispersal of transgenic pollen into the environment is widely considered as undesirable and the ability to produce transgenic plants that do not produce pollen would facilitate the commercial use of such translines in other countries. This will be a major problem for the environment.

The debate on Genetically Modified Organisms (GMOs) has until now largely focused on agricultural crops and much less on genetically modified trees. This is understandable, given the fact that there are already several GM crops being commercially grown in many places of the world and given that many often them are aimed at directly or indirectly feeding human beings, whose health is thus potentially threatened. However, that does not mean that GM trees are less dangerous. On the contrary, the potential dangers posed by GM trees are in some ways even more serious than those posed by GM crops. Trees live longer than agricultural crops, which means that changes in their metabolism may occur many years after they are planted. At the same time, trees are also different from crops in that they are largely undomesticated and scientists' knowledge about forest ecosystems is poor. This implies that the ecological and other potential risks associated with GM trees are far greater than in the case of crops.

There are many criticisms by the media throughout the world that forestry scientists argue that by genetically modifying trees to have less lignin they have found a way of making pulp mills less polluting. The ultimate threat to forests portion of the pulp and paper making process, from both an economic and environmental perspective, is attributable to the removal of lignins. Therefore, it is highly desirable to develop means by which lignin content is decreased, or make lignins more extractable.

The risks associated with reduced-lignin GM trees include trees which are weakened structurally and which are more vulnerable to storms. Reduced lignin trees are more susceptible to viral infections. Reducing lignin can reduce trees' defences to pest attack, leading to increased pesticide use. Low-lignin trees will rot more readily, with serious impacts on soil structure and ecology. If reduced-lignin GM trees were to cross with forest trees these impacts would not be limited to plantations. Although reduced lignin GM trees might be less competitive than native trees, the GM trees would be planted in vast numbers. If the plantation was near to a small population of native trees of the same species, the GM trees could overwhelm the reproduction of same-species native trees. Trees that cannot resist storms and which are at risk from attack by pests and viral infections could take over ecosystems and wipe out same species of native trees locally. They could also lead to a rapid increase in insect populations. Focusing narrowly on lignin as the cause of pollution from pulp mills, GM proponents can argue that reducing the amount of lignin in trees is a reasonable solution. They overlook other possible solutions such as using crops like hemp which have lower levels of lignin than trees. Growing plantations of GM trees with reduced lignin fail to address any of the environmental and social problems that industrial plantations cause to local communities. Rather than asking questions about the nature of the global pulp and paper industry for which they are working, forestry scientists are asking whether genetically modifying trees for reduced lignin will work.

Genetic engineering involves profound ethical questions. The fundamental issue is that genetic engineering modifies the very 'code of life' through an artificial, asexual process. We must ask ourselves whether we have the right to do such things to ourselves or to any other living things. From a moral point of view it is equally important to weigh the likely benefits of this technology against the potential risks – and to assess which groups stand to gain or lose out. This is not an attempt to argue that science is wrong or that everything new is automatically bad. However, when scientists announce that a new discovery or process is "safe" we would be wise to ask questions about the validity of the claim, particularly when the scientists are funded by the industry that stands to benefit from the new discovery. Criticisms of research into GM trees are not directed at a personal level at the researchers or their lifestyles. They are directed at an economic and politic system and a model of forestry that together are responsible for massive destruction of the world's forests and the livelihoods of local communities.

PROBLEMS OF PUBLIC ACCEPTANCE

Public acceptance of transgenic trees is highly variable on a global scale. Before the release of transgenic conifer trees to the field, we have to achieve environmental and public acceptance (Mullin and Bertrand 1998; Rogers and Parkes 1995). In India, both central and state governments have not approved transgenic trees and even field trials are also strongly restricted. In the rest of the world, the same problem exists but to some extent field trials are renewed annually only by special application to the government. The feasibility of managing gene flow and minimizing the risks of genetic pollution appear only to have been considered in the US and Japan, and only under certain conditions (Strauss and Bradshaw 2001). This trend is still continuing up to 2007. Therefore, no field trials are allowed in any country unless a special permission from the government is provided. All the experimentation concerning GM trees are under the strict control of greenhouse conditions for research purposes only. If social acceptance is of sufficient importance, then gauging public attitudes toward any new technology becomes an important step in market assessment (James 2003). This will lead to the justification of financial investment to conduct research and development. People express their preferences directly in the market place. Public perceptions of biotechnology are extremely complex and can

not be generalized easily (Mullin and Bertrand 1998). New technology brings risk and benefit, both of which have some degree of uncertainty before introduction to society and environment. To protect the interests of the greater population, assessment of risk is necessary before release of new technologies. Transgenic super trees possess all the characteristics of a good weed and risk becoming invasive, and very fast growing nutrient demanding plantations operated on short rotations could drive inappropriate plantation development. Risk can be defined as the probability that a substance or situation will produce harm under specified conditions. Risk is a function of the probability that an adverse event will occur, and the consequences of that adverse event. Therefore, there is some degree of risk in taking an action, and in not taking action. We must accept that there will always be risk as a consequence of decision-making. Although the questions about the potential ecological risks of introducing genetically engineered trees into the environment have been very complicated. Long-term field studies should be designed to examine not only novel genes stability and transgenic behavior but also tree-crop-induced fluxes in soil nutrient status and soil water availability.

CONCLUSIONS

Both particle bombardment and *Agrobacterium*-mediated transformation have been successfully used for a wide range of conifers. Production of genetically engineered conifers with commercially useful traits such as herbicide, insect and pathogen resistance has been accomplished. Both of the methods remain a uniquely advantageous transformation method, and indeed the only one available for many species. The absence of biological constraints (host-range, genotype dependence) and the ability to target any cell type, even in intact organized tissues, means that the method is uniquely versatile. Therefore, in some pines elite superior genotypes are amenable to transformation without extensive backcrossing, which is normally required in other transformation systems to introgress genes from amenable model varieties into elite defined parents. The ability to target organized tissues reduces or eliminates the requirement for tissue culture, and therefore, limits the occurrence of somaclonal variation, which can lead to infertility and morphological abnormalities in transgenic plants. Refinements of the technology to produce clean transgene loci have demonstrated clearly that this is not the case of gene silencing, and the particle bombardment has many advantages for the production of commercial transgenic plants that perform well in the field and comply with all relevant regulatory processes. It is concluded that particle bombardment and *Agrobacterium*-mediated genetic transformation are likely to continue to play an important role in plant biology and forest biotechnology for many years into the future. At presently traditional breeding programs proceed at a slow rate due to long maturation times and the slow growth rate of trees; however, biotechnological approaches have the potential to provide significant improvement in tree growth and quality. If these problems can be addressed, commercial forestry gain more importance and emerge as a profitable business sector.

REFERENCES

Aggarwal H, Goyal D (2007) Phytoremediation of some heavy metals by agronomic crops. *Developments in Environmental Sciences* **5**, 79-98

Alpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding* **15**, 305-327

Aronen TS, Haggman HM, Hohtola A (1994) Transient *β*-glucuronidase expression in Scots pine tissues derived from mature trees. *Canadian Journal of Forest Research* **24**, 2006-2011

Aronen TS, Haggman HM, Salonen M (1996) Rooting of Scots pine fascicular shoots by *Agrobacterium rhizogenes*. *Forest Genetics* **3**, 13-22

Aronen TS (1997) Interactions between *Agrobacterium tumefaciens* and coniferous defence compounds α-pinene and *trans*-stilbene. *European Journal of Forest Pathology* **27**, 55-67

Aronen TS, Nikkanen TO, Haggman HM (1998) Compatibility of different pollination techniques with microprojectile bombardment of Norway spruce and Scots pine. *Canadian Journal of Forest Research* **28**, 79-86

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

Aronen TS, Ryynanen L, Malabadi RB (2007) Somatic embryogenesis of Scots pine: initiation of cultures from mature tree explants and enhancement of culture system [Abstract]. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIX. 2

Asante D, Yakovlev IA, Fossdal CG, Johnson O, Junttila O (2007) Differential gene expression during early development in Norway spruce related to cold and warm maternal environment and short day bud set induction. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIVIII. p 16

Barrell PJ, Yongjin S, Cooper PA, Conner AJ (2002) Alternative selectable markers for potato transformation using minimal T-DNA vectors. *Plant Cell, Tissue and Organ Culture* **70**, 61-68

Becwar MR, Blush TD, Brown DW, Chesick EE (1991) Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly pine seeds. *Plant Cell, Tissue and Organ Culture* **26**, 37-44

Bekkaoui F, Datla RSS, Pilon M, Tautorus TE, Crosby WL, Dunstan DI (1990) The effects of promoter on transient expression in conifer cell lines. *Theoretical and Applied Genetics* **79**, 535-539

Bekkaoui F, Pilon M, Laine E, Raju DSS, Crosby WL, Dunstan DL (1988) Transient gene expression in electroporated *Picea glauca* protoplasts. *Plant Cell Reports* **7**, 481-484

Bennett LE, Burkhead JL, Hale KL, Terry N, Pilon M, Elizabeth A, Pilon-Smits H (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. *Journal of Environmental Quality* 32, 432-440

Bergmann BA, Stomp AM (1992) Effect of host plant genotype and growth rate on *Agrobacterium tumefaciens*-mediated gall formation in *Pinus radiata*. *Phytopathology* **82**, 1457-1462

Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* **304**, 184-187

Birch RG, Bower R (1994) Principles of gene transfer using particle bombardment. In: Yang N-S, Christou P (Eds) *Particle Bombardment Technology for Gene Transfer*, Oxford University Press, New York, pp 3-37

Birch RG (1997) Plant transformation: Problems and strategies for practical applications. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 297-326

Bishop-Hurley SL, Zabkiewicz RJ, Grace L, 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

Boerjan W (2007) Lignin: biosynthesis, polymerization and engineering. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIV. p 1 (Abstract)

Bommineni VR, Chibbar RN, Datla RSS, Tsang EWT (1993) Transformation of white spruce (*Picea glauca*) somatic embryos by microprojectible bombardment. *Plant Cell Reports* **13**, 17-23

Bonga JM, Pond SE (1991) Adventitious shoot formation in cultures of 30-year old *Larix deciduas*, *L. leptolepis*, and *L. laricina* trees. *Plant Cell, Tissue and Organ Culture* **26**, 45-51

Bonga JM, von Aderkas P (1993) Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro*. In: Ahuja MR, Libby WJ (Eds) *Clonal Forestry I, Genetics and Biotechnology*, Springer-Verlag, Berlin, pp 182-199

Bonga JM (1996) Frozen storage stimulates the formation of embryo-like structures and elongating shoots in explants from mature *Larix deciduas* and *L.* × *eurolepos*. *Plant Cell, Tissue and Organ Culture* **51**, 195-200

Bonga JM (2004) The effect of various culture media on the formation of embryo-like structures in cultures derived from explants taken from mature *Larix deciduas*. *Plant Cell, Tissue and Organ Culture* **77**, 43-48

Bower R, Elliot AR, Potier BAM, Birch RG (1996) High efficiency, microprojectile-mediated co-transformation of sugarcane, using visible or selectable markers. *Molecular Breeding* **2**, 239-249

Brukhin V, Clapham D, Elfstand M, von Arnold S (2000) Basta tolerance as a selectable and screening marker for transgenic plants of Norway spruce. *Plant Cell Reports* **19**, 899-903

Burke DT, Carle GF, Olson MV (1987) Cloning of large segments of exogenous DNA into yeast using artificial-chromosome vectors. *Science* **236**, 806-812

Burke DT (1990) YAC cloning: Options and problems. *GATA* **7**, 94-99

Campbill MA, Kinlaw CS, Neale DB (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-Johson P (2002) Stable

transformation of *Pinus radiata* embryogenic tissue by *Agrobacterium tumefaciens*. *Plant Cell, Tissue and Organ Culture* **70**, 251-257

Cervera M, Juarez J, Navarro A, Pina JA, Duran-Vila N, Navarro L, Peña L (1998) Genetic transformation and regeneration of mature tissue of woody fruit plants bypassing the juvenile stage. *Transgenic Research* **7**, 51-59

Cervera M, Pina JA, Juarez J, Peña L (2000) A broad exploration of a transgenic population of citrus: Stability of gene expression and phenotype. *Theoretical and Applied Genetics* **100**, 670-677

Charest PJ, Devantier Y, Ward C, Jones C, Schaffer U (1991) Transient expression of foreign chimeric genes in gymnosperm hybrid *larch* following electroporation. *Canadian Journal of Botany* **69**, 1731-1736

Charest PJ, Calero N, Lachance D, Datla RSS, Duchesne LC, Tsang EWT (1993) Microprojectile-DNA delivery in conifer species: Factors affecting assessment of transient gene expression using the *β*-glucuronidase reporter gene. *Plant Cell Reports* **12**, 189-193

Charest PJ, Devantier Y, Lachance D (1996) Stable genetic transformation of *Picea mariana* (black spruce) *via* microprojectile bombardment. *In Vitro Cellular and Developmental Biology – Plant* **32**, 91-99

Charity JA, Holland L, Donaldson SS, Grace LJ, Walter C (2002) *Agrobacterium*-mediated transformation of *Pinus radiata* organogenic tissue using vacuum infilteration. *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

Cherian S, Oliveira MM (2005) Transgenic plants in phytoremediation: recent advances and new possibilities. *Environmental Science and Technology* **39**, 9377-9390

Chiang VL (2002) From rags to riches. *Nature Biotechnology* **20**, 557-558

Choi S, Creelman RA, Mullet JE, Wing RA (1995) Construction and characterization of bacterial artificial chromosome library of *Arabidopsis thaliana*. *Weeds World* **2**, 17-20 and *Plant Molecular Biology Reporter* **13**, 124-128

Christou P, Ford TL, Kofron M (1992) Rice genetic engineering: a review. *Trends in Biotechnology* **10**, 239-246

Clapham DH, Ekberg I (1986) Induction of tumors by various strains of *Agrobacterium tumefaciens* on *Abies nordmanniana* and *Picea abies*. *Scandinavian Journal of Forest Research* **1**, 453-437

Clapham DH, Demel P, Elfstrand M, Koop HU, Sabala I, von Arnold S (2000) Gene transfer by particle bombardment to embryogenic cultures of *Picea abies* and the production of transgenic plantlets. *Scandinavian Journal of Forest Research* **15**, 151-160

Crossway A, Oaks JV, Irvine JM, Ward B, Knauf VC, Shewmaker CK (1986) Integration of foreign DNA following microinjection of tobacco obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Molecular Breeding* **7**, 25-33

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beuchy RN, Fauquet C (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Molecular Breeding* **7**, 25-33

Dandekar AM, Gupta PK, Durzan DJ, Knauf V (1987) Transformation and foreign gene expression in micropropagated Douglas fir (*Pseudotsuga menziesii*). *Bio/Technology* **5**, 587-590

Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile Indica-rice plants recovered from protoplasts. *Bio/Technology* **8**, 736-740

de Buck S, Jacobes A, van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggests that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. *The Plant Journal* **20**, 295-304

de Buck S, de Wilde C, van Montagu M, Depicker A (2000) Determination of the T-DNA transfer and T-DNA integration frequencies upon cocultivation of *Arabidopsis thaliana* root explants. *Molecular Plant-Microbe Interactions* **6**, 658-665

Diner AM, Karnosky DF (1987) Differential responses of two conifers to *in vitro* inoculation with *Agrobacterium rhizogenes*. *Europian Journal of Forest Pathology* **17**, 211-216

Diner AM (1999) Genetic transformation of *Pinus palustris* (longleaf pine). In: Bajaj YPS (Ed) *Transgenic Trees*. *Biotechnology in Agriculture and Forestry* (Vol 44), Springer, Berlin, pp 185-192

Doblin MS, Kurek I, Jacob-Wilk D, Delmer DP (2002) Cellulose biosynthesis in plants: From genes to rosettes. *Plant Cell Physiology* **43**, 1407-1420

Dodueva IE, Frolova NV, Lutova LA (2007) Plant tumorigenesis: different ways for shifting systemic control of plant cell division and differentiation. *Transgenic Plant Journal* **1**, 17-38

Doty SL, James CA, Moore AL, Vajzovic A, Singleton GL, Ma C, Khan Z, Xin G, Kang JW, Park JY, Meilan R, Strauss SH, Wilkerson J, Farin F, Strand SE (2007) Enhanced phytoremediation of volatile environmental pollutants with transgenic trees. *Proceedings of the National Academy of Sciences USA* **104**, 16816-16821

Drake PMW, Chargelegue D, Vine ND, van Dolleweerd CJ, Obregon P, Mao JK (2002) Transgenic plants expressing antibodies: A model for phytoremediation. *The FASEB Journal* **16**, 1855-1860

Duchesne LC, Charest PJ (1991) Transient expression of the *β*-glucuronidase gene in embryogenic callus of *Picea mariana* following microprojection. *Plant Cell Reports* **10**, 191-194

Duchesne LC, Lelu MA, Aderkas PV, Charest PJ (1993) Microprojectile-mediated DNA delivery in haploid and diploid embryogenic cells of *Larix* spp. *Canadian Journal of Forest Research* **23**, 312-316

Eapen S, D'Souza SF (2005) Prospects of genetic engineering of plants for phytoremediation of toxic metals. *Biotechnology Advances* **23**, 97-114

Ebinuma H, Sugita K, Matsunga E, Yamakado M (1997) Selection of marker-free transgenic plants using the isopentyl transferase gene. *Proceedings of the National Academy of Sciences USA* **94**, 2117-2121

Ebinuma H, Sugita K, Matsunga E, Yamakado M, Komamine A (2001) Systems for the removal of a selection marker and their combination with a positive marker. *Plant Cell Reports* **20**, 383-392

Elfstrand M, Fossdal CG, Sitbon F, Olsson O, Lonneborg A, von Arnold S (2001) Overexpression of the endogenous peroxidases-like gene spi 2 in transgenic Norway spruce plants results in increased total peroxidase activity and reduced growth. *Plant Cell Reports* **20**, 596-603

Ellis D, Roberts D, Sutton B, Lazaroff W, Webb D, Flinn B (1989) Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens*. *Plant Cell Reports* **8**, 16-20

Ellis DD, McCabe DE, Russsell D, Martinell B, McCown BH (1991) Expression of inducible angiosperm promoters in gymnosperm, *Picea glauca* (white spruce). *Plant Molecular Biology* **19**, 19-27

Ellis DD, McCabe DE, McInnis S, Ramachandran R, Russell DR, Wallace KM, Martinell BJ, Roberts DR, Raffa KF, McCown BH (1993) Stable transformation of *Picea glauca* by particle acceleration. *Bio/Technology* **11**, 84-89

Fang YD, Akula C, Altpeter F (2002) *Agrobacterium*-mediated barley (*Hordeum vulgare* L.) transformation using green fluorescent protein as a visual marker and sequence analysis of the T-DNA: Genome DNA junctions. *Journal of Plant Physiology* **159**, 1131-1138

Fenning TM, Gershenzon J (2002) Where will the wood come from? Plantation forests and the role of biotechnology. *Trends in Biotechnology* **20**, 291-296

Fernando DD, Owens JN, Misra S (2000) Transient gene expression in pine pollen tubes following particle bombardment. *Plant Cell Reports* **19**, 224-228

Fladung M (1999) Gene stability in transgenic aspen-*Populus* I. Flanking DNA sequences and T-DNA structure. *Molecular General Genetics* **260**, 574-581

Flavell RB, Dart E, Fuchs RL, Fraley RT (1992) Selectable marker genes: safe for plants. *Biotechnology* **10**, 141-144

Fillatti JJ, Sellmer J, McCown B, Haissig B, Comai L (1987) *Agrobacterium*-mediated transformation and regeneration of *Populus*. *Molecular and General Genetics* **206**, 192-199

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811

Fourré JL, Berger P, Niquet L, André PA (1997) Somatic embryogenesis and somaclonal variation in Norway spruce: Morphogenetic, cytogenetic and molecular approaches. *Theoretical and Applied Genetics* **94**, 159-169

Frame BR, Drayton PR, Bagnall SV, Lewnau CJ, Bullock WP, Wilson HM, Dunwell JM, Thompson JA, Wang K (1994) Production of fertile transgenic maize plants by silicon-carbide whisker-mediated transformation. *The Plant Journal* **6**, 941-948

Fromm M, Taylor L, Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. *Nature* **319**, 791-793

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 223-256

Goldfrab B, Strauss SH, Howe GT, Zaerr JB (1991) Transient gene expression of microprojectile-introduced DNA in Douglas-fir cotyledons. *Plant Cell Reports* **10**, 517-521

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

Gonçalves S, Simoes M, Oliveira MM, Miguel C (2007a) PpRAB1, a small RAB-like GTPase is involved in early embryogenesis of Maritime pine. In: IUFRO Tree Biotechnology conference, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIVIII. p 4 (Abstract)

Goncalves S, Cairney J, Rodriguez MP, Canovas FM, Oliveira M, Miguel C (2007b) PpRab1, a Rab GTPase from maritime pine is differentially expressed during embryogenesis. *Molecular Genetics and Genomics* **278**, 273-282

Gould JH, Zhou YX, Padmanabhan V, Magallanes-Cedeno ME, 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

Gupta PK, Dandekar AM, Durzan DJ (1988) Somatic embryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts. *Plant Science* **58**, 85-92

Haggman HM, Aronen TS, Nikkanen TO (1997) Gene transfer by particle bombardment to Norway spruce and Scots pine pollen. *Canadian Journal of Forest Research* **27**, 928-935

Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proceedings of the National Academy of Sciences USA* **93**, 9975-9979

Hamilton CM (1997) A binary-BAC system for plant transformation with high molecular weight DNA. *Gene* **200**, 107-116

Hamilton CM, Frary A, Xu Y, Tanksley SD, Zhang HB (1999) Construction of tomato genomic DNA libraries in a binary-BAC (BIBAC) vector. *The Plant Journal* **18**, 223-229

Hansen G, Das A, Chilton MD (1994) Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*. *Proceedings of the National Academy of Science USA* **91**, 7603-7607

Hawkins S, Samaj J, Lauvergeat V, Boudet A, Grima-Pettenati J (1997) Cinnamyl alcohol dehydrogenase: Identification of new sites of promoter activity in transgenic poplar. *Plant Physiology* **113**, 321-325

Heiser W (1992) Optimization of biolistic transformation using the helium-driven PDS-1000/He system. *Bio-Rad Bullitein* **1688**, 1-7

Herschbach C, Kopriva S (2002) Transgenic trees as tools in tree and plant physiology. *Trees* **16**, 250-261

Hobbs SLA, Warkentin TD, Delong CMO (1993) Transgene copy number can be positively or negatively associated with transgene expression. *Plant Molecular Biology* **21**, 17-26

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort A (1983) A binary plant vector strategy based on separation of *vir* and T-region of *Agrobacterium tumefaciens* Ti plasmid. *Nature* **303**, 179-180

Hoffmann K, Verbeek M, Romano A, Dullemans AM, van den Heuvel JFJM, van der Wilk F (2001) Mechanical transmission of poleroviruses. *Journal of Virology Methods* **91**, 197-201

Holland L, Gemmell JE, Charity JA, Walter C (1997) Foreign gene transfer into *Pinus radiata* cotyledons by *Agrobacterium tumefaciens*. *New Zealand Journal of Forest Science* **27**, 289-304

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, Diner AM, Karnosky DF (1991) *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of conifer: *Larix deciduas*. *In Vitro Cellular and Developmental Biology – Plant* **27**, 201-207

Humara JM, Lopez M, Ordas RJ (1999) *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

James R, DiFazio SP, Brunner A, Strauss SH (1998) Environmental effects of genetic engineering of woody biomass crops. *Biomass and Bioenergy* **14**, 403-414

James C (2003) Preview: Global status of commercialized transgenic crops: 2003. ISAAA Briefs No. 30. ISAAA, Ithaca, NY, pp 21-37

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reports* **5**, 387-405

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: *β*-glucuronidase as sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6**, 3901-3907

Joersbo M, Okkels FT (1996) A novel principle for selection of transgenic plant cells: Positive selection. *Plant Cell Reports* **16**, 219-221

Johnston SA, Anziano PQ, Shark K, Sanford JC, Butow RA (1988) Mitochondrial transformation in yeast by bombardment with microprojectiles. *Science* **240**, 1538-1541

Klimaszewska K, Devantier Y, Lachance D, Lelu MA, Charest PJ (1997) *Larix laricina* somatic embryogenesis and genetic transformation. *Canadian Journal of Forest Research* **27**, 538-550

Klimaszewska K, Lachance D, Pelletier G, Lelu M-A, Seguin A (2001) Regeneration of transformed *Picea glauca*, *P. mariana*, and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. *In Vitro Cellular and Developmental Biology – Plant* **37**, 748-755

Klimaszewska K, Lachance D, Bernier-Cardou M, Rutledge RG (2003) Transgene integration patterns and expression levels in transgenic tissue lines of *P. mariana*, *P. glauca* and *P. abies*. *Plant Cell Reports* **21**, 1080-1087

Kolosova N, Ralph S, Breuil C, Ritland K, Bohlman J (2007) Conifer defense against bark beetle-associated blue-stain fungi and white pine weevil. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SII. p 2 (Abstract)

Kramer U (2005) Phytoremediation: Novel approach to cleaning up-polluted soils. *Current Opinion in Biotechnology* **16**, 133-141

Kumar S, Fladung M (2001) Gene stability in transgenic aspen (*Populus*). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen. *Planta* **213**, 731-740

Kumpatla SP, Teng W, Bucholz WG, Hall TC (1997) Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. *Plant Physiology* **115**, 361-373

Kurita M, Watanabe A, Ohmiya Y, Taniguchi T, Kondo T (2007) Characterization of expressed genes associated with male strobilus development in *Cryptomeria japonica*. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIVIII. p 18 (Abstract)

Le VQ, Belles-Isles J, Dusabenyagasani M, Tremblay FM (2001) An improved procedure for production of white spruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens*. *Journal of Experimental Botany* **52**, 2089-2095

Lelu MA, Pilate G (2000) Transgenic in *Larix*. In: Jain SM, Minocha SC (Eds) *Molecular Biology of Woody Plants* (Vol 2), Kluwer Academic Publishers, Dordrecht, pp 119-134

Leple 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

Levee 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

Levee V, Garin E, Klimaszewska K, Seguin A (1999) Stable genetic transformation of white pine (*Pinus strobus* L.) after cocultivation of embryogenic tissues with *Agrobacterium tumefaciens*. *Molecular Breeding* **5**, 429-440

Li YH, Tremblay FM, Seguin A (1994) Transient transformation of pollen and embryogenic tissues of white spruce (*P. glauca*) resulting from microprojectile bombardment. *Plant Cell Reports* **13**, 661-665

Li L, Zhou YH, Cheng XF, Sun JY, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proceedings of the National Academy of Sciences USA* **100**, 4939-4944

Lindroth AM, Gronroos 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

Lopez M, Humara JM, Rodriguez R, Ordas RJ (2000) Factors involved in *Agrobacterium tumefaciens* mediated gene transfer into *Pinus nigra* Arn. ssp. Salzmannii (Dunal) Franco. *Euphytica* **114**, 195-203

Loopstra CA, Stomp AM, Sederoff RR (1990) *Agrobacterium*-mediated DNA transfer in sugar pine. *Plant Molecular Biology* **15**, 1-9

Lucier AA, Hinchee M, McCullough RB (2001) Biotechnology and the forest products industry. In: Strauss SH, Bradshaw HD (Eds) *Proceedings of the First International Symposium on Ecological and Society Aspect of Transgenic Plantations*, College of Foresty, Oregon State University, pp 57-61

Macek T, Mackova M, Kas J (2000) Exploitation of plants for the removal of organics in environmental remediation. *Biotechnology Advances* **18**, 23-34

Malabadi RB (2003) Protoplast isolation, culture and plant regeneration in Butterfly pea (*Clitoria ternatea* Linn.). *Indian Journal of Genetics and Plant Breeding* **63**, 243-246

Malabadi RB (2006a) *Agrobacterium*-mediated genetic transformation of *Vigna unguiculata*. *Journal of Phytological Research* **19**, 1-4

Malabadi RB (2006b) Effect of glutathione on maturation of somatic embryos derived from vegetative shoot apices of mature trees of *Pinus roxburghii*. *Journal of Phytological Research* **19**, 35-38

Malabadi RB, Choudhury H, Tandon P (2002) Plant regeneration via somatic embryogenesis in *Pinus kesiya* (Royle ex. Gord). *Applied Biological Research* **4**, 1-10

Malabadi RB, Choudhury H, Tandon P (2003) Effect of gelling agent, carbon sources and sterilization methods on initiation and establishment of embryogenic cultures in Khasi pine (*Pinus kesiya* Royle ex. Gord). *Applied Biological Research* **5**, 1-8

Malabadi RB, Choudhury H, Tandon P (2004) Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord) promoted by partial desiccation and Gellan gum. *Scientia Horticulturae* **102**, 449-459

Malabadi RB, Hills PN, van Staden J (2006) RAPD assessment of clonal identity of somatic seedlings derived from the vegetative shoot apices of mature *Pinus patula* trees. *South African Journal of Botany* **72**, 181-183

Malabadi RB, Mulgund GS, Nataraja K (2005) Plant regeneration *via* somatic embryogenesis in *Pinus kesiya* (Royle ex. Gord.) influenced by triacontanol. *Acta Physiologiae Plantarum* **27**, 531-537

Malabadi RB, Nataraja K (2003) Alkaloid biosynthesis influenced by *Agrobacterium rhizogenes* mediated transformation and bioreactor in *Clitoria ternatea* (Linn.). *Plant Cell Biotechnology and Molecular Biology* **4**, 169-178

Malabadi RB, Nataraja K (2006a) Cryopreservation and plant regeneration *via* somatic embryogenesis using shoot apical domes of mature *Pinus roxburghii* Sarg. trees. *In Vitro Cellular and Developmental Biology – Plant* **42**, 152-159

Malabadi RB, Nataraja K (2006b) RAPD detect no somaclonal variation in cryopreserved cultures of *Pinus roxburghii* SARG. *Propagation of Ornamental Plants* **6**, 114-120

Malabadi RB, Nataraja K (2007a) Gene transfer by particle bombardment of embryogenic tissue derived from vegetative shoot apices of mature trees of *Pinus roxburghii*. *American Journal of Plant Physiology* **2**, 90-98

Malabadi RB, Nataraja K (2007b) A biolistic approach for the production of transgenic plants using embryogenic tissue in *Pinus kesiya* Royle ex. Gord. (Khasi pine). *Biotechnology* **6**, 86-92

Malabadi RB, Nataraja K (2007c) Stable transformation and recovery of transgenic plants by particle bombardment in *Pinus wallichiana* A. B. Jacks (Himalayan blue pine). *Biotechnology* **6**, 105-111

Malabadi RB, Nataraja K (2007d) Genetic transformation of *Vanilla planifolia* by *Agrobacterium-tumefaciens* using shoot tip sections. *Research Jour-*

nal of Botany 2, 86-94

Malabadi RB, Nataraja K (2007e) *Agrobacterium-tumefaciens* mediated genetic transformation of *Vigna aconitifolia* and stable transmission of the genes to somatic seedlings. *International Journal of Agriculture Research* **2**, 450-458

Malabadi RB, Nataraja K (2007f) Plant regeneration *via* somatic embryogenesis using secondary needles of mature trees of *Pinus roxburghii* Sarg. *International Journal of Botany* **3**, 40-47

Malabadi RB, Nataraja K (2007g) Isolation of cDNA clones of genes differentially expressed during somatic embryogenesis of *P. roxburghii. American Journal of Plant Physiology* **2**, 333-343

Malabadi RB, Nataraja K (2007h) Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* A. B. Jacks. *Journal of Plant Sciences* **2**, 45-53

Malabadi RB, Nataraja K (2007i) Production of transgenic plants *via Agrobacterium*-mediated genetic transformation in *Pinus wallichiana* (Himalayan blue pine). *Transgenic Plant Journal* **1**, 376-383

Malabadi RB, Nataraja K (2007j) Spatio-temporal accumulation of peroxidases and assessment of clonal identity of somatic seedlings by ISSR and RAPD in *Pinus roxburghii*. *American Journal of Plant Physiology* **2**, 356-366

Malabadi RB, Nataraja K (2007k) Influence of triacontanol on somatic embryogenesis of *Pinus roxburghii* Sarg. *Baltic Forestry* **13**, 39-44

Malabadi RB, Nataraja K (2007l) 24-epibrassinolide induces somatic embryogenesis in *Pinus wallichiana* A. B. Jacks. *Journal of Plant Science* **2**, 171-178

Malabadi RB, Nataraja K (2007m) Putrescine influences somatic embryogenesis and plant regeneration in *Pinus gerardiana*. *American Journal of Plant Physiology* **2**, 107-114

Malabadi RB, Raghvendra S, Vijay Kumar S (2007) Production of cellulase-free xylanases from a novel yeast strain used for biobleaching in paper industry. *Research Journal of Microbiology* **2**, 24-33

Malabadi RB, van Staten J (2003) Somatic embryos can be induced from shoot apical domes of mature *Pinus patula* trees. *South African Journal of Botany* **69**, 450-451

Malabadi RB, van Staden J (2005a) Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. *Tree Physiology* **25**, 11-16

Malabadi RB, van Staden J (2005b) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. *In Vitro Cellular and Developmental Biology – Plant* **41**, 181-186

Malabadi RB, van Staden J (2005c) Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. *Plant Cell, Tissue and Organ Culture* **82**, 259-265

Malabadi RB, van Staden J (2006) Cold-enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium. *South African Journal of Botany* **72**, 613-618

Mathews JH, Campbell MM (2000) The advantages and disadvantages of the application of genetic engineering to forest trees: A discussion. *Forestry* **73**, 371-380

Matsunaga E, Sugita K, Ebinuma H (2002) Asexual production of selectable marker-free transgenic woody plants, vegetatively propagated species. *Molecular Breeding* **10**, 95-106

Matzke AJM, Neuhuber F, Park YD, Ambros PF, Matzke MA (1994) Homology dependent gene silencing in transgenic plants: Epistatic silencing loci contain multiple copies of methylated transgenes. *Molecular and General Genetics* **244**, 219-229

Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biology* **1**, 142-148

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

McLean MA, Charest PJ (2000) The regulation of transgenic trees in North America. *Silvae Genetica* **49**, 233-239

Merkle SA, Dean JFD (2000) Forest tree biotechnology. *Current Opinion in Biotechnology* **11**, 298-302

Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: Applications, alternatives and biosafety. *Journal of Biotechnology* **107**, 193-232

Milhinhos A, Miguel C, Oliveira MM, Tereso S (2007) Transformation of *P. pinaster* for studying nitrogen metabolism regulation. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIX. p 9 (Abstract)

Morris JW, Castle LA, Morris RO (1989) Efficacy of different *Agrobacterium tumefaciens* strains in transformation of pinaceous gymnosperms. *Physiology and Molecular Plant Pathology* **34**, 451-461

Moyle R, Moody J, Phillip L, Walter C, Wagner A (2002) Isolation and characterization of a *Pinus radiata* lignin biosynthesis-related *O*-methyltransferase promoter. *Plant Cell Reports* **20**, 1052-1060

Mullin TJ, Bertrand S (1998) Environmental release of transgenic trees in Canada-potential benefits and assessment of biosafety. *Forest Chronicle* **74**, 203-219

Nap JP, Metz PL, Excaler AJ (2003) The release of genetically modified crops into the environment. Part I. Overview of current status and regulations. *The Plant Journal* **33**, 1-18

Newton RJ, Bloom J, Bivans DH, Jain SM (2001) Stable genetic transformation of conifers. *Phytomorphology* **Golden Jubilee Issue**, 421-434

Nigro SA, Makunga NP, Jones NB, van Staden J (2004) A biolistic approach towards producing transgenic *Pinus patula* embryonal suspensor masses. *Plant Growth Regulation* **44**, 187-197

OECD (1999) Concensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. *Series on Harmonization of Regulatory Oversight in Biotechnology*, No-11

Parasharami VA, Naik VB, von Arnold S, Nadagauda 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

Pena L, Seguin A (2001) Recent advances in the genetic transformation of trees. *Trends in Biotechnology* **19**, 500-506

Peterson D, Nelson CD, Islam-Farida MN, Main DS, Tomkins JP, Avery A, Carlson JE, Chouvarine P, Magbanua Z, Mukherjee D, Robertson L, Saha S, Thummasuwan S (2006) Towards physical mapping and genome sequencing of Loblolly pine. In: *International Plant and Animal Conference*, Jan. 14-18th, San Diego, CA, USA (poster) XII-13

Peuke AD, Rennenberg H (2005) Phytoremediation with transgenic trees. *Zeitschrift fur Naturfrschung* **60c**, 199-207

Pierce JC, Sauer B, Sternberg N (1992) A positive selection vector for cloning high molecular weight DNA by the bacteriophage P1 system: Improved cloning efficacy. *Proceedings of National Academy of Science USA* **89**, 2056-2060

Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leple JC, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Cornu D, Halpin C (2002) Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnology* **20**, 607-612

Pilon-Smits P, Elizabeth A, Marinus P (2002) Phytoremediation of metals using transgenic plants. *Critical Reviews in Plant Science* **21**, 439-456

Popelka JC, Altpeter F (2003) *Agrobacterium-tumefaciens*-mediated genetic transformation of rye (*Secale cereale*). *Molecular Breeding* **11**, 203-211

Qu S, Coaker G, Francis D, Zhou B, Wang GL (2003) Development of a new transformation-competent artificial chromosome (TAC) vector and construction of tomato and rice TAC libraries. *Molecular Breeding* **12**, 297-308

Regan S, Bourquin V, Tuominen H, Sunderg B (1999) Accurate and high resolution *in situ* hybridization analysis of gene expression in secondary stem tissues. *The Plant Journal* 19, 363-369

Robertson D, Weissinger AK, Ackley R, Glover S, Sederoff RR (1992) Genetic transformation of Norway spruce (*Picea abies*) using somatic embryo explants by microprojectile bombardment. *Plant Molecular Biology* **19**, 925-935

Rogers HJ, Parkes HC (1995) Transgenic plants and the environment. *Journal of Experimental Botany* **46**, 467-488

Ruaud JN, Bercetche J, Paques M (1992) First evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. *Plant Cell Reports* **11**, 563-566

Ruaud JN (1993) Maturation and conversion into plantlets of somatic embryos derived from needles and cotyledons of 7-56-day-old *Picea abies*. *Plant Science* **92**, 213-220

Rugh CL, Senecoff JF, Meagher RB, Merkle SA (1998) Development of transgenic yellow poplar for mercury phytoremediation. *Nature Biotechnology* **16**, 925-928

Rugh CL (2001) Mercury detoxification with transgenic plants and other biotechnological breakthrough for phytoremediation. *In Vitro Cellular and Developmental Biology – Plant* **37**, 321-325

Sederoff R, Stomp AM, Chilton WS, Moore LW (1986) Gene transfer into loblolly pine by *Agrobacterium tumefaciens*. *Biotechnology* **4**, 647-649

Sharma K, Degen B, von Wuehlisch G, Singh NB (2007) An assessment of heterozygosity and fitness in Chir pine (*P. roxburghii* Sarg.) using isozymes. *New Forests* **34**, 153-162

Sheng JS, Citovsky V (1996) *Agrobacterium*-plant cell DNA transport: Have virulence proteins, will travel. *Plant Cell* **8**, 1699-1710

Shibata D, Liu YG (2000) *Agrobacterium*-mediated plant transformation with large DNA fragments. *Trends in Plant Science* **5**, 354-357

Shin DI, Podila GK, Huang Y, Karnosky DF (1994) Transgenic larch expressing genes for herbicide and insect resistance. *Canadian Journal of Forest Research* **24**, 2059-2067

Smith EF, Towsend CO (1907) A plant tumor of bacterial origin. *Science* **25**, 671-673

Smith DR (1997) The role of *in vitro* methods in pine plantation establishment: The lesson from New Zealand. *Plant Tissue Culture Biotechnology* **3**, 63-73

Stomp AM, Loopstra C, Chilton WS, Sederoff RR, Moore LW (1990) Extended host range of *Agrobacterium tumefaciens* in the genus *Pinus*. *Plant Physiology* **92**, 1226-1236

Stomp AM, Weissinger A, Sederoff RR (1991) Transient expression from microprojectile-mediated DNA transfer in *Pinus taeda*. *Plant Cell Reports* **10**, 187-190

Strauss SH, Rottmann WH, Brunner AM, Sheppard LA (1995) Genetic engineering of reproductive sterility in forest trees. *Molecular Breeding* **1**, 5-26

Strauss SH, Bradshaw HD (Eds) (2001) *Proceedings of the First International Symposium on Ecological and Societal Aspect of Transgenic Plantations*,

College of Forestry, Oregon State University, pp 21-23
Strauss SH, DiFazio SP, Meilan R (2001) Genetically modified *Poplars* in context. *Forest Chronicle* **77**, 271-279
Sul IW, Korban SS (1998) Influence of bombardment with BA-coated microprojectiles on shoot organogenesis from *Phlox paniculata* L. and *Pinus pinea* L. tissues. *In Vitro Cellular and Developmental Biology – Plant* **34**, 300-302
Suresh B, Ravishankar GA (2004) Phytoremediation – A novel and promising approach for environmental clean up. *Critical Reviews in Biotechnology* **24**, 97-124
Sutton B (2002) Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. *Annals of Forest Science* **59**, 657-661
Tang W (2001) *Agrobacterium*-mediated transformation and assessment of factors influencing transgenic expression in loblolly pine (*Pinus taeda* L.) *Cell Research* **11**, 237-243
Tang W, Sederoff R, Whetten R (2001a) Regeneration of transgenic loblolly pine (*Pinus taeda* L.) from zygotic embryos transformed with *Agrobacterium tumefaciens*. *Planta* **213**, 981-989
Tang W, Guo ZC, Ouyang F (2001b) Plant regeneration from embryogenic cultures initiated from mature loblolly pine zygotic embryos. *In Vitro Cellular and Developmental Biology – Plant* **37**, 558-563
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 (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, Newton RJ, Lin J, Charles TM (2006) Expression of a transcription factor from *Capsicum annuum* in pine calli counteracts the inhibitory effects of salt stress on adventitious shoot formation. *Molecular Genetics Genomics* **276**, 242-253
Tang W, Lin J, Newton RJ (2007) Okadaic acid and trifluoperazine enhance *Agrobacterium*-mediated transformation in eastern white pine. *Plant Cell Reports* **26**, 673-682
Tautorus TE, Bekkaoui F, Pilon M, Datla RSS, Crosby WL, Fowke LC, Dunstan DI (1989) Factors affecting transient gene in electroporated black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) protoplasts. *Theoretical and Applied Genetics* **78**, 531-536
Taylor NJ, Fauquet CM (2002) Microparticle bombardment as a tool in plant science and agricultural biotechnology. *DNA and Cell Biology* **21**, 963-977
Tereso S, Miguel C, Zoglauer K, Valle-Piquera C, Oliveira MM (2006) Stable *Agrobacterium*-mediated transformation of embryogenic tissues from *Pinus pinaster* Portuguese genotypes. *Plant Growth Regulation* **50**, 57-68
Tian L, Seguin A, Charest PJ (1997) Expression of the green fluorescent protein gene in conifer tissues. *Plant Cell Reports* **16**, 267-271
Tian L, Levee V, Mentag R, Charest PJ, Seguin A (1999) Green fluorescent protein as a tool for monitoring transgene expression in forest tree species. *Tree Physiology* **19**, 541-546
Tian L, Charest PJ, Seguin A, Rutledge RG (2000) Hygromycin resistance is an effective selectable marker for biolistic transformation of black spruce (*Picea mariana*). *Plant Cell Reports* **19**, 358-362
Tian L, Jordan M, Miki B (2006) Marker and selector genes for plant transformation. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books, Isleworth, UK, pp 9-20
Tian L (2006) Marker gene removal from transgenic plants. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books, Isleworth, UK, pp 26-29
Tibbits JFG, Spokevicius AV, Rodrigues J, Alves A, Warrener D, Kasel S, Bossinger G (2007) GUS expression alters lignin composition in secondary cell walls of poplar tree stems. In: IUFRO tree biotechnology conference, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIV. p 3 (Abstract)
Tinland B, Schoumacher F, Gloeckler V, Bravo AM, Angel M, Hohn B (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. *The EMBO Journal* **14**, 3585-3595
Tinland B (1996) The integration of T-DNA into plant genomes. *Trends in Plant Science* **1**, 178-184
Tong YP, Kneer R, Zhu YG (2004) Vacuolar compartmentalization: a second generation approach to engineer plants for phytoremediation. *Trends in Plant Sciences* **9**, 7-9
Trontin JF, Harvengt L, Garin E, Lopez-Vernaza M, 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
Trontin JF, Michel R, Germain A, Pillet-Emanuel H, Canlet F, Harvengt L (2007) *Bar* genetic transformation of maritime pine. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIX. p 20 (Abstract)
Tuominen H, Puech L, Regan S, Fink S, Olsson O, Sundberg B (2000) Cambial-region-specific expression of the *Agrobacterium iaa* genes in transgenic aspen visualized by linked *uid*A reporter gene. *Plant Physiology* **123**, 531-541
Twyman RM, Stoger E, Kohli A, Capell T, Christou P (2002a) Selectable and screenable markers for rice transformation. *Molecular Methods and Plant Analysis* **22**, 1-17
Twyman RM, Stoger E, Kohli A, Christou P (2002b) Foreign DNA: integration and expression in transgenic plants. In: Setlow JK (Ed) *Genetic Engineering Principles and Practices* (Vol 24), Kluwer Academic/Plenum Publishers, NY, pp 107-136
Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: Host system and expression technology. *Trends in Biotechnology* **21**, 570-578
Tzfira T, Yarnitzky O, Vainstein A, Altman A (1996) *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis*. *Plant Cell Reports* **16**, 26-31
Tzfira T, Zuker A, Altmann A (1998) Forest-tree biotechnology: Genetic transformation and its application to future forests. *Trends in Biotechnology* **16**, 439-446
Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends in Cell Biology* **12**, 121-129
van den Elzen PJM, Townsend J, Lee KY, Bedbrook JR (1985) A chimaeric hygromycin resistance gene as a selectable marker in plant cells. *Plant Molecular Biology* **5**, 299-302
van Raemdonck D, Jaziri M, Boerjan W, Baucher M (2001) Advances in the improvement of forest trees through biotechnology. *Bulgarian Journal of Botany* **134**, 64-78
Vaucheret H, Beclin C, Elmayan T, Feuerbach F, Gordon C, Morel JB, Mourrain P, Palaugui JC, Vernhettes S (1998) Transgene-induced silencing in plants. *The Plant Journal* **16**, 651-659
Villalobos-Amador E, Rodriguez-Hernandez G, Perez-Molphe-Balch E (2002) Organogenesis and *Agrobacterium rhizogenes*-induced rooting in *P. maximartinezii* and *P. pinceana*. *Plant Cell Reports* **20**, 779-785
Wagner A, Moody J, Grace LJ, Walter C (1997) Stable transformation of *Pinus radiata* based on selection with hygromycin B. *New Zealand Journal of Forest Science* **27 (3)**, 280-288
Walter C, Smith DR, Connett MB, Grace L, White DWR (1994) A biolistic approach for the transfer and expression of a *uidA* reporter gene in embryogenic cultures of *P. radiata*. *Plant Cell Reports* **14**, 69-74
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, Grace LJ, Donaldson SS, Moody J, Gemmell JE, van der Mass S, Kvaalen H, Lonneborg A (1999) An efficient biolistic transformation protocol for *Picea abies* embryogenic tissue and regeneration of transgenic plants. *Canadian Journal of Forest Research* **29**, 1539-1546
Walter C (2002) Genetic engineering as promising tool in forest biotechnology. In: Pandalai SG (Ed) *Recent Research Developments in Plant Biology* (Vol 2), Research Signpost, Kerala, India, pp 245-260
Walter C, Charity J, Grace L, Hofig K, Moller R, Wagner A (2002) Gene technologies in *Pinus radiata* and *Picea abies*: Tools for conifer biotechnology in the 21st century. *Plant Cell Tissue and Organ Culture* **70**, 3-12
Walter C, Fenning T, Carson M (2007) The benefits of tree biotechnology to science and society. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SV. p 1 (Abstract)
Warwick SI, Beckie H, Small E (1999) Transgenic crops: new weed problems for Canada. *Phytoprotection* **80**, 71-84
Wegner J (2006) Plant chimeras. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol I), Global Science Books, Isleworth, UK, pp 197-202
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
Westcott RJ (1994) Production of embryogenic callus from nonembryonic explants of Norway spruce *Picea abies* (L.) Karst. *Plant Cell Reports* **14**, 47-49
WHO (1993) Health aspects of markers in genetically modified plants. Report of a WHO Workshop, WHO, Geneva, 45 pp
Wilson SM, Thorpe TA, Moloney MM (1989) PEG-mediated expression of GUS and CAT genes in protoplasts from embryogenic suspension cultures of *Picea glauca*. *Plant Cell Reports* **7**, 704-707
Woo SS, Jiang J, Gill BS, Paterson AH, Wing RA (1994) Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*. *Nucleic Acids Research* **22**, 4922-4931
Zhang HB, Choi S, Woo S-S, Li Z, Wing RA (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Molecular Breeding* **2**, 11-24