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

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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
Biomediation ............................................................. 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
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 productivity and disturbing effects of conifer forests are some 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 competitiveness 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 as-
says, 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 2001a, 2001b, 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). Filatti 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 kesiyai (Malabadi and Nataraja 2007b), Pinus roxburghii (Malabadi and Nataraja 2007a), Pinus wallichiana (Malabadi and Nataraja 2007c, 2007d, 2007f, 2007h) provides the best opportunities to produce transgenic plants in a number of species that will lead to their application in commercial forestry (1992) (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 as phenotypically dominant genes for improving 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

Selective 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 have incorporated the DNA, and 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 not. There are many 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 is based on a condition 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 nptII 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 (Twyma et al. 2002;
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; Malabadi et al. 2006). In corn and confiers, a large number 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. 2001; 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; Tereus et al. 2007i; Tang et al. 2007i; Tang et al. 2007). This enzyme utilizes the external substrate 4-methyl umbelliferone glucuronide for measurement 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 nptII allows the visualization of transformation in addition to selection (Cerda et al. 2000; Charity et al. 2005; Malabadi and Nataraja 2007j). 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 plant 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. Electro- transfection of P. radiata protoplasts with a plasmid containing a firefly luciferase reporter gene driven by a 355 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 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; Tian et al. 2006)

Bacterial aminoglycoside 3-phosphotransferase II, also known as neomycin phosphotransferase II (nptII), was shown to be effective as a selectable marker tested in many plant species including conifers (Miki and McHugh 2004). The nptII 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 2007j). 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 nptII gene has generally achieved 10,000 copies per cell and can accumulate up to 1% of total protein (Miki and McHugh 2004). Further a
From 52 hygromycin-resistant lines obtained, 47 showed levels resulting in plant cell death (OECD 1999; Miki and for biolistic transformation of black spruce (Picea mariana) hygromycin resistance was an effective selectable marker for selection after kanamycin in conifers (Tian et al. 2000; Wagner et al. 2007). This enzyme, when used as a selectable marker with nptII was not found to be effective (Twymon et al. 2002). Hygromycin B is the second most frequently used antibiotic for tissue-culture-related regeneration difficulty (Birch 1997; Tang and Newton 2003; Nigro et al. 2004; Altpeter et al. 2005). 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 has been the most widely for generating transgenic conifer trees, and the delivery of transgene into embryogenic tissues by particle bombardment remains the principal 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 contamination, and occurred transgene integration in 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 nptII gene driven by the nptII 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 encoding chimeric GUS and CAT constructs, using the CaMV 3S promoter, was introduced into P. glauca protoplasts using polyethylene glycol-mediated transient expression was detected by histochemical 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 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 has been the most widely for generating transgenic conifer trees, and the delivery of transgene into embryogenic tissues by particle bombardment remains the principal 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 contamination, and occurred transgene integration in 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).

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expression (Walter et al. 1998; Malabadi and Nataraja 2007a, 2007b, 2007c). In Pines, 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 uidA 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 bombardment 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 biotic 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 a 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 uidA or gfp is used routinely to compare different expression constructs and identify those with the most appropriate activity in pines for example, Larix decidua (Duchesne et al. 1993), Larix leptopeles (Duchesne et al. 1993), Larix leptoeuropaeae (Duchesne et al. 1993), Larix eurolepis × hybrid larch (Duchesne et al. 1993), P. glauca (Ellis et al. 1991; Tian et al. 1994).

**Table 1 Transformation of conifers by particle bombardment.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Bombarded tissue</th>
<th>Plasmid vectors</th>
<th>Gene expression</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>P. roxburghii</td>
<td>Embryogenic tissue from mature trees</td>
<td>pAHC25</td>
<td>stable expression</td>
<td>Malabadi and Nataraja 2007a</td>
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<td>Embryogenic cells</td>
<td>pRT99GUS</td>
<td>transient expression</td>
<td>Duchesne et al. 1993</td>
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<td>L. leptoeuropaeae</td>
<td>Embryogenic cells</td>
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<td>L. leptoeuropaeae</td>
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<td>L. (entrelapos × hybrid larch)</td>
<td>Embryogenic cells</td>
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<td>Duchesne et al. 1993</td>
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<td>pTUBT41100</td>
<td>stable expression</td>
<td>Ellis et al. 1993</td>
</tr>
<tr>
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<td>pRT99GUS</td>
<td>stable expression</td>
<td>Robertson et al. 1992</td>
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<td>P. radiata</td>
<td>Embryogenic cells</td>
<td>pB112, pCAMVLAN</td>
<td>transient expression</td>
<td>Campbell et al. 1992</td>
</tr>
<tr>
<td>P. mariana</td>
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<td>pRT99GUS, pB113bp</td>
<td>transient expression</td>
<td>Duchesne and Charest 1991</td>
</tr>
<tr>
<td>P. glauca</td>
<td>Embryos, seedlings</td>
<td>pUC19</td>
<td>transient expression</td>
<td>Ellis et al. 1991</td>
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<td>P. taeda</td>
<td>cotyledons</td>
<td>pB112</td>
<td>transient expression</td>
<td>Stomp et al. 1991</td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>cotyledons</td>
<td>pTVBTGS</td>
<td>transient expression</td>
<td>Goldfrab et al. 1991</td>
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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 of 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 (Vincent et al. 1993; Charis 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 developing cells in 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 transgenotypes 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 of Agrobacterium in plant 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 in Altpeter et al. 2005).

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. 2003). This resulted in transient transformation frequencies varying from 15 to 45% 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 pre-treatment, 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 24 h to improve 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; Ragan et al. 1999; Tuominen et al. 2000). In another biolistic method of pollen transformation of three species of white 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; in another biolistic method of pollen transformation, 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 ensures higher probability of hitting cells. For trees other than conifers, 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 (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, cry1Ac, were produced by means of biolistic transformation of embryonic 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 the fact that a single somatic cell is programmed towards somatic 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 2007b). Therefore, the transgenic trees 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-requrement 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 enhancers may be required for efficient DNA delivery (Malabadi and Nataraja 2007a). 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. 2000; 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 (Elfrstrand 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 genetic information. But due to this, it 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. kesiyai and P. wallichiana (Malabadi and Nataraja 2007b, 2007c), L. laricina (Klimaszewska et al. 1997), P. abies (Robertson et al. 1992; Walter et al. 1999; Clapham et al. 2000; Bishop-Hurley et al. 2001; Elfrstrand et al. 2001), P. glauca (Bommi-neni 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, cryIAc, were produced by means of biotic transformation of embryogenic tissue (Grace et al. 2005). Using the selectable marker gene nptII 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 larvae of the painted apple moth, Tetia anartoides, demonstrated that transgenic conifer transgenic lines exhibited 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 confers 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) assessed the production of Basta-resistant plantlets from 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. mari-ana 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 pR99Gus (Robertson et al. 1992). Stable transformation of P. glauca by biolistic and transgenic regeneration 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. kesiyai, 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 metabolic activity (Malabadi et al. 2005). 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). P. kesiyai, the introduction of a bar-GUS cassette under the control of the ubiquitin promoter was achieved through biotic gene transfer (Malabadi and Nataraja 2007b). Expression of positive histochemical GUS activity (39%) in the bombarded embryogenic tissue was observed. PCR analysis of bar transgenes (46%) transformation efficiency indicated successful genetic modifications of P. kesiyai embryogenic tissue by using the pAH25 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 biotic 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 pAH25 plasmid. This was achieved using the embryogenic tissue derived from the vegetative shoot apic-
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 events sets 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 virB, 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. strobos (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 transgenic lines. 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, genetin-resistant lines recovered was 0.5 per 200 mg fresh weight bombarded tissue. Expression of the uidA reporter gene was detected histochemically and fluorimetrically 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. The uidA gene containing nptII 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 uidA 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, chromosomal or genetic. Genomic mutations affect the number of genes and can be caused by 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, 2007)). It is also important to remember that mutations can occur on nuclear as well as on mitochondrial (mt) or chloroplast (cp) DNA (Foure et al. 1997). Southern hybridization analysis of buds from transgenic trees of P. taeda developed from polyploid transformation experiment confirmed the presence of transgenes and their copy number was determined (Walter et al. 1998). Radiata pine explants transformed with the nptII and the uidA 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 by bioistics, 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 biotic transformation of embryogenic tissue (Tereso et al. 2004). Over 200 plants regenerated from ten transgenic lines were successfully transferred to soil. Bioassays with the larvae of the painted apple moth, *Teia amaroides*, 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 control in trees, particularly since expression in transgenic conifers are not yet available (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. In an attempt to separating the T-DNA from the DNA, Southern analysis showed that truncation/rearrangements are due to the CaMV 3SS 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 3SS promoter (Grant et al. 2004), and recombination events clustered within the T-regions. These 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 *nptII*, *uidA* and *bar* genes in up to ten plants of each individual line (Charity et al. 2005). Molecular analysis revealed the expression of the *nptII* gene varied among the ten lines, while within ten replicates of the same line, *nptII* 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 *nptII* genes in the nine transgenic lines tested (Charity et al. 2005). Hence the relation between gene copy number and gene expression in transgenic conifers 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 biotic- or Agrobacterium-mediated transformation the activity of the promoter determining expression in *P. radiata* 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). Tang et al. (2007) reported the influence of okadaic acid and trifluoperazine on *Agrobacterium*-mediated transformation in eastern white pine (*P. strobos*). Stable integration of *uidA* gene in the plant genome of eastern white pine was confirmed by PCR/Southern/Northern analysis, and concluded that zygotic embryos are excellent targetting 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 of the *bar* gene in *P. wallichiana* (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 bio-Listics 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 obtained from a molecule of the T-DNA from excision from the plant genome, plants were regener-ated 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. strobos*). 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minded (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 pnpII 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 bombardment 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.

<table>
<thead>
<tr>
<th>Features</th>
<th>YAC</th>
<th>BAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Configuration</td>
<td>Linear</td>
<td>Circular</td>
</tr>
<tr>
<td>Host</td>
<td>Yeast</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Copy number/cell</td>
<td>1</td>
<td>1-2</td>
</tr>
<tr>
<td>Cloning capacity</td>
<td>Unlimited</td>
<td>Up to 350 kb</td>
</tr>
<tr>
<td>Transformation</td>
<td>Spheroplast (&lt;10⁷ T/ug)</td>
<td>Electroporation (&lt;10⁷ T/ug)</td>
</tr>
<tr>
<td>Chimerism</td>
<td>Up to 40%</td>
<td>None to low</td>
</tr>
<tr>
<td>DNA isolation</td>
<td>Pulsed-field-gel-electrophoresis-Gel isolation</td>
<td>Standard plasmid miniprep</td>
</tr>
<tr>
<td>Insert stability</td>
<td>Unstable</td>
<td>Stable</td>
</tr>
</tbody>
</table>

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(259)
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 BAC technology demonstrated the feasibility 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. pennelli*, 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). *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 (virA, virB, virD and virE) (Tzfira and Citovsky 2002). The process of gene transfer from *A. tumefaciens* to plant cells implies several essential steps. 1. Bacterial colonization. 2) Introduction of bacterial Ti-plasmid. 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. decidua (Huang et al. 1991), L. decidua (Shin et al. 1994; Humara et al. 1996; L. simplonianaMcAfee et al. 1993), L. laricina (McAfee et al. 1993), L. decurrens (Stomp et al. 1990), P. abies (Wenck et al. 1999; Klimaszewska et al. 2001), P. engelmannii (Ellis et al. 1989), P. glauca (Ellis et al. 1989; Le et al. 2001; Klimaszewska 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. elderica (Stomp et al. 1990), P. elliottii (Stomp et al. 1990), P. halepensis (Tzfra 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. picea (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. wallachiana (Himalayan blue pine) (Malabadi and Narataraj 2007).

*A. tumefaciens* is more than the causative agent of crown gall disease among 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 species. This advance has both theoretical 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. 2001b). 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-
formed cells. It has also been 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 2007; 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

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Agrobacterium strain</th>
<th>Plasmid vectors</th>
<th>Gene expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. palustris (A. t.) EHA105</td>
<td>pBi121</td>
<td>stable expression</td>
<td>Malabadi and Nataraja 2007i</td>
<td></td>
</tr>
<tr>
<td>P. pinaster (A. t.) C58/pMP90</td>
<td>pCAMBIA1301</td>
<td>stable expression</td>
<td>Trontin et al. 2007</td>
<td></td>
</tr>
<tr>
<td>P. pinaster (A. t.) C58/pMP90</td>
<td>pPCV6NFGUS</td>
<td>stable expression</td>
<td>Milhinhos et al. 2007</td>
<td></td>
</tr>
<tr>
<td>P. strobus (A. t.) GV3101</td>
<td>pCAMBIA1301</td>
<td>Stable expression</td>
<td>Tang et al. 2007</td>
<td></td>
</tr>
<tr>
<td>P. pinaster (A. t.) C58/pMP90</td>
<td>pPCV6NFGUS</td>
<td>stable expression</td>
<td>Tereso et al. 2006</td>
<td></td>
</tr>
<tr>
<td>P. radiata (A. t.) EHA105</td>
<td>pTOK47</td>
<td>stable expression</td>
<td>Charity et al. 2005</td>
<td></td>
</tr>
<tr>
<td>P. radiata (A. t.) AG1L, LBA4404, EHA101</td>
<td>pAK64</td>
<td>stable expression</td>
<td>Grant et al. 2004</td>
<td></td>
</tr>
<tr>
<td>P. mariana (A. t.) C58/pMP90</td>
<td>pBi1</td>
<td>stable expression</td>
<td>Klimaszewska et al. 2001, 2003</td>
<td></td>
</tr>
<tr>
<td>P. glauca (A. t.) C58/pMP90</td>
<td>pBi121</td>
<td>stable expression</td>
<td>Klimaszewska et al. 2001, 2003</td>
<td></td>
</tr>
<tr>
<td>P. abies (A. t.) EHA105, LBA4404, GV3101</td>
<td>pBi121</td>
<td>stable expression</td>
<td>Le et al. 2001</td>
<td></td>
</tr>
<tr>
<td>P. abies (A. t.) C58/pMP90</td>
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<td>stable expression</td>
<td>Klimaszewska et al. 2003, 2001</td>
<td></td>
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<tr>
<td>P. taeda (A. t.) EHA101, 105</td>
<td>pGUSr, pSSLa3</td>
<td>stable expression</td>
<td>Gould et al. 2002</td>
<td></td>
</tr>
<tr>
<td>P. radiata (A. t.) LBA4404</td>
<td>pBi121</td>
<td>stable expression</td>
<td>Cerdà et al. 2002</td>
<td></td>
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<tr>
<td>P. radiata (A. t.) AG1L</td>
<td>pSKY1</td>
<td>stable expression</td>
<td>Charity et al. 2002</td>
<td></td>
</tr>
<tr>
<td>P. maximowiczii (A. t.) A4</td>
<td>pRi4A</td>
<td>stable expression</td>
<td>Villalobos-Amador et al. 2002</td>
<td></td>
</tr>
<tr>
<td>P. maximowiczii (A. t.) A4</td>
<td>pRi4A</td>
<td>stable expression</td>
<td>Villalobos-Amador et al. 2002</td>
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<td>P. taeda (A. t.) LBA4404</td>
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<td>Tang 2000</td>
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<tr>
<td>P. taeda (A. t.) GV3101</td>
<td>pCV6NHpyGUSINT</td>
<td>stable expression</td>
<td>Tang et al. 2001</td>
<td></td>
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<tr>
<td>P. taeda (A. t.) LBA4404, GV3101</td>
<td>pWWS006</td>
<td>stable expression</td>
<td>Wenck et al. 1999</td>
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<tr>
<td>P. contorta (A. r.) LBA9402</td>
<td>pHRGpIII-GUS</td>
<td>stable expression</td>
<td>Lindroth et al. 1999</td>
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<tr>
<td>P. pinea (A. t.) EHA105, GV3850, LBA4404, C58</td>
<td>P35S:GUSINT</td>
<td>transient expression</td>
<td>Humara et al. 1999</td>
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<tr>
<td>P. abies (A. t.) EHA105, LBA4404, GV3101</td>
<td>pWWS006</td>
<td>stable expression</td>
<td>Wenck et al. 1999</td>
<td></td>
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<td>P. palastris (A. t.) A4; (A. t.) 208</td>
<td>pRi4A, wild Ti</td>
<td>stable expression</td>
<td>Diner et al. 1999</td>
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<tr>
<td>L. kaempferi × L. deciduas (hybrid larch) (A. t.) C58/pMP90/pMReK07Km</td>
<td>P35S:ndT1-p19S-ndT1</td>
<td>stable expression</td>
<td>Leeve et al. 1997</td>
<td></td>
</tr>
<tr>
<td>P. halepensis (A. r.) LBA9402</td>
<td>P35S:GUSINT</td>
<td>stable expression</td>
<td>Tzfira et al. 1996</td>
<td></td>
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<tr>
<td>L. deciduas (A. r.) 11325</td>
<td>pCGN1133/pWB139</td>
<td>stable expression</td>
<td>Shin et al. 1994</td>
<td></td>
</tr>
<tr>
<td>L. laricina (A. r.) A4/R100</td>
<td>A4/pRi4Ab</td>
<td>stable expression</td>
<td>McAfee et al. 1993</td>
<td></td>
</tr>
<tr>
<td>P. banksiana (A. r.) A4/R100</td>
<td>A4/pRi4Ab</td>
<td>stable expression</td>
<td>McAfee et al. 1993</td>
<td></td>
</tr>
<tr>
<td>P. monticola (A. r.) A4/R100</td>
<td>A4/pRi4Ab</td>
<td>stable expression</td>
<td>McAfee et al. 1993</td>
<td></td>
</tr>
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<td>L. deciduas (A. r.) pTH1325</td>
<td>pBi11325</td>
<td>stable expression</td>
<td>Huang et al. 1991</td>
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<tr>
<td>P. radiata (A. r.) C27/4/54</td>
<td>C2/74/54</td>
<td>Gall formation</td>
<td>Bergman and Stomp 1992</td>
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<tr>
<td>Tsuga heterophylla (A. t.) A136</td>
<td>A136</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
<td></td>
</tr>
<tr>
<td>P. menziesii (A. t.) A281/542</td>
<td>A281/542</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
<td></td>
</tr>
<tr>
<td>P. virginiana (A. t.) 542/M2/73/U3</td>
<td>542/M2/73/U3</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
<td></td>
</tr>
<tr>
<td>P. sylvestris (A. t.) 542/M2/73/U3</td>
<td>542/M2/73/U3</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<tr>
<td>P. elliotii (A. t.) U3</td>
<td>U3</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<td>P. eldarica (A. t.) U3</td>
<td>U3</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<td>P. jeffreyi (A. t.) C58</td>
<td>C58</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<tr>
<td>Libocedrus decurrens (A. t.) C58/M2/73</td>
<td>C58/M2/73</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<td>P. lambertiana (A. t.) pTH1325</td>
<td>pBi11325</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<tr>
<td>P. menziesii (A. t.) A281/542</td>
<td>A281/542</td>
<td>Gall formation</td>
<td>Stomp et al. 1990</td>
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<tr>
<td>Pseudotsuga menziesii (A. t.) A281/542</td>
<td>pEDDK/pLUX2</td>
<td>Gall formation</td>
<td>Ellis et al. 1998</td>
<td></td>
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<tr>
<td>L. deciduas (A. t.) ATCC12</td>
<td>ATCC12</td>
<td>Tumor formation</td>
<td>Diner and Karnosky 1987</td>
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</tr>
<tr>
<td>P. taeda (A. t.) M2/73/U3</td>
<td>M2/73/U3</td>
<td>Gall formation</td>
<td>Sederoff et al. 1986</td>
<td></td>
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<tr>
<td>Abies nordmanniana (A. t.) C58/pV3304/pV3851/pV2298</td>
<td>C58/pV3304/pV3851/pV2298</td>
<td>Tumor formation</td>
<td>Clapham and Ekberg 1986</td>
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A. t. = Agrobacterium tumefaciens; A. r. = Agrobacterium rhizogenes
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, sonication at higher 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). Lower (25-80 μM) or high concentrations of acetosyringone (more than 100-150 μM) severely negatively affected the 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 and trifluoperazine 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 2007). Genetic transformation was achieved using Agrobacterium strain EHA105 fused with a binary vector pBI121 containing nptII as a selectable marker gene and uidA 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 2007). 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 suspension just before infection (2000 mg/L in PW39 line, 36% in PW120 line and 14% in PW and PW10 lines) the transformation efficiency in P. wallichiana (Malabadi and Nataraja 2007). 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 2007). Further, 470 mg/l of acetoxyime was crucial for the recovery of embryogenic tissue after co-cultivation, and 35 mg/l 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 2007).

The method of transformation in P. wallichiana had no effect on the integration pattern of T-DNAs (Malabadi and Nataraja 2007). 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 2007). 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 explants of young growths. Mature culture 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; Leli-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 media, and agrobacterium dependent transient 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 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 our study of P. wallichiana, 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 2007). 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 mg/l timentin, which was critical for the recovery of transgenic tissues. Cerda et al. (2007) 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 nptII 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 (Trentin et al. 2007). They also noticed the low maturation yields of transgenic tissue related with ageing tissues during the whole maturation process in P. pinaster (Trentin et al. 2007). In another Agrobacterium study of P. pinaster, the omission of casein hydrolysate 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
Genetic transformation of conifers. Malabadi and Nataraja

conifers, an increase in transformation efficiency of embryogenic tissues by adding acetosyringone was reported, such as in *P. strobos* (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 2007). However, there was no effect of acetosyringone in a few conifers such as *L. kaempferi* × *L. decidualis* (Leeve et al. 1997) and French genotypes of *P. pinaster* (Charity et al. 2001). 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 hydrolysate supplemented with 400 mg l⁻¹ timentin in *P. pinaster* (Levee et al. 2002). In one protocol, the bacteria 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 hydrolysate supplemented with 400 mg l⁻¹ timentin in *P. pinaster* (Tereso et al. 2002). In one protocol, the bacterial drops were repeated twice, following 4 ml of cells which were transferred to plates containing 400 mg l⁻¹ timentin and 10 mg l⁻¹ kanamycin (Wenck et al. 1999). In our own study of *Agrobacterium* transformation in *P. wallichiana*, the lower concentrations of cetoxafin (100-400 mg l⁻¹) and kanamycin (5-30 mg l⁻¹) were not effective (Malabadi and Nataraja 2007). Therefore, on the basis of a literature survey, the optimum concentration of cetoxafin (in our own study of *P. wallichiana*) or other antibiotics such as chloramphenicol, streptomycin, ampicillin, carbencillin, neomycin, and tetracyclin (350-470 mg l⁻¹) and kanamycin (25-35 mg l⁻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 2007). Further these antibiotic concentrations and the type of antibiotic used in the transformation study, vary from species to species or from genotype to genotype in plants including conifers. A. *tumefaciens-mediated* transformation protocol was developed for embryogenic cell cultures of *P. radiata* (Charity et al. 2005). 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 suspension cultures by increasing the growth of *tumefaciens* cells and/or by promoting 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* (Bryant 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 genetin (15 mg l⁻¹) 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 biologically-transgenic plants by subculturing on the bacteria-free medium containing geneticin (10 mg/l) in *P. radiata* (Charity et al. 2005). As with other transformation protocols for conifers (Leeve 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 delaying 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. Aro nen (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 was competent for T-DNA transfer or not. 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. (2005) who recovered 100% shoot from a shoot from an apical meristem 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) proposed that the physical plasmid status of the transformation vector is 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 ds subculture, provides suitable material to get a high frequency of transformation in white spruce, additionally, cotransformation 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 \textit{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 \textit{A. tumefaciens} with plant cells, including those of conifers. It was also reported that coculture of explants with \textit{A. tumefaciens} induces rapid necrosis in tissue (Grant et al. 2004). Another factor that influences infectivity is the extracellular matrix deposits and sugars and a low pH. However, in our own study of transformation with \textit{P. wallichiana} these factors did not affected the rate of transformation (Malabadi and Nataraja 2007). 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 \textit{Agrobacterium} (Malabadi and Nataraja 2007)). These studies demonstrated that several factors are important for the application of \textit{Agrobacterium}-mediated gene transfer in conifers and other plants. Among them is the rate of infection. Infectivity varies among different \textit{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 collection marker genes and selection agents and promoters used (Ellis et al. 1989; Bergmann and Stomp 1992; Wenc et al. 1999), Humara et al. (1999) reported the transfer and expression of foreign chimeric genes in cotyledons of \textit{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 \textit{Agrobacterium}-mediated gene transfer has been widely used in many plant species of conifers. Transgenic regenerated plantlets were obtained with \textit{L. decidua}, \textit{P. halepensis} and \textit{P. menziesii} (Dandekar et al. 1987; Huang et al. 1991; Tzfira et al. 1996, 1998), and hybrid larch was transformed by \textit{A. tumefaciens} (Levee et al. 1997). However, large-scale use of this \textit{Agrobacterium} in conifer transformation has been limited due to difficult propagation of explant material, selection inefficiencies and low transformation frequency (Wenc 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 \textit{Agrobacterium} strains (Wenc et al. 1999). It was also a useful tool for large-scale generation of transgenic Norway spruce. Both family and species types are important for the application of \textit{Agrobacterium}-mediated gene transfer in conifers. Embryos of 24 open-pollinated families of loblolly pine (\textit{P. taeda} L.) were used as explants to conduct in vitro regeneration (Tang et al. 2001). \textit{A. tumefaciens} was used to transform mature zygotic embryos of seven families of loblolly pine. Ninety transgenic plants were regenerated from large-scale culture of transgenic proplasts (Tang 2001; Tang et al. 2001). In Pinus, transgenic plants of three \textit{Picea} species, i.e. \textit{P. glauca}, \textit{P. mariana} and \textit{P. abies} were produced by this method of transformation. Transgenic plants were regenerated for all three \textit{Picea} species (Klimaszewska et al. 2001). In \textit{P. radiata}, Charity et al. (2002) demonstrated that an \textit{Agrobacterium}-mediated transformation protocol could be developed for detached cotyledon explants and embryos. Transient expression of firefly luciferase 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 \textit{A. tumefaciens} (Charity et al. 2002).

A method for \textit{A. tumefaciens}-mediated transformation of \textit{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 \textit{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 \textit{nptII} gene when analyzed by PCR. This is the first report of transgenic radiata pine plantlets from isolated cotyledons using \textit{A. tumefaciens}-mediated transformation (Grant et al. 2003). An efficient protocol for the transformation of white spruce \textit{(P. glauca)} embryogenic tissue was developed using \textit{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 \textit{P. radiata} embryogenic tissue by \textit{Agrobacterium} was also reported by Cerda et al. (2002). Further Charity et al. (2005) reported the consistent and stable expression of the \textit{nptII}, \textit{uidA}, and \textit{bar} genes in transgenic \textit{P. radiata} after \textit{A. tumefaciens}-mediated transformation using nurse cultures. Tang et al. (2007) recently reported that oka-daic acid and trifluoperazine enhance \textit{Agrobacterium}-mediated transformation in eastern white pine (\textit{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 comparison 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 \textit{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 \textit{P. glauca} (Bekkaoui et al. 1988; Wilson et al. 1989), \textit{P. mariana} (Tautorus et al. 1989), \textit{P. taeda} (Gupta et al. 1988), and \textit{Larix × eurolepis} (Charest et al. 1991), and from non-embryogenic cultures of \textit{P. radiata} (Campbell et al. 1992), and \textit{P. banksiana} (Tautorus et al. 1989). According Tautorus et al. (1989), transient expression of the chlorophenolic acetate esterase gene were established \textit{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 \textit{P. glauca}, \textit{P. mariana} and \textit{P. banksiana} protoplasts is depend on the promoter transient electron 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; Agrawal 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 groundwater 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) 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 Lobolly 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 to uptake trichloroethylene (TCE) and dichloroethylene (DCE) and reduce them to ethane (Boerjan 2007). Molecular insight into these pleiotrophic effects is essential if we are to use cell wall engineering in the future use of such plants with or without transgenic modification.

One of the most successful phytoremediation efforts has been the demonstration that transgenic poplars are able to remove ethylenedibromide from water (Tibbits et al. 2007). Their 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 source and industrial material (Chiang 2002; Doebler 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, 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. 1998). 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). Confiers 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 quality for example conversion of plant cell walls to biofuels (Boerjan 2007). Molecular insight into these pleiotrophic effects is essential if we are to use this cell wall expression in the future use of such plants with or without transgenic modification.

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 Transformed Somatic Sector Analysis (TSSA) 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-glucosyl-
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 fungus pathogen associated with MPB, while *Leptogium obetium* 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 ofSitka spruce and Norway spruce in North America (Kolosova et al. 2007). Europen shoot-tip moth (*Rhyacionia buoliana*), a pest endemic to Chilean forests, is responsible for damage to approximately 50% 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 *cry1Ac*, 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 bio-listic 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 *nptII* gene and uidA 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 xylegious tissue (Moyle et al. 2002). Transgenic roots were induced by infection with *Agrobacterium rhizogenes* strain LBA9402, harboring a binary plasmid construct that contained one of the following promoters such as; *Ubi-1* from *Zea mays*, 35S from CaMV, *cdc2a* and *sam-1* from *A. thaliana*, *HRGP* 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. Elsfstrand et al. (2001) have studied the effects of an endogenous peroxidase-like gene from *P. abies*, *spr2*, on the development and growth of Norway spruce somatic embryo plants. Overexpression of *spr2* 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 *Agrobacterium* virulence and T-DNA transfer to disarmed strains of *A. tumefaciens* increased transformation efficiencies for embryogenic *P. abies* (Wench 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 *Florcicaula* from *P. radiata* and black spruce (*Picea mariana*). Bishop-Hurley et al. (1999). Studies on *P. abies* showed 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 (Elsstrand 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 *PpRab1* from *P. pinaster*. In so-matic embryos, *PpRab1* appears to be expressed abundantly in stage T4 and decreasing towards the maturation of somatic embryos and the subcellular localization of *PpRab1* protein by green fluorescent technology and to develop genetic
complementation assays to prove the function of PpRab1 protein has been analyzed in P. pinaster (Goncalves 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 many countries where tree biotechnology leads to the development 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 CO2 sequestration indicate that trees, and in particular those enhanced using biotechnology, will make a large difference in the near future. 

Secondary needles as the starting material. An embryogenic bryogenic suspension masses derived either from immature or mature explant in many plant species, including conifers. All the explants such as pollen grains, cotyledon, mature and immature tissues can be used as starting material in the tissue culture protocol before thinking of genetic transformation. Tissue culture is genotype dependent since the type of the explants used as starting material in the tissue culture protocol before thinking of genetic transformation. Tissue culture is essential steps will improve the current transformation studies and helps in solving the problems of traditional tree breeding.

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 regenerative since current tissue culture protocols in a few conifers 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 protocols for conifers. Agrobacterium of many plant species in conifers in a few conifer 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. 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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 technologies used. 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. Other factors behind the genetic inclusion 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 this risk of funding use 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
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 and restricted by GM trees. 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 also other limitations by 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 are unlikely to cross with native trees for this purpose has not been tested, 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 political 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 restricted by GM trees. 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.
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 ope- rated 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. Also, new technologies 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 coni- fers with commercially useful traits such as herbicide, in- sect 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 transforma- tion systems to introgress genes from amenable model varieties into elite defined parents. The ability to target or- ganized tissues reduces or eliminates the requirement for tissue culture, and therefore, limits the occurrence of soma- clonal variation, which can lead to infertility and morpholo- gical abnormalities in transgenic plants. Refinements of the technology to produce clean transgene loci have been demonstrated clearly that this is not the case of gene silencing, and the particle bombardment has many advantages for the pro- duction of commercial transgenic plants that perform well in the field and comply with all relevant regulatory pro- cesses. It is shown that particle bombardment and Agrobac- terium-mediated genetic transformation are likely to continue to play an important role in plant biology and for- est biotechnology for many years into the future. At pre- sently 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 poten- tial to provide significant improvement in tree growth and quality. If these problems can be addressed, commercial for- estry gain more importance and emerge as a profitable busi- ness sector.

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