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Tuija Aronen

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Cover illustration: Crown galls in a Scots pine seedling. Photo by Jouko Lehto.

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To my parents

ABSTRACT

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Genetic transformation provides a method for broadening the genetic variation available for breeders, as well as means for studying gene expression, but in most forest tree species transformation techniques are still under development. This is also true in Scots pine (*Pinus sylvestris* L.) which has been involved in the forest tree breeding schemes in Finland as one of the major tree species since the beginning of the breeding activities. The aim of the present study was to test different gene transfer methods in Scots pine, and to look for potential applications of genetic engineering in Scots pine breeding. The transformation techniques studied were *Agrobacterium*-mediated gene transfer using either *A. tumefaciens* or *A. rhizogenes* strains, and particle bombardment. Different types of Scots pine tissues, such as vegetative buds, calli, suspension cultures and pollen, as well as in vivo and in vitro seedlings of various ages and fascicular shoots, were used as target material.

The transient transformation of Scots pine was demonstrated. The best transformation frequencies, up to 55 %, were achieved in pollen. Of the tested explants, pollen also proved to be the best target for aiming at stable transformation and regeneration of transgenic Scots pines via controlled pollinations with bombarded pollen. Whether this approach will work, remains to be seen in the near future due to the long development of Scots pine cones and seeds. Of the tested transformation methods, particle bombardment is more applicable for genetic engineering of Scots pine than the *Agrobacterium*-vectors, since the chemical defence of Scots pine seems to be able to interfere with the gene transfer process of agrobacteria. Nevertheless, *Agrobacterium* inoculations could be utilized for improving the potential to propagate Scots pine vegetatively, through the increased rooting frequency of Scots pine cuttings.

Gene transfer experiments performed in the present study resulted in much information on the factors affecting the transformation efficiency and transgene expression in Scots pine. It was evident that both the characteristics of the explants, i.e. genotype, age and physiological stage, and the transferable gene construct, especially promoter sequences, had remarkable influence on the success of transformation. The present study revealed a yearly trend in the transgene expression under the control of the CaMV 35S promoter, indicating that endogenous mechanisms of the species are also involved in the regulation of foreign gene expression. Moreover, the competence of bud tissues derived from 50-year-old trees for transformation was demonstrated. This is the first time that transgene expression has been reported in explants originating in such old gymnosperms, suggesting that the functioning of maturation-related genes could be studied by introducing them into the bud tissues of both juvenile and mature Scots pines.

As regards the breeding aims of Scots pine, the most important single gene effects that could be introduced into the breeding material would be the ones related to improved timber quality. The Scots pine genotype E1101, "Kanerva pine", represents a natural mutant, in which many desired crown characteristics are combined with vigorous stem growth and a high harvest index. The "Kanerva phenotype" has been suggested to be caused by pleiotropic effects of a single dominant allele, but the gene has not been identified or isolated. The corresponding gene from pendulous Norway spruce has already been mapped. When the spruce pendula gene is available, it will be interesting to introduce it into Scots pine and study whether it causes advantageous effects comparable to "Kanerva allele", and whether these are accompanied by a tortuous stem that is typical of the original "Kanerva phenotype". Other traits, which may be genetically engineered in Scots pine, include reproductive sterility, altered lignin content of wood, and potentially also resistance against pests and pathogens, if global warming causes drastic changes in the existing ecological balance.

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Punkaharju, April 1996

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numerals. All the papers are reprinted with the permission of the publishers.

I. Aronen, T. & Häggman, H. 1995. Differences in *Agrobacterium* infections in silver birch and Scots pine. European Journal of Forest Pathology 25:197-213.

II. Aronen, T. 1996. Interactions between *Agrobacterium tumefaciens* and coniferous defence compounds α-pinene and stilbene. Manusript, submitted.

III. Aronen, T., Häggman, H. & Salonen, M. 1996. Rooting of Scots pine fascicular shoots by *Agrobacterium rhizogenes*. Forest Genetics 3: 15-24. In press.

IV. Aronen, T., Häggman, H. & Hohtola, A. 1994. Transient β-glucuronidase expression in Scots pine tissues derived from mature trees. Canadian Journal of Forest Research 24:2006-2011.

V. Aronen, T., Hohtola, A., Laukkanen, H. & Häggman, H. 1995. Seasonal changes in the transient expression of a 35S CaMV-GUS gene construct introduced into Scots pine buds. Tree Physiology 15:65-70.

VI. Häggman, H., Aronen, T. & Nikkanen, T. 1996. Transformation of Norway spruce and Scots pine pollen by particle bombardment. Manuscript, submitted.

CONTENTS:

1. INTRODUCTION

Scots pine has been involved in the forest tree breeding schemes in Finland as one of the major tree species since the beginning of the breeding activities in the 1940's. The breeding has been based on the phenotypic selection of plus trees from natural stands and on their evaluation by progeny tests. The main target characteristics have been volume growth or wood production in southern Finland, and good climatic adaptation in northern Finland. At the moment, the first results from the progeny trials have been attained, and the establishment of the actual first generation breeding population is going on (Anon. 1989). On the basis of young progeny tests, the phenotypic plus tree selection in southern breeding zones has resulted in better growth and viability, and the estimates of the gains in wood production vary between 5 and 10 percent (Mikola 1992, Venäläinen et al. 1994).

In the Long-Term Tree Breeding Programme for Finland (Anon. 1989) the distinction between short-term and long-term objectives is emphasized. While the long-term breeding focuses on assuring the genetic variability among the breeding materials, the short-term activities are intended as a response to new needs and challenges in a changing forestry environment. In the case of Scots pine, the breeding of quality traits on the basis of the ideotype concept has been mentioned as an example of a specific short-term breeding program. The Long-Term Tree Breeding Programme for Finland also recognizes the possibilities of biotechnology. In the working plan for the years 1990-1999, studies on the potentials of genetic engineering in Scots pine are included as one goal.

Genetic transformation permits the transfer of genes from unrelated species, modified regulation of specific gene expression, and down-regulation of the expression of existing genes (Jones & Cassells 1995). Most of the applications aim at improved resistance against viruses, insects, and herbicides, but also characteristics affecting the product quality are abundant among the target traits. The exponentially expanding number of genetic transformation applications in the agricultural crop improvement results from the great input in the basic research, i.e. the biochemical and genetic studies of target traits, and the development of transformation and regeneration techniques. Although the applications of genetic engineering concentrate on certain crops, practically all the important crop plant species can be transformed at the moment (Law 1995).

Forest tree species differ from agricultural crops as a breeding target in many ways. First of all, the industrial forestry species have been domesticated very recently compared with the agricultural crops, so that in the most advanced programmes in *Eucalyptus grandis* and in *Pinus taeda, P. radiata,* and *P. caribaea* only three or four breeding generations have now been achieved (Haines 1994). On the other hand, most forest tree species still have a tremendous genetic variability, especially for adaptability characteristics. Trees have a long life span with a prolonged juvenile period before reaching reproductive maturity, and the juvenile-mature correlations for example for growth characteristics are often poor. This makes quick breeding

programmes difficult to carry out. Also the physical size of the trees can cause problems in measurement, crossing and seed collection (Zobel & Talbert 1984).

The relatively short history of trees as breeding targets, i.e. less amount of time and man-power put into research, is also reflected in the achievements of biotechnological research. In most of the existing transformation techniques the regeneration of transgenic plantlets is based on tissue culture, which in many forestry species, especially in conifers, is still the stumbling block. The number of propagatable species is, however, increasing, and it is highly probable that with sufficient research effort successful protocols will be developed for all the important species (Haines 1994). Restructuring of nuclear or organellar DNA by genetic engineering also requires knowledge of tree genomes. Information on molecular structure of forest trees has been rather limited, but is rapidly expanding owing to new molecular techniques available. Most of the research is done in conifers. Conifers are unique in having a large nuclear genome, of which a relatively big portion consists of repeated sequence DNA, much with unknown or no function. However, repetitive sequences and many multigene families have already been cloned and to some extent characterized (Kriebel 1993). Also the numerous mapping projects are progressing well (Haines 1994). The prerequisites for genetic engineering of forest trees are thus improving all the time.

Transformation techniques for different forest tree species are currently under active development. All the major transformation techniques, i.e. *Agrobacterium*-mediated transformation, direct gene transfer to protoplasts, and particle bombardment, have been tested with both angiosperms and gymnosperms. Of these techniques, the protoplasts have had least success, mainly due to regeneration problems. Many deciduous tree species have been transformed by *Agrobacterium* (for reviews see Fenning & Gartland 1995, Häggman & Aronen 1996), but so far the only conifer in which the regeneration of transgenic plantlets after *Agrobacterium*-mediated transformation has been published, is European larch (*Larix decidua*) (Huang et al. 1991). The latest technique, particle bombardment, on the other hand, has been applied especially to conifers. At the moment, the production of stably transformed plantlets via particle bombardment has been reported in a few species, i.e. white spruce (*Picea glauca*) (Ellis et al. 1993), black spruce (*Picea mariana*), tamarack (*Larix laricina*) (Charest et al. 1995), and radiata pine (*Pinus radiata*) (Walter & Smith 1995).

The aim of the present study was to test the usefulness of different gene transfer methods in Scots pine, and to look for potential applications of genetic engineering in Scots pine breeding. The transformation techniques studied were the *Agrobacterium*-mediated gene transfer using either *A. tumefaciens* or *A. rhizogenes* strains, and particle bombardment. Different types of Scots pine tissues, such as vegetative buds, calli, suspension cultures and pollen, as well as in vivo and in vitro seedlings of various ages and fascicular shoots, were used as a target material. Genotypic variation in the competence for genetic transformation was also studied. The suitability of the tested techniques for Scots pine transformation and the possibilities of applying gene transfers to future breeding programs are discussed in the light of the results achieved in the present work.

2. GENETIC TRANSFORMATION

2.1. Theoretical background behind genetic engineering

Genetic transformation or manipulation of plant genomes by using recombinant DNA technology, is based on the theory that the genetic code stored in the trinucleotides or codons of DNA is universal. This means that genetic material can be transferred between different species, even between prokaryotes and eukaryotes, still remaining functional. Some exceptions from the universal genetic code are, however, found in unicellular protozoa and in the mitochondria of yeast, invertebrates, and vertebrates, but not in plants. A change that has occured several times is that one of the three signals for terminating protein synthesis, UGA, has come to represent tryptophan, normally uniquely encoded by UGG (Lewin 1990).

Even though the genetic code itself is universal, the organization and regulation of structural genes vary among species. This fact has its implications in plant genetic transformation, because the transferred gene constructs are usually chimeric - built up by joining together regulative and structural DNA sequences from quite different sources. Prokaryotic genes are often expressed as operons coding for more than one protein, while the eukaryotic mRNAs are monocistronic with methylated cap at the 5' end and poly-A tail at the 3' end. Contrary to typical prokaryotes, many eukaryotic genes are interrupted by introns, i.e. additional sequences that lie within the coding region and are removed from mRNA before protein synthesis (Lewin 1990). Moreover, the codon usage differs between bacteria, plants and other eukaryotes. Abundance of rare codons within the coding sequence can thus lead to inefficient expression of transgenes (Fütterer 1995). Each species has its own promoter and enhancer sequences, but they share common modules that are recognized by transcriptional factors, and can therefore be functional in foreign species as well (Lewin 1990). To date, however, the expression signals used for regulating transgenes have been derived from endogenous plants genes, from plant viruses or from the T-DNA of agrobacteria (Fütterer 1995).

In conclusion, a translationally optimized gene construct for plant transformation consists, besides a promoter, of a 5' untranslated leader region, a start codon for translation in optimal sequence context, a coding region without rare codons, and a 3' untranslated region containing transcription terminator and polyadenylation signal from a highly expressed plant gene (Fütterer 1995).

2.2. Transferable genes

The function of chimeric genes in plants has been well demonstrated since the pioneer work of Luis Herrera-Estrella and co-workers in the beginning of 1980´s (Herrera-Estella et al. 1983a, - 1983b). The most commonly used promoter, 35S, originates from cauliflower mosaic virus (Odell et al. 1985), and the prevalent selectable marker genes and reporter genes from bacteria. The selectable marker genes

typically code for resistance to chemical agents, such as antibiotics or herbicides, which are normally inhibitory to plant development. The reporter genes for their part code for products which are directly detectable or catalyze specific reactions whose products can easily be detected (Jefferson et al. 1986, Herrera-Estrella et al. 1988, Schrott 1995). Transgenes, which contribute to characteristics which are interesting from the breeding point of view (Table 1), have usually been cloned either from other plant species or from bacteria (Dale & Irwin 1995).

Table 1. Target traits for plant genetic manipulation (modified from Law 1995 and Dale & Irwin 1995). All the selected examples of modified characteristics, except the ones in italics, have already been demonstrated in agricultural crop plants.

Category	Examples of traits conferred by transgenes
Reduced inputs in cultivation	Herbicide resistance Pest resistance Pathogen resistance *Improved nutrient uptake* *Improved photosynthetic efficiency* *Nitrogen fixation in non-legumes*
Product quality	Improved storage Modified composition or higher content of - sugars and starch - storage proteins - fatty acids - *fibres* Flavour enhancement Flower colour
Environmental stress tolerance	Frost protection Cold tolerance Heavy metal uptake and accumulation
Breeding system	Male sterility

The genes transferred to forest tree species are mainly selectable marker genes and reporter genes, due to the fact that the development of transformation techniques is still going on. Model species for tree transformation are poplars, into which several different genes coding for resistance to herbicides (Fillatti et al. 1987, De Block 1990, Brasileiro et al. 1991, - 1992, Leple et al 1992), the Bt-toxin gene conferring resistance to lepidopteran pests (McCown et al. 1991), and an endogenous peroxidase gene (Kajita et al. 1994) have successfully been introduced. Examples of regenerated

transgenic conifers are few. The T-DNA of *Agrobacterium rhizogenes* has been transferred into European larch, and the transformants were shown to produce opines (Huang et al. 1991). The transfer and expression of the reporter gene β-glucuronidase (GUS) and the selectable marker gene neomycine phosphotransferase (NPT) have been reported in radiata pine (Walter & Smith 1995), in black spruce, and in tamarack (Charest et al. 1995). Transformation of white spruce has resulted not only in the GUS and NPT activity, but also in the expression of *Bacillus thuringiensis cryIA* endotoxin gene in the regenerants (Ellis et al. 1993).

2.3. Function and inheritance of transgenes

When plant genetic engineering is viewed from the breeder's standpoint, one of the most important aspects is the long-term stable activity of introduced genes. In the case of the long-living forest trees this aspect is even more critical than in crops having shorter rotation times. Plants, due to their open developmental systems and to the high frequency of somatic mutations and epigenetic changes, such as sequence amplification and methylation processes, can tolerate drastic changes in genome organization better than animals (Buiatti & Bogani 1995). However, variation in the transgene expression and inactivation of transgenes is often observed.

The inactivation of introduced genes can be caused by several factors (for reviews see Finnegan & McElroy 1994, Matzke & Matzke 1995, Meyer 1995). Integration of multiple copies of the same foreign gene is one reason for transgene silencing, but also retransformation with independent genes sharing homology with firstly introduced constructs may result in *trans*-inactivation. The introduction of genes homologous to endogenous plant genes can cause the co-suppression of both the transgenes and the endogenous genes. The integration of the transgenes into the genome segments having a different base content than the introduced DNA and the variations in the codon usage may lead to the recognition of the transgene as an alien sequence. Or the integration site is simply located in the chromosomal region having a low transcription capacity. The actual mechanisms behind the transgene silencing include the methylation of transgenes or their promoters and post-transcriptional regulation, most probably the degradation of mRNAs.

The stability of transgene expression may vary owing to environmental stimuli and endogenous factors, such as plant age. This phenomenon has been connected to the degree of the DNA methylation (Meyer 1995). Transgenic plants may also suffer from the pleiotropic effects of transgenes. The complex network of plant physiological processes is easily disturbed, especially if the target genes are involved in important metabolic and developmental pathways. The most common pleiotropic consequence of foreign gene introduction is the reduction of yield. All the problems associated with transgene expression can, however, be at least partly compensated for by careful screening and selection of transformants (Buiatti & Bogani 1995, Jones & Cassells 1995).

The data on the function of transgenes in tree species is mainly based on the transient expression results, as well as on the results from stably transformed, but non-

regenerable tissues. The number of species, in which transgenic plantlets have been produced, is still relatively small, and the regeneration of the first transformants was not achieved until the end of the 1980's. Transgenic apples, however, displayed a stable expression of the introduced nopaline synthase and NPT genes in the fruit flesh seven years after the initial transformation. The overall phenotypes of the apple transformants were variable, representing both normal and abnormal growth habits and leaf morphology. In the same study, a Mendelian 1:1 segregation of transgenes was evidenced among the R1 progeny (James et al. 1995). The field trials with transgenic *Populus* hybrids and white spruce have shown a general trend - the highest and least variable expression levels of the enhanced 35S-GUS construct occured *in vitro*, and the most variable and lowest levels in the field grown transgenics. In poplar, the stability of GUS activity was connected to the age of leaves, and in both species yearly trends in the transgene expression were observed. The total suppression of the transgene was noted in some lines of both species and was not restored after three years in the field (Ellis et al. 1995). The Bt-toxin gene introduced into poplar, on the other hand, proved to confer insect resistance also under the field conditions, but on a lower level than achieved in the greenhouse grown transformants (Kleiner et al. 1995).

2.4. *Agrobacterium*-mediated transformation

2.4.1. General gene transfer mechanism

Agrobacteria are common soil inhabitants belonging to the family *Rhizobiaceae*. Phytopathogenic agrobacteria, a small precentage of the total soil populations, carry the so called Ti (tumour inducing)- or Ri (root inducing)- plasmid that has an extraordinary capability to transfer a DNA segment called T-DNA into a plant cell and integrate it into the plant genome. The T-DNA is delimited by 25-bp direct repeats, and any DNA between these borders will be transferred to a plant cell. Wild-type T-DNAs encode enzymes for the synthesis of plant growth regulators and opines, which are nitrogen-containing compounds catabolized by agrobacteria. The insertion and expression of these oncogenes in host plant cause symptoms of either a grown gall or hairy root disease (Clare 1990, Zambryski 1992, Zupan & Zambryski 1995).

The separate virulence (*vir*-) region of the Ti- or Ri-plasmids provides most of the *trans*-acting products for the transfer of T-DNA. It is organized into six main operons, *virA, virB, vir G, virC, virD,* and *virE,* which all have their specific functions during the *Agrobacterium*- mediated DNA transformation, as seen in Table 2. In addition to the *vir*-genes some chromosomal genes are involved in the attachment of agrobacteria to plant cells, in the induction of *vir*-genes, and in the regulation of *vir*-gene expression (reviewed by Pan et al. 1995). The expression of the *vir*-genes is controlled through a two-component regulatory systems consisting of the VirA and VirG proteins (reviewed by Winans 1991). Following the *vir*-gene induction, the T-DNA transfer begins with the generation of T-strand, a single stranded copy of the T-DNA. The T-strand is coated with proteins, and it travels through the bacterial membranes, the bacterial cell wall, the plant cell wall and the nuclear membrane, piloted by the VirD2-leader protein. Recent studies have shown that the T-DNA transfer is evolutionarily related to

the conjugative DNA transfer in bacteria, but the mechanisms for the T-DNA entering into the plant cell still remain unclear (Zupan & Zambryski 1995).

Table 2. The virulence (vir) gene operons of the Ti-plasmid and their functions during the T-DNA transfer from agrobacteria to a plant cell.

Locus	Characteristics of gene products	Reference
virA	A transmembrane protein, recognizes signal molecules from wounded plant cells, such as phenolics and monosaccharides, and activates the VirG protein by phosphorylating it.	Winans 1991
virB	11 proteins, involved in forming a membrane structure or a pore required for exporting the T-DNA out of bacteria.	Zambryski 1992 Thorstenson et al. 1993 Finberg et al. 1995
virG	A transcriptional activator of all *vir*-genes.	Winans 1991
virC	2 proteins enhancing the VirD-mediated cleavage of the T-strand at the T-DNA borders.	Toro et al. 1988
virD	5 proteins, of which the VirD1 and the VirD2 catalyze the nicking of the T-strand at the T-DNA borders. The VirD2 binds covalently to the 5' end of the T-DNA, leads it into plant cell nucleus, and participates in precise integration of the T-DNA into the plant genome.	Lin & Kado 1993 Scheiffele et al. 1995 Tinland et al. 1995 Zupan & Zambryski 1995
virE	A ss nucleic acid -binding protein, coats the T-strand during the forming of the T-complex. Unfolds and extends the T-DNA, and protects it from nucleases. Possibly assists in the nuclear transport of the T-complex.	Zupan & Zambryski 1995

The T-DNA integration into the host plant chromosome is suggested to occur by illegitimate recombination (Mayerhofer et al. 1991). The 5' end of the T-strand joins a nick in the plant DNA, and the 3' end pairs with another plant DNA region nearby. Then probably the plant's own enzymes for DNA repair and recombination join the 3' end covalently to the plant DNA. This results in the introduction of the T-DNA into one strand of the plant DNA, causing a nick into the opposite strand. Then gap repair and DNA synthesis by using the T-DNA as template result in the final integration (Sonti et al. 1995, Zupan & Zambryski 1995). The T-DNA integrations in plant chromosomes are randomly distributed, but the potentially transcribed regions are preferred. Due to the protecting protein coat, the T-DNA is frequently integrated

without large internal deletions, but deletions, inversion and duplications occur often within the target area. It has been estimated that, on an average, probably one in ten transformants contains a single T-DNA copy integrated only at one locus (Meyer 1995, Ohba et al. 1995). There exists, however, some evidence of the *Agrobacterium*-mediated transformation being used also for gene targeting - for example, for restoring a defective locus in the plant chromosome via nonreciprocal homologous recombination with the T-DNA insert (Offringa et al. 1993).

Agrobacterium-mediated transformation can be performed by using several methods. Inoculations with the strains carrying the wild-type Ti- or Ri-plasmids are usually conducted to test the infection ability of particular strains and host genotype - bacterial strain interactions. Co-cultivation is the technique used most often for actual genetic engineering. In this technique disarmed *Agrobacterium*-vectors cured of the oncogenes and carrying transferable genes within the T-DNA borders are cultivated together with the explants for a short time and then killed by antibiotics. The transformed plant cells are selected, and further on, regenerated into plantlets via tissue culture procedures. The transferable genes may be included either in co-integrating or binary form, that is, either in the Ti-plasmid carrying also the *vir*-genes (Rogers et al. 1988) or in a separate compatible replicon. In the latter case the *vir* functions are still encoded by the Ti-plasmid serving as a helper (An et al. 1988). The latest modification of *Agrobacterium*-mediated transformation is called co-inoculation. This technique utilizes a specific *A. tumefaciens* strain allowing a spontaneous shoot development from the tumorous tissues induced by the bacterial oncogenes. The transferable genes are provided in another, disarmed strain carrying a binary plasmid, and the bacterial strains are mixed to form the inoculum suspension. The co-inoculation method was developed in poplar by Brasileiro and co-workers (1991) in order to avoid regeneration problems related to tissue culture.

2.4.2. Applications in conifers

The greatest problem in the application of the *Agrobacterium* sp. for genetic transformation of coniferous forest trees lies in the fact that gymnosperms are not natural hosts for the phytopathogenic agrobacteria. There are only a few reports on naturally occuring crown galls (Brown & Evans 1933, Smith 1935, White & Millington 1954, Phillips & Burdekin 1982), even though many conifers have been shown to be susceptible to *Agrobacterium* under experimental conditions (Diner & Karnosky 1987, Ellis et al. 1989, Hood et al. 1990, Loopstra et al. 1990, Stomp et al. 1990, Han et al. 1994, Huang & Tauer 1994). Also in artificial inoculations the infection rates are frequently low. Another main difficulty in the production of transgenic conifers is caused by the recalcitrance of many conifers in tissue culture.

The only report on the regenerated transgenic conifer produced by *Agrobacterium*-mediated technique is in European larch, *Larix decidua* (Huang et al. 1991). The hypocotyls of young seedlings were inoculated with *A. rhizogenes* carrying the wild-type Ri plasmid. The symptoms at the wound sites included prominent swelling, hairy roots, gall-like tissues, and adventitious buds. These buds were elongated and rooted *in vitro*, and then transferred to greenhouse conditions, where they grew normally.

Transformants were shown to produce opines, and the transformation was confirmed by Southern blot analysis.

The reasons for the low *Agrobacterium* infection frequencies in conifers have been studied to some extent, but an overall cause for incompetence between gymnosperms and agrobacteria has not been found. It has been proved that agrobacteria are able to attach to coniferous cells at the wound site in a manner similar to that described in susceptible dicotyledonous angiosperms (Huang et al. 1993). Also a natural *vir*-inducer, a phenolic compound coniferin, has been isolated from a pinaceous gymnosperm *Pseudotsuga menziesii* (Morris & Morris 1990). The wound reaction of the host plant plays an important role in *Agrobacterium*-mediated transformation. It has been supposed that the ability of wounded plant cells to enter and carry out one or more cycles of cell divisions is a prerequisite for the successful T-DNA transfer, and in the incompatible species the cells around the wound site lignify or sclerify in the absence of apparent cell divisions (Binns 1990, van Wordragen & Dons 1992).

2.4.3. Experiments in Scots pine

In the present work, the *Agrobacterium*-mediated gene transfer was studied in Scots pine by examining the host-pathogen relationship from several points of view (I, II). In the inoculation experiments, the competence of Scots pine seedlings for the wild type *A. tumefaciens* strain A281 and its genetically engineered derivatives A281(pTVK291) and A281(pRT45) containing either extra copies of the *virA, virB, virG* and *virC* loci or a binary plasmid with reporter genes, respectively, was tested. The host plant material consisted of seedlings from 2 to 16 weeks of age, growing both under *in vitro* and greenhouse conditions (I). Also the infection ability of the strain A281(pSY204) carrying a constitutive mutant of the *virG* gene was studied with 2-week-old *in vitro* seedlings (II).

Previously, both the host plant age and the environmental conditions have been observed to affect the infection rates in conifers (Ellis et al. 1989, Hood et al. 1990, Stomp et al. 1990). Genetic engineering of agrobacteria, i.e. T-DNA configuration - cointegrating versus binary- and the inclusion of several plasmids, have also been shown to have an effect on transformation frequencies (Rempel & Nelson 1995). Moreover, the constitutive expression of the *vir*-genes or multiple copies of the *virG* has been demonstrated to enhance the *Agrobacterium*-mediated transformation in several crop plant species (Liu et al. 1992, Hansen et al. 1994), and this approach has been proposed as one solution for developing a functional transformation protocol for gymnosperms as well.

The wound reaction of gymnosperms involves a localized accumulation of oleoresin and the accelerated *de novo* biosynthesis of its components, monoterpenes and resin acids (Gref & Ericsson 1985, Marpeau et al. 1989, Lewinsohn et al. 1991, Sjöström 1993, Funk et al. 1994). Phenolic constituents form another group of defence compounds present in conifers (Sjöström 1993). In the present work, the interactions between coniferous defence compounds, a monoterpene α-pinene and a phenolic constitutent trans-stilbene, and *A. tumefaciens* were examined by studying their effects

on the induction of the *vir*-genes, as well as on the later stages of the bacterial infection (II).

Besides studying the host-pathogen relationship between agrobacteria and Scots pine, the potential applications of *Agrobacterium*-mediated transformation in forest tree breeding were also looked for in the present work. The most realistic application seemed to be the utilization of *A. rhizogenes* for root induction in cuttings. The wild-type *A. rhizogenes* strains typically induce the proliferation of numerous roots at the infection site, and this ability has successfully been used for rooting the cuttings or *in vitro* shoots of several gymnosperms, such as *Larix laricina, Pinus banksiana, P. monticola* (McAfee et al. 1993), and *P. contorta* (Yibrah et al. 1996). In the present work (III), the *A. rhizogenes* strain A4 and the *A. tumefaciens* strain C58 carrying the Ri-plasmid A4b were tested for their root induction capability in Scots pine cuttings. Fascicular shoots, induced by cytokinin sprayings, were used as plant material, and a range of different Scots pine genotypes were evaluated for their competence for *Agrobacterium* infection.

2.5. Particle bombardment

2.5.1. Bombardment devices and their function

Particle bombardment was developed by John Sanford and co-workers at Cornell University to overcome the transformation barriers in species that are incompetent with other gene transfer techniques (Klein et al. 1987, Sanford 1988). The essence of this physical gene transfer method is to accelerate DNA-coated particles directly into plant cells. The original device used a gunpowder charge to accelerate a nylon macroprojectile towards a stop-plate, through which the DNA-coated microprojectiles from the front of the macroprojectile continue to hit the target tissue at high velocity. Nowadays a number of different instruments based on various accelerating mechanisms are available. These include the original gunpowder model, an improved version of it utilizing compressed helium, an apparatus based on electric discharge, a pneumatic instrument, and a device based on flowing helium (Birch & Franks 1991, Klein et al. 1992, Christou 1995). Also a system for micro-targeting the transferable DNA in very small areas of the plant tissue has been developed (Sautter et al. 1991, Potrykus 1992).

There are a number of parameters to be considered carefully in particle bombardment. The particles or microprojectiles should be of sufficiently high mass to be able to penetrate the target tissue, and chemically inert to prevent adverse reactions in plant cells. Also their size, shape, agglomeration and dispersion properties are important. The common choice is either gold or tungsten particles. The nature (single-stranded versus double-stranded), form (linear or circular) and concentration of the transferable DNA should also be considered, as well as the procedure for coating the metal particles with the DNA. Targeting the appropriate cells competent both for transformation and regeneration is of major importance. Thus the depth of particle penetration, and the injuries caused in the target tissues should be adjustable. Besides the physical parameters, the environmental conditions and the characteristics of the

explant are also significant for successful transformation. The explants should, of course, be competent for transformation and, furher on, for regeneration. Their pre- and post-bombardment culture conditions have to be optimized (Christou 1995). In order to achieve high transformation frequencies, the DNA-coated particles should be directed to the nucleus of the target cells (Yamashita et al. 1991). The removal or reduction of the vacuole, osmotic treatments of the target cells resulting in plasmolysis, and the bombardment at the mitotic phase of the cell cycle, have been suggested for improving the transformation frequencies (Iida et al. 1991, Yamashita et al. 1991, Vain et al. 1993, Clapham et al. 1995)

How the actual integration of foreign genes into the plant genome after particle bombardment actually occurs, is not known. It seems to be totally random concerning the distribution of the insertions within the target genome. For avoiding the failures in the transgene expression due to unsuitable genomic matrix, it has been suggested that recombinant genes should be transferred together with chromosomal flanking regions (Scaffold attachment regions) to isolate them from the influences of the integration site. Moreover, it has been observed that the particle acceleration-mediated transformation often results in the integration of multiple and / or truncated copies of the transgene. This is probably caused by the high amounts of DNA used in the technique, and by the fact that the transferred DNA is not coated with the protecting proteins as in the case of the T-complex (Jones & Cassells 1995, Meyer 1995).

2.5.2. Advantages in conifer transformation

In recent years, particle bombardment has been applied for transforming many coniferous species. The technique allows the introduction of foreign DNA into intact plant cells, thus avoiding the host range limitations of *Agrobacterium*-mediated transformation, and the problems of plant regeneration from protoplasts.

Many reports in which the transient expression of reporter genes has been observed in the target tissues following particle bombardment have been published recently. The targets in these studies have usually been young tissues, such as whole embryos or cotyledons of *Picea abies* (Newton et al. 1992), *Picea glauca* (Ellis et al. 1991), *Pinus taeda* (Stomp et al. 1991), *Pinus radiata* (Rey et al. 1995), and *Pseudotsuga menziesii* (Goldfarb et al. 1991), or embryogenic cultures of *Picea abies* (Robertson et al. 1992, Yibrah et al. 1994, Clapham et al. 1995), *Picea glauca* (Charest et al. 1993, Li et al. 1994), *Picea mariana* (Charest et al. 1993, Bommineni et al. 1994), *Picea rubens* (Charest et al. 1993), *Pinus radiata* (Walter et al. 1994), and *Larix* spp. (Charest et al. 1993, Duchesne et al. 1993). Also suspension cells of *Pinus radiata* (Campbell et al. 1992) and differentiating wood of *Pinus taeda* (Loopstra et al. 1992) have been successfully transformed transiently. The latest target for transformation in gymnosperms has been pollen, in which the transient expression of the reporter genes has been achieved in *Picea abies, Pinus pinaster* (Martinussen et al. 1994, Martinussen et al. 1995), *Picea glauca* (Li et al. 1994), *Picea mariana, Pinus contorta, Pinus banksiana, Tsuga heterophylla* and *Chamaecyparis nootkatensis* (Hay et al. 1994). The main focus in the above mentioned studies has been in optimizing the transformation

protocol and in examining the effects of various promoter sequences on the reporter gene expression.

Application of particle bombardment in conifer transformation has also gained some reports on regenerated transgenic plantlets. The first published work was the stable transformation of white spruce, *Picea glauca* (Ellis et al. 1993). In this work, somatic embryos were used as targets for bombardment, and regeneration of transformants occured through embryogenic callus induced in bombarded material. The introduced plasmid DNA contained the GUS and NPT genes together with the Bt-endotoxin gene. The spruce budworm feeding trials with embryogenic calli and transgenic plantlets indicated a low, sublethal expression level of the Bt-toxin gene. Recently, the stable transformation and regeneration of black spruce (*Picea mariana*), tamarack (*Larix laricina*) (Charest et al. 1995) and radiata pine (*Pinus radiata*) (Walter & Smith 1995) have been reported. Mature somatic embryos and embryogenic cultures were used as transformation targets also in these works, and the transferred constructs contained the GUS and NPT genes.

2.5.3. Experiments in Scots pine

The particle bombardment device used in the present work was the helium gas-driven Biolistic® particle delivery system PDS-1000/He, which has been considered as a gentle and effective device (Russell et al. 1992). To avoid the undesired cell injuries due to the toxicity of the microprojectiles (Russell et al. 1992), gold particles were used instead of tungsten ones. The bombardment parameters were optimized for the various types of explant material, including the vegetative buds of Scots pines from 5 to 50 years in age (IV), bud-derived calli and suspension cultures (IV), and pollen (VI).

The competence of different tree genotypes for transformation (V, VI), as well as the effect of the growth regulator pretreatments (IV, V) on the transformation efficiency in Scots pine were tested. Clonal differences in the compatibility for transformation via particle bombardment have been observed earlier in various spruce species (Duchesne & Charest 1991, Newton et al. 1992, Charest et al. 1993, Yibrah et al. 1994), in Douglas fir (Goldfarb et al. 1991), and in larch (Duchesne et al. 1993), but until now not reported in pines.

The gene constructs introduced into Scots pine pollen contained the GUS reporter gene driven by various promoters (VI). The choice of promoter has a remarkable effect on the level of transgene expression, as the experiments with reporter genes have shown in many coniferous species (Duchesne & Charest 1991, Loopstra et al. 1992, Charest et al. 1993, Walter et al. 1994, Yibrah et al. 1994, Clapham et al. 1995, Rey et al. 1995). Promoters, which have frequently given high transient GUS expression results in the above mentioned studies, include the 35S-35S with or without various enhancer sequences, the abscisic acid inducible promoter of the wheat EM gene, and the promoter of the sunflower polyubiquitin gene. All these, as well as the single 35S promoter with and without the AMV enhancer element from the alfa-alfa mosaic virus were tested in Scots pine (VI).

Older Scots pines are recalcitrant for regeneration through tissue culture procedures, but their tissues would be a valuable material for studying the genetics of characteristics, such as maturation or floral development, which are not expressed at the young age. One of the aims in the present work was to examine the competence of tissues derived from mature Scots pines for genetic transformation (IV), in order to see if this technique could be used for studying the genetic functioning in older trees. Subsequently, the 35S-GUS gene construct was introduced into Scots pine buds during different seasons (V). It is known that the natural gene expression level of pine buds varies according to season (Häggman 1986, Nuotio et al. 1990), and it was studied whether a foreign gene, driven by a constitutive promoter, would be expressed in a similar way to native genes.

Keeping in mind the potential applications of genetic transformation in forest tree breeding, the particle bombardment experiments were also performed in Scots pine pollen (VI). The regeneration of transgenic plantlets is required for transferring interesting traits into breeding material. The *in vitro* propagation of Scots pine via somatic embryogenesis is still under development (Hohtola 1995), and the micropropagation based on organogenesis has its own limitations (Supriyanto & Rohr 1994, Häggman et al. 1996). Pollen, on the other hand, is a natural vector for the transfer of genetic material, also providing a regeneration method independent of *in vitro* techniques. In the present work, the Scots pine pollen was transformed and used for controlled pollinations in order to evaluate the potential to produce transgenic progenies (VI).

2.6. Comparison of *Agrobacterium*-vectors and particle bombardment in Scots pine transformation

2.6.1. Transformation frequencies

When comparing the overall transformation frequencies achieved by the *Agrobacterium*-mediated techniques and by particle bombardment in Scots pine, it is clearly seen that particle bombardment was more effective as a gene transfer method than the *Agrobacterium* vectors. The results varied, however, depending on several factors, such as the genotype and the age of the explants, pretreatments, and environmental conditions. It should also be noticed that the stable transformation was not confirmed at a biochemical or molecular level in either of the tested methods. All the bombarded material was tested histochemically only for the transient expression of the GUS reporter gene (IV, V, VI). In the *Agrobacterium* inoculation experiments both tumorous tissues (I, II) and roots (III) were produced at the inoculation sites. Part of the *in vitro* developed gall tissues were able to grow on tissue culture media in the absence of growth regulators, suggesting that the bacterial hormone genes were introduced and expressed in the tissue (I). However, the attempts to prove the integration of the T-DNA genes either by opine analysis or by PCR tests failed both in the case of the gall tissues (I) and the roots (III) formed after the *Agrobacterium* inoculations.

The highest gall formation frequency observed in the Scots pine inoculation experiments with the *A. tumefaciens* strain A281 and its derivatives was slightly over

20 %, and generally the infection frequencies were around or less than 10 % (I, II). The reported infection rates of A281 in other conifers have been higher, such as 75 % in *Picea glauca* (Ellis et al. 1989), 31 % in *Picea abies* (Hood et al. 1990), from 13 to 75 % in various *Pinus* species (Stomp et al. 1990), 93 % in *Larix decidua* (Huang et al. 1993), and 86 % in *Pinus taeda* (Huang & Tauer 1994), but until the present study this strain has not been tested in Scots pine.

In the experiments with the *A. rhizogenes* strain A4 or the *A. tumefaciens* strain C58 carrying Ri-plasmid (called R1600), the bacterial inoculation enhanced the rooting of the Scots pine fascicular shoots. At best, more than 80 % of the cuttings rooted after *A. rhizogenes* treatment and 30-40 % in the control treatment (III). In *Larix decidua* seedlings, the strain A4 has caused a formation of roots in 83 % of the hypocotyl inoculations (Huang et al. 1993). The strain R1600 has been reported to induce hairy root development in 2-3 % of the inoculated hypocotyls of the *Picea abies, Pinus sylvestris,* and *Pinus contorta* seedlings (Magnussen et al. 1994). McAfee and co-workers (1993) have made actual rooting experiments by using the strains A4 and R1000, which is closely related to the strain R1600. In their study they found that both strains improved remarkably the rooting ability of the *Pinus monticola* adventitious shoots (from 1 % in the control, up to 77 %) and the de-rooted seedlings of *Pinus banksiana* (from 64 to 88 %) and *Larix laricina* (from 82 to 100 %). They were, however, not able to show that the roots formed after the bacterial inoculations were transgenic, as was true also in the case of Scots pine (III).

The transformation frequencies after particle bombardment varied according to the target material used, being highest in pollen. In the vegetative buds (IV, V) and bud-derived calli (IV) of Scots pine the transient GUS expression levels were low - usually one or two expression units per bud. In the bombarded suspension cultures of Scots pine, on an average, 0.12 % of the viable cells expressed the reporter gene, and at best 0.23 % (IV). Comparing this result with the studies published on the bombarded suspension cultures of other gymnosperms is difficult, mainly due to the common tendency of the authors to report their results as expression units per disk, per bombardment or per fresh weight unit of the target tissue. Campbell and co-workers (1992) have, however, observed from 3 to 10 GUS expressing cells per 10^7 bombarded cells of *Pinus radiata*, meaning the transformation frequency of 0.0001%, much lower than found in Scots pine (IV).

The best transformation frequencies in Scots pine were found in the bombarded pollen. At the maximum, as high proportion as 55 % of the pollen grains on an average were shown to express the GUS reporter gene (VI). Hay and co-workers (1994) reported in their study with pollen of *Pinus contorta, Pinus banksiana, Picea mariana, Tsuga heterophylla*, and *Chamaecyparis nootkatensis* the maximum transformation frequency in the best species, *P. mariana*, to be 8.7 %. In the pollen of *Picea abies* the proportion of the transformed pollen grains has been less than one percent, based on the data presented by Martinussen and co-workers (1994).

2.6.2. Factors affecting the transformation efficiency and transgene expression

The genotype of the explants affected significantly the competence of Scots pine both for *Agrobacterium* infections and for the transformation via particle bombardment. This was demonstrated in 32 different genotypes tested for root induction by *A. rhizogenes* (III), in the vegetative buds of three 50-year-old individuals bombarded during various seasons (V), and in the bombarded pollen lots collected from 10 different plus trees (VI). The results emphasize the careful choice of the target material, or alternatively, the transformation techniques should be developed further to obtain methods for genotype-independent gene transfer. According to Christou (1995) the limitations set by the target plant genotype are minor in the particle bombardment when compared with the *Agrobacterium*-mediated techniques, but this does not seem to be the case in Scots pine. Keeping in mind the potential applications for breeding, it should be realized that the most interesting genotypes or the best elite trees may have poor competence for transformation, at least by using the current techniques.

The age of the target plant also proved important both in the *Agrobacterium* experiments and in particle bombardment -mediated gene transfer in Scots pine. It was interesting to observe that under the greenhouse conditions the older Scots pine seedlings seemed to be more competent for *A. tumefaciens* infections than the younger ones (I), while in the bombardment experiments the buds from the younger trees showed higher transgene expression levels than the buds obtained from the more mature individuals (IV, V). The highest transformation frequencies were achieved in pollen (VI) which can also be considered the youngest material, representing the gametophytic generation prior to formation of the youngest possible of the diploid cells and tissues, the zygote. The differences in the transgene activity in the tissues derived from different-aged trees may be related to the level of DNA methylation. In Norway spruce, the degree of methylation of cytosine nucleotides has been found to increase with ageing of trees (Westcott 1988). On the other hand, there are qualitative differences in the gene expression between the gametophytic and sporophytic generations in plants (Okamuro & Goldberg 1989), which may also explain the remarkable variation in the transgene expression found between the pollen and vegetative tissues of Scots pine.

Besides the age of the target plant or tissue, the changes in the endogenous or exogenous phytohormone supply can affect the transformation efficiency or the expression of the transgenes. The infection rates after *Agrobacterium* inoculations were measured as gall or root formation frequencies, which are phytohormone-dependent processes (Clare 1990, Gelvin 1990). In the Scots pine seedlings treated with *A. tumefaciens* strains, the development of visible tumours was often not seen until the end of the first growing season or the beginning of the second one after the original inoculations (I). Likewise, the Scots pine fascicular shoots that had already stopped their growth were more competent for rooting after the *A. rhizogenes* treatment than the spring cuttings (III). In both cases, the phenomenon may be related to the seasonal changes in the phytohormone balance of Scots pine. For example, the abscicic acid (ABA) levels increase in Scots pine during the autumn (Oden & Dunberg 1984), and in the grape plants inoculated with the strain A281 the re-activation of the tumourigenesis has been connected with the elevated ABA levels (Pu & Goodman 1992). Moreover,

complex interactions between plant hormones and the introduced phytohormone genes of the T-DNA origin have been demostrated in bean, in which exogenously supplied auxin downregulates the accumulation of the *ipt* mRNAs (Song et al 1995). The endogenous auxin levels in Scots pine cambium have been found to be higher during the growing season than in dormancy (Sandberg & Ericsson 1987), suggesting that also the changes in the endogenous auxin levels could have affected the expression of the introduced T-DNA genes in Scots pine.

When looking for potential explanations for the delayed gall formation observed in the inoculated Scots pine seedlings (I), the possibility of delayed T-DNA transfer should also be considered. Under the *in vitro* conditions, the agrobacteria were observed to grow on the inoculation sites at least one month after the original inoculation (I). It has been shown that agrobacteria can function as an intracellular infectious agent, performing the whole T-DNA transfer process inside the plant cell (Escudero et al. 1995). There is, however, no evidence that this would have happened in Scots pine in the present work.

The effect of exogenous growth regulator pretreatments on the transgene expression was clearly seen in the bombarded Scots pine material. The vegetative buds, cultured for one week prior to bombardment on the tissue culture media containing auxin and cytokinin, showed significantly higher levels of transient GUS expression than the ones bombarded without the pretreatments (IV, V). The same pretreatments were also found to cause an increase in arginine decarboxylase (ADC) activity in the buds (V). The high activity of polyamine biosynthetic enzymes, to which ADC also belongs, characterizes the rapidly dividing tissues, and the enhanced nucleic acid and protein synthesis are correlated with increased polyamine biosynthesis, i.e. with the ADC activity (Egea-Cortines & Mizrahi 1991). Thus the enhanced GUS expression levels in the pretreated Scots pine buds (IV, V) were probably related to the increased mitotic frequency, as also reported in tobacco by Iida and co-workers (1991). This hypothesis is further supported by the fact that the bombarded Scots pine calli expressing low GUS levels also grew slowly (IV).

In the bombarded suspension cultures of Scots pine (IV), the interactions between exogenous growth regulators, the growth rate of the suspensions, and the level of transgene expression were also evident. The growth regulator combination, which was optimal for the growth of the cultures, was also characterized by the highest GUS expression levels. The transgene expression levels in the Scots pine suspensions were affected by the time of sub-culture (IV), which may reflect the variation in the mitotic activity of the cultures. Alternatively, the expression of the introduced 35S-GUS construct could vary owing to the changes in the availability of transcription factors, or the cell-cycle dependent acitivity of 35S promoter, as suggested for explanations for the same kind of variation observed in the bombarded embryogenic suspension cultures of Norway spruce (Yibrah et al. 1994) and larch (Duchesne et al. 1993).

The configuration of the transferable foreign DNA has been reported to affect the success of transformation both in *Agrobacterium*-mediated technique and in particle bombardment. In the suspension cells of tobacco, the activity of GUS reporter gene was three times higher when the transgene was included as a cointegrating vector

compared with the strain having a binary configuration of the T-DNA. Moreover, when the agrobacteria used in the tobacco study also carried an additional plasmid for monitoring the *vir*-gene induction, the transformation frequency was reduced 97 % (Rempel & Nelson 1995). In the Scots pine inoculation experiments of the present work, such an effect could not be seen (I, II). On the other hand, the chromosomal matrix of the bacterial strain was observed to contribute to the success of rooting in the Scots pine fascicular shoots treated with *Agrobacterium*. When the strains A4 and C58, both carrying the same A4Ri-plasmid as a root inducing agent, were used for inoculating the cuttings, the strain A4 doubled root formation compared with the strain C58 (III).

Walter and co-workers (1994) optimized the bombardment parameters for radiata pine transformation, and they found linearised DNA to produce higher transient GUS expression levels than circular plasmid DNA. This kind of comparison was not performed in Scots pine material, but the transformation frequencies achieved in pollen with the plasmids introduced in a circular configuration were remarkably high (VI) when compared with the published results generally obtained in coniferous material. To date, a higher level of transgene expression has been reported only in electroporated lily pollen protoplasts, in which 70 % of the cells expressed the GUS reporter gene (Miyoshi et al. 1995). The influence of different promoter sequences on the transgene activity in the Scots pine pollen was, however, highly significant (VI). The observations on the promoter effects are further discussed in Chapter 3.2.

2.6.3. Chemical defence of Scots pine against agrobacteria

Poor results of the Scots pine inoculation experiments can be at least partly explained by the interactions between coniferous defence compounds and *Agrobacterium tumefaciens*. Monoterpenes and phenolic constitutents are known to have bactericidal effects (Frykholm 1945, Sheers 1971, Kartnig et al. 1991, Himejima et al. 1992, Sjöström 1993), and of the defence chemicals studied in the present work, a monoterpene, α-pinene proved to prevent the growth of agrobacteria in doses equal to the contents found in wounded trees (I, II). In nature, i.e. in trees, the constituents tested now occur with a huge number of other defence chemicals, and thus it is most probable that Scots pine is often able to kill invaders, such as agrobacteria, effectively.

The induction of the *vir*-genes is a prerequisite for *Agrobacterium* infection (Ankerbauer & Nester 1990, Song et al. 1991, Winans 1991). Under the experimental conditions it is usually assured by supplying *vir*-inducing agents, such as asetosyringone (Sheikholeslam & Weeks 1987, Shimoda et al. 1990, Godwin et al. 1991), as was done also in the case of Scots pine seedlings (I). When the defence compounds of Scots pine were included in the experiments, it appeared that neither α-pinene nor trans-stilbene had any inhibitory or inducing effect on the virulence gene induction in agrobacteria (II).

Both defence compounds tested in the present work, i.e α-pinene or trans-stilbene, caused a reduction in gall formation frequency when applied after the vir-induction on birch seedlings used as susceptible woody model plants. This effect was smaller with

the strain A281(pTVK291) containing extra copies of the *virA, virB, virG*, and *virC* genes than with the wild type strain A281 (II). It seems feasible that extra copies of the *virB* and *virC* in the strain A281(pTVK291) could partly compensate for the interfering influence of α-pinene and trans-stilbene on the transformation events, assuming that due to higher copy number there are more VirB and VirC proteins present in the A281(pTVK291) than in the wild type strain A281. There is a parallel example of the VirD proteins: extra copies of the *virD1* and the *virD2* genes were found to increase the amount of corresponding proteins, thus accelerating T-strand formation (Wang et al. 1990). The efficiency of Agrobacterium-mediated transformation in Scots pine could not be enhanced by using the strains containing a constitutive mutant of *virG* gene or extra copies of *virA* and *virG* (I, II). The results suggest that inefficiency of *Agrobacterium* to infect Scots pine is not caused by insufficient *vir*-gene induction but rather by failures to accomplish the later stages of the T-DNA transformation, i.e. T-DNA processing, transportation, or integration.

2.6.4. Possibilities to obtain transgenic Scots pines

When the results of the *Agrobacterium* experiments in the present study are examined critically, it can be concluded that there are hardly realistic possibilities to produce transgenic Scots pines through *A. tumefaciens* -mediated transformation. The infection frequencies with the wild type strain A281 and its genetically engineered derivatives were so low (I, II) that the development of co-cultivation techniques is not worth of trying, at least by using the disarmed derivatives of the A281. Some other strains, might, of course, work better in Scots pine than the A281, although it has proved to be one of the best in several other *Pinus* species (Stomp et al. 1990, Huang & Tauer 1994). New phytopathogenic *Agrobacterium* strains carrying novel Ti-plasmids have recently been isolated from naturally induced galls on fig trees (*Ficus benjamina*) (Bouzar et al. 1995, Vaudequindransart et al. 1995). These strains were distinct from the known plant pathogens belonging to *Agrobacterium*, i.e. *A. tumefaciens, A. rhizogenes, A. vitis,* and *A.rubi*, so it will be interesting to see whether they have a broader host plant range than the strains utilized up to date.

Agrobacterium-mediated transformation by co-cultivaltion technique also requires a reliable *in vitro* regeneration method for transformed cells. In the micropropagation procedures developed for Scots pine either the cotyledons of the embryo (Häggman et al. 1996) or the adventitious buds formed on the needles of the *in vitro* induced axillary shoots (Supriyanto & Rohr 1994) are used as explants. The methods are based on organogenesis, and neither of them has been tested for competence for co-cultivation with *Agrobacterium*. In the method using cotyledons as explants, the differentiation of shoots begins near the tip of the cotyledons, not at the excised end (Häggman et al. 1996), also limiting the use of agrobacteria as gene transfer vectors. From this point of view, the Supriyanto's and Rohr's (1994) procedure, providing subculturable organogenic calli, could be more suitable for obtaining regenerable transformed cells through the co-cultivation with *Agrobacterium*.

Genetic transformation of Scots pine by *A. rhizogenes* could not be confirmed in the present work. Agrobacteria were, however, able to improve the rooting of the Scots

pine fascicular shoots (III). Potentials of this rooting technique and its applications in Scots pine breeding are further discussed in Chapter 3.4.

By using particle bombardment the transient expression of the GUS reporter gene was achieved in the various types of Scots pine material (IV, V, VI). Transient expression results can be used for obtaining information on the gene expression of the given species (as discussed in Chapters 3.1. and 3.2.), and as a guide in the development of systems for the stable transformation. Nevertheless, it should be remembered that optimization or maximization of transient activity does not necessarily result in optimal or even any stable transformation. Of course, if no transient activity is observed following bombardment experiment, it is also unlikely that stable transformants will be recovered (Christou 1995).

On the basis of the transient expression levels observed in the different types of Scots pine material after particle bombardment (IV, V, VI), the best targets for obtaining transgenic trees would be either suspension cultures or pollen samples. Of these, pollen should be preferred for several reasons. First of all, at the moment there is no method for regenerating plantlets from Scots pine suspension cells, but if the transformed pollen is used for pollinating female strobili, no artificial tissue culture procedures are needed for recovering the seedlings. Transient transgene activity in Scots pine pollen was remarkably high with several of the introduced gene constructs (VI), much higher than achieved in suspension cultures (IV), reflecting a good general competence of pollen for transformation. As a target material pollen is also easy to handle, and natural pollen supply is practically unlimited.

By utilizing a modification of the liquid pollination technique developed for radiata pine (Sweet et al. 1992, - 1993) it is possible to use bombarded pollen for controlled pollinations in Scots pine, as it was done in the present work (VI). The bombardment itself did not affect the germination ability of the pollen, but it was reduced when the very high levels of foreign gene expression were observed. All the results in the present work were, however, counted by including only the viable, germinated pollen grains in order to get a realistic view on the possibilities of producing transgenic progenies. In Scots pine, the development of the cones reflects the success of pollination. Insufficient pollination causes deterioration of the female strobilus, and it drops during the first growing season or at the beginning of the second one (Sarvas 1962). In the present work, 21 % of the female strobili pollinated with the bombarded pollen developed normally (VI), and one can assume that in these cases the liquid pollination technique and the bombarded pollen have been functional. Generally, approximately one fifth of the Scots pine conelets drop even if the pollen catch of the female strobili has been sufficient (Sarvas 1962).

The high transformation frequencies of Scots pine pollen, together with the normal development of relatively large proportion of the female strobili pollinated with bombarded pollen are promising steps towards the production of transgenic Scots pine seedlings. Fertilization in Scots pine, however, occurs in the second growing season after the pollination (Sarvas 1962), meaning that in the present study the seeds having the bombarded pollen as the paternal parent will be available by the end of the year 1996. Thus the proportion of transgenic progenies and their characteristics can be

examined only after the stratification period of the seeds, in the year 1997. In tobacco, pollinations with the bombarded pollen samples, in which 3 % of the pollen grains showed transient transgene expression, resulted in raising two transgenic plants from 295 000 seeds (van der Leede-Plegt et al. 1995). In Scots pine, much higher transient activities in the pollen used for the controlled pollinations, up to 22 % (VI), provide the means for more frequent recovery of transgenic seedlings, too.

The fact that coniferous pollen is multicellular consisting of a big tube cell and a smaller cell called either generative or antheridial cell (Moitra & Bhatnagar 1982) complicates the assessment of possibilities of producing transgenic progenies through pollen transformation. The generative cell, which in Scots pine divides into two male gametes or sperm nuclei in the beginning of the second growing season, prior to fertilization (Sarvas 1962), should be transformed in order to get transgenic seedlings. It was, however, impossible to distinguish whether the blue stain in the histochemical GUS assay of the bombarded and germinated pollen grains (VI) originated in the tube cell or in the generative cell. The generative cell is known to remain inside the pollen grain during the germination (Sarvas 1962), but the diffusion of a GUS intermediate from one cell to another may happen before the final insoluble blue product, dichloro-dibromoindigo, is formed. In fact, only the examination of the progenies will provide definitive proof of the transformation of the generative pollen cells.

Alternative approaches for producing transgenic Scots pines, in addition to the ones studied in the present work, can most probably be developed. To date, the best method for producing transgenic coniferous plantlets has been particle bombardment using embryogenic cultures as explants (Ellis et al. 1993, Charest et al. 1995, Walter & Smith 1995). In Scots pine, the micropropagation via somatic embryogenesis is actively being studied (Hohtola 1995, Keinonen-Mettälä et al. 1995), and when the procedure is available, it will also provide good target material for particle bombardment. On the other hand, the *in vitro* regeneration methods based on organogenesis (Supriyanto & Rohr 1994, Häggman et al. 1996) could be utilized. The preliminary results of the bombardment experiments, by using regenerable cotyledons from Scots pine embryos as explants, have revealed that also this kind of target material is competent for transformation (Häggman & Aronen 1994).

3. APPLICATIONS OF GENETIC TRANSFORMATION IN FOREST GENETICS AND IN TREE BREEDING

3.1. Goals set for genetic engineering

Investments in the new biotechnology should be seen as an addition to the classical breeding work. When the utilization of the new techniques and the conventional breeding programmes are properly integrated, the adoption of biotechnology is a part of an increased commitment to the genetic improvement of forest trees, rather than a switch of effort away from classical breeding. Biotechnology can, in fact, be considered *ipso facto* a commitment to greater domestication of forest trees (Burdon 1994). Of the new techniques included in the concept "biotechnology", genetic transformation has its own place in forest genetics and in tree breeding. Genetic engineering offers tools both for transferring interesting or important traits into breeding material, as well as for studying the gene expression in forest trees.

It is important that genetically engineered genotypes are of high quality with respect to other traits as well. For this reason, genetic transformation is most appropriately conducted with species in which breeding programmes are well established and advanced, and in which clonal forestry can realistically be achieved. According to the FAO's list, globally the most important target traits for the genetic engineering of forest trees are the following: the improved insect resistance in poplars and some tropical hardwoods, the reduction of lignin biosynthesis in species used for pulp production, the introduction of herbicide resistance to environmentally benign chemicals, the improved cold tolerance in eucalypts, and the reproductive sterility for the prevention of the escape of transgenes into wild populations (Haines 1994). Traits which have been considered valuable goals for tree transformation include also drought tolerance (Newton et al. 1991) and phytoremediation of hazardous wastes (Stomp et al. 1993). In Finland, the most potential targets for the genetic engineering of forest trees are presumably related to improved timber quality and high harvest index, i.e. to crown characteristics.

Long rotation times of forest trees set specific requirements for their genetic engineering. A good example is the improvement of insect resistance by transferring the toxin gene from *Bacillus thuringiensis* or proteinase inhibitor genes from other plant species, which has successfully been done not only in deciduous *Populus* species (McCown et al. 1991, Cornu et al. 1995), but also in a gymnosperm, white spruce (Ellis et al. 1993). In this case, a big environmental risk associated with the use of transgenic trees is the counter-evolution of the insects to overcome the introduced resistance. Unlike the situation in annual crops, where new genotypes may be substituted as problems develop, this is not a practical solution in forestry. The long life span of forest trees should be taken into account already when producing transgenic trees by using multiple genes for resistance, and by inserting transgenes into a wide variety of genotypes to maintain the genetic diversity in forest (Strauss et al. 1991). Also the accurate regulation of transgene expression, for example in a wound-

inducible manner (Bradshaw et al. 1991), would be needed during the decades of the tree's life cycle.

Increased knowledge on the physiology and genetics of forest trees will undoubtedly benefit tree breeding as well. Genetic transformation has potential for research applications which cannot be performed in any other way. These include the over-expression or suppression of selected genes encoding for biosynthetic key enzymes, and modification of the expression of transcriptional activators. The over-expression of a specific gene may be obtained by introducing this gene under the control of a strong or constitutive promoter. Alternatively, an endogenous gene can be suppressed by inserting an antisense construct of the same gene, leading to the situation, in which the complementary mRNAs hybridize making each other unfunctional. Besides gaining information on the genetic control and interactions of biochemical pathways, these techniques can be used for modifying the metabolism of plants (Kishore & Somerville 1993, Kooter & Mol 1993, Nessler 1994). Moreover, the differentiation or development of a specific organ or cell type can be prevented by introducing a gene construct carrying a cytotoxin gene driven by the appropriate promoter. From the tree breeding point of view, interesting genes are those involved in floral differentiation (Strauss et al. 1995), lignin biosynthesis (Whetten & Sederoff 1991), and maturation (Hutchison & Greenwood 1991, Mikola 1992).

Genetic transformation is an excellent tool for investigating the regulation of gene expression. Various reporter genes can be introduced under the control of different regulative sequences, such as promoters and enhancers, and the function of these factors in the cell and tissue types of a given species can easily be examined by following either the transient or stable transgene activity (Herrera-Estrella et al. 1988, Schrott 1995). A wide range of regulative sequences, including various inducible promoters from angiosperms (Ellis et al. 1991), have proved to be active in gymnosperms. This is important from the genetic engineering point of view, since up to date only few promoters originating from conifers have been available. Specific promoters would, however, be needed for modifying, for example, the lignin biosynthesis (Whetten & Sederoff 1991) and reproductive sterility (Strauss et al. 1995) in forest trees. Search for suitable promoters and transcription factors for these purposes is going on (Loopstra et al. 1992, Campbell et al. 1995, Feuillet et al. 1995).

Genetic transformation may also be a useful technique for isolating novel plant genes and promoters. T-DNA tagging can be used for over-expressing or inactivating the flanking plant genes causing either dominant or recessive mutations, which will be identified as specific phenotypes directly or after selfing of primary transformants (Fritze & Walden 1995). Alternatively, in the method called "promoter-trapping" a promoterless reporter gene is introduced into the genome of the target plant, and appearing transgene activity shows that the integration of the reporter gene has occured in the vicinity of a functioning regulative sequence (Teeri et al. 1986, Fobert et al. 1991, Lindsey et al. 1993). This approach has already been used for isolating a cambium-specific promoter from a tree species, hybrid aspen (*Populus tremula x P. tremuloides*) (Stenberg et al. 1995).

3.2. Information gained on the transgene functioning in Scots pine

In the present study the main goal was to test the usefulness of *Agrobacterium*-mediated transformation and particle bombardment in the genetic engineering of Scots pine, and to look for potential breeding applications. At the same time, however, a lot of information was gained on the expression of the transgenes in the species.

An interesting observation was the competence of bud tissues originating from 50-year-old Scots pine individuals for expressing a foreign gene, although at a low level (IV, V). This is the first report confirming transgene expression in explants which were derived from mature gymnosperms. Previously, transient foreign gene expression has been reported in the differentiating xylem of 7- to 8-year-old loblolly pines (*Pinus taeda*) (Loopstra et al. 1992), but trees of this age are still considered juvenile (Zobel & Talbert 1984). According to Hutchison and Greenwood (1991), maturation causes a stable genetic change in the meristematic tissues of plants. They suggest that the genetic control of maturation could be related to the level of DNA methylation, alterations in chromatin structure, or DNA rearrangements. Genes which are believed to be regulated in a maturation-related manner have already been cloned from non-woody species (Hutchison & Greenwood 1991). On the other hand, the present results show that the bud tissues of mature Scots pines are still competent for transformation (IV, V). This means that it may be possible to study the functioning of the maturation-related genes and their promoters by introducing them into meristems of both juvenile and mature trees. Later, if the regulation of maturation in conifers including Scots pine could be modified, it would greatly benefit both tree breeding and forestry. For example, long generation intervals could be reduced by accerelating maturation, or rejuvenation of adult trees would make them suitable for effective vegetative propagation (Hutchison & Greenwood 1991, Mikola 1992).

The transient expression of a 35S CaMV-GUS gene construct introduced into Scots pine buds proved to be dependent on the season. The vegetative buds were bombarded for the first time in the early autumn, in August, then in the winter time, in December, in the early spring, in March, and finally in the late spring, in May. The highest amount of transgene expression was found in March, and the lowest both at the beginning and at the end of the growing season (V). This variation in the transgene expression resembles the variation in the levels of native gene expression of Scots pine buds (Häggman 1986, Nuotio et al. 1990), suggesting that the introduced chimeric gene construct was under the genetic control of the species. The 35S promoter of the cauliflower mosaic virus was originally considered constitutive (Odell et al. 1985), but later on it has shown variable activity in different plant tissues, developmental stages, and environmental conditions. This phenomenon has been observed also in several tree species (Ellis et al. 1991, Nilsson et al. 1992, Wilde et al. 1992, Bommineni et al. 1994, Diouf et al. 1995, Ellis et al. 1995). In transgenic hybrid aspen trees, the activity of the 35S promoter was connected to the metabolic activity of the cell (Nilsson et al. 1992). Also in Scots pine buds, the time of the highest transgene expression level in the early spring (V) corresponds with the transition from dormancy period to renewed metabolic activity (Kupila-Ahvenniemi 1985).

The present discovery that a yearly trend in the transgene expression is possible in a forest tree species (V), is further confirmed by the observations of Dave Ellis and co-workers (1995). They found seasonal changes in the reporter gene activity in the transgenic poplar and spruce plants growing in the field. In their study, the highest activity of an enhanced 35S-GUS construct was expressed in the spring. Also their preliminary results with a wound-inducible promoter suggest that seasonal variation of transgene expression is not unique to the 35S CaMV promoter. Moreover, the promoter of the *Agrobacterium rolC* gene has shown seasonal variation in activity in transgenic hybrid aspens in a study in which the 35S driven transgenes were expressed similarly during a complete cycle of growth, dormancy and reactivation (Nilsson 1995). These reports together with the results of the present study (V) clearly emphasize the importance of studying the transgene expression throughout the year when transgenic perennial plants, such as forest trees, are produced.

The functioning of various regulative sequencies was studied in the Scots pine pollen. Of the tested promoters, the highest transient GUS expression frequency was achieved with the sunflower polyubiquitin UbB1 promoter. The second best promoter, the abscisic acid inducible wheat EM promoter, yielded approximately half of the transgene expression obtained with the UbB1 promoter, and all the derivatives of the 35S even less. The original 35S proved to be better than the 35S with the AMV enhancer or the double 35S with the AMV (VI). Both the UbB1 and EM promoters have shown high activities also in other conifers (Duchesne & Charest 1991, Loopstra et al. 1992, Hay et al. 1994, Clapham et al. 1995, Rey et al. 1995).

Ubiquitin is a small, highly conserved protein present universally in eukaryotic cells, whose function is to mark other proteins for degration by attaching covalently to them. Ubiquitinylation of the histone proteins appears to contribute both to active transcription and repair of DNA (Wilkinson 1995), and increased ubiquitin mRNA levels have been observed after mechanical pertubation (Galaud et al. 1995). Thus the high activity of the UbB1 promoter in Scots pine pollen (VI) is probably related to the conserved nature of the ubiquitin genes between the *Pinus* sp. and other plants (Carter et al. 1995), as well as to cell damage caused by penetrating gold particles. The activity of the abscisic adic (ABA) inducible EM promoter in Scots pine pollen (VI) can also be related to the stress caused by the bombardment, since ABA is known to be abundant in the plant tissues under stress conditions (Bray 1991, Taiz & Zeiger 1991, Trewavas & Jones 1991). Moreover, ABA stimulates an increase in cytosolic free calsium (McAinsh et al. 1991), and a Ca^{2+} influx together with a Ca^{2+} gradient are needed for pollen tube growth during the germination (Pierson et al. 1994, Malhó et al. 1995).

3.3. Interesting traits to be transferred into Scots pine breeding material

Gene transfer methods together with the breeding goals fix the limits of traits, which could be transferred into Scots pine breeding material. The capacity of the current transformation methods allows, at most, the transfer of a few genes at the same time. On the other hand, many traits, such as growth, yield and adaptability, which are important for forest tree improvement, are quantitatively inherited. From the genetic

engineering point of view, the genetic background of these characteristics is still poorly understood, even though the relationships between different yield components have been studied extensively (Mikola 1985, Velling 1988, Albrektson et al. 1995). Besides the increased volume growth, the characteristics improving timber quality, e.g. fine-branchiness together with perpendicularity, small number, and good natural pruning of branches, are included among the breeding goals of Scots pine in Finland (Velling 1982, Anon. 1989). The most interesting applications of genetic transformation in Scots pine breeding could undoubtedly be achieved, if the introduction of single gene effects contributing to these quality components were possible.

The genotype E1101, called "Kanerva pine", represents many characteristics which have been considered ideal in Scots pine breeding. It has a narrow crown, short and thin branches at a perpendicular angle, minimal tapering, abundance of foliage, vigorous stem height growth and a high harvest index. The "Kanerva pine" has, in fact, inspired greatly the development and definition of the ideotype concept for Scots pine improvement (Kärki 1985, Kärki & Tigerstedt 1985, Velling 1988, Pöykkö 1993). In the progenies of the E1101 the specific crown form segregates close to 1:1, suggesting that this tree type could be a result of one single dominant allele (K), the "Kanerva pine" being heterozygous for the allele (Kk) and the wild-type trees being recessive homozygotes (kk). Unfortunately, the ideal characteristics of the E1101 are connected with a tortuosity of the stem, and often also inherited together with it (Mikola 1985, Pöykkö & Velling 1993). Usually, the branch thickness, and the angle and number of branches per whorl correlate positively with growth in Scots pine (Velling 1982). If the advantageous effects of the "Kanerva allele" could be separated from the crooked stem form, it would be the number one choice for the gene to be transferred into Scots pine breeding material.

Naturally occuring mutants having a specific tree form, such as the "Kanerva pine", have been found also in Norway spruce. These trees called pendula phenotypes have exceptionally narrow crowns with thin branches and strong apical dominance (Pulkkinen & Pöykkö 1990, Pöykkö & Pulkkinen 1990). The progeny trials have proved the pendula characteristics to be controlled by a single dominant gene (P) (Lepistö 1985), parallel to the case of the "Kanerva gene" in Scots pine. There are some observations that the narrow crowned phenotype also in spruce would be correlated with a reduced straightness of the stem. This phenomenon may be caused by fast height growth, and variation both among different families and sites exist (Pöykkö 1996, pers. comm.). While no efforts have been made to isolate the "Kanerva gene", the pendula gene of spruce has recently been mapped by using random amplified polymorphic DNA (RAPD) markers (Lehner et al. 1995). This is an important step towards the ultimate identification and cloning of the gene. When the pendula gene of Norway spruce is available, it will be extremely interesting to introduce it into Scots pine material in order to see whether it causes advantageous effects comparable to the "Kanerva allele", and whether these are accompanied by a tortuous stem or not.

Approximately half of the Scots pine roundwood produced in Finland, 9.5 million m^3 in 1994, is used for chemical pulping (Aarne 1995). The main purpose of pulping is to liberate the wood fibres by removing the lignin as completely as possible. In the

sulfate (kraft) process, the lignin content of Scots pine is reduced from the original 27 % in roundwood to 3 % in pulp, while for example in birch the corresponding reduction is from 20 % to 2 %. During delignification in pulping also part of the cellulose yield is lost (Sjöström 1993). If the lignin content of Scots pine could be reduced to the same level as in hardwood (birch), the yield in pulping would increase from 48 % to 50 %, meaning annually 20-30 million FIM more profit per a large pulp mill producing 500 000 tn pulp per year (Paavilainen, J. Pöyry Ltd, 1994, pers. comm.). In addition, the consumption of bleaching chemicals, as well as effluents, would be reduced. The reduction of the lignin biosynthesis in the species used for pulping is globally one of the main goals set for tree genetic engineering (Haines 1994), but up to date the interest in this approach in Finland has been minor. The situation may, of course, change if, for example, the price of energy increases remarkably.

If interest in the genetic engineering of lignin content rises, the possibilities to carrying it out are rather good, since the biosynthetic pathway for lignin production is fairly well known. The key enzymes are phenylalanine ammonia lyase (PAL) governing the entry into the lignin precursor pathway, and at the other end of the pathway, cinnamyl alcohol dehydrogenase (CAD) catalyzing the formation of coniferyl and synapyl alcohols, the actual lignin precursors (Trotter 1990). Polymerization of these precursors is supposed to be catalyzed at least partly by laccase (polyphenoloxidase) enzymes (Dean & Eriksson 1992). The reduction of the Scots pine lignin content could be performed either by reducing the amount of precursors or by reducing the level of precursor polymerization. The genes encoding PAL and CAD have been isolated and characterized from a pine species, *Pinus taeda* (O'Malley et al. 1992, Whetten & Sederoff 1992), allowing the introduction of the antisense constructs also into Scots pine. It has been estimated that reductions of 10-15 % in total lignin content should be attainable without a loss of biomass yield (Dean & Eriksson 1992), but the modifications should be expressed only in xylem in order to avoid problems in the resistance against pests, for example (Whetten & Sederoff 1991). Otherwise, the lignin content is considered to have minor importance for the adaptability of Scots pine (Ståhl & Ericson 1991).

The introduction of genes conferring the resistance against pests or pathogens is not relevant in the case of Scots pine. Even though Scots pine is a host for many fungal pathogens and insect species (Kurkela 1990, Stephan 1991), the damage caused by these agents or mammal herbivores do not have a major role in forestry. This is also seen in the Long-Term Tree Breeding Programme for Finland (Anon. 1989), in which the resistance against biotic agents is not mentioned among the breeding goals of Scots pine. The most problematic single damage agent in Scots pine is Scleroderris cancer caused by a fungus *Gremmeniella abietina* (*Ascocalyx abietina*) (Kurkela 1990), but only 1.2 % of the forest land area is attacked so seriously that the stand quality is reduced (Aarne 1995). Global warming or climatic change may, of course, facilitate the invasion of both existing or new pests and pathogens, and under such circumstances re-evaluation of the breeding aims might be necessary.

Whichever transgenes are introduced into Scots pine, a careful consideration whether these genes can disperse to the surrounding natural populations has to be

appended to the genetic engineering programme. The release of transgenic plants has been discussed a lot during the last few years (for example, see Seidler & Levin 1994, Tomlin 1994, Dale 1995, Rogers & Parkes 1995), and especially the ecological and non-target effects of transgenes in agriculture, silviculture and natural ecosystems have worried both scientists and the public. The pollen dispersal in Scots pine is effective (Koski 1970), probably causing a high rate of transgene flow to natural populations. On the other hand, the potential ecological effects of the transgenes depend on the characteristics of these genes. For example, the dispersal of the spruce pendula gene or the "Kanerva allele" to the wild populations of Scots pine may not necessarily have any harmful effects. Narrow crowned Scots pine individuals already exist naturally, and moreover, their flowering abundance and fitness is lower than that of trees with a wide crown and thick branches (Nikkanen & Velling 1987, Hertel & Kohlstock 1994). If the dispersal of the introduced foreign genes to natural populations in every case is considered undesirable, transgenic Scots pines should also be engineered to carry reproductive sterility.

Possibilities of introducing either male or complete sterility into forest trees have recently been reviewed by Strauss and co-workers (1995). The two primary options include the ablation of floral tissues via floral promoter-cytotoxin fusions and the disruption of floral gene expression by various methods of gene suppression. Genes involved in the development of the flowers have mostly been studied in non-woody plants and in angiosperm trees. A recent Norway spruce study has, however, proved that at least some genes controlling floral development in a conifer are homologues of the corresponding angiosperm genes, despite the great differences in the structure of reproductive organs (Tandre et al. 1995). This discovery undoubtedly facilitates the genetic engineering of sterility in gymnosperms, including Scots pine. When the introduction of the reproductive sterility is examined from the practical point of view, a prequisite for it is naturally an effective vegetative propagation method for the given species. On the other hand, the stimulation of faster wood production has been mentioned as a potential positive consequence arising from the introduced sterility in trees (Strauss et al. 1995).

3.4. How to make practical use of transgenic trees ?

Integration of genetic engineering into forest tree breeding programmes can be performed in several different ways. In the agricultural crop approach the foreign genes are introduced into commercially proven genotypes, and these are then tested in the field for stable transgene integration and expression, and propagated vegetatively for commercial purposes. Alternatively, juvenile material from selected families could be transformed, and the clonal testing conducted at the same time with the testing for the novel trait expression. This approach is perhaps more realistic, taking into account the general recalcitrance of older trees for regeneration and the savings in time when the same material is not tested twice in the field (Haines 1994). The above mentioned methods, however, are applicable only for the species in which clonal forestry can be carried out on a practical scale. Other possibilities to integrate transgenic trees into breeding programmes involve their use as parents in the controlled crossings and in the seed orchards, and the transformation of pollen. If transgenic trees are used as parents.

they cannot be sterile, and the transgenes are likely to escape to natural populations. Transgenic pollen grains, on the other hand, could be used for producing either fertile or sterile progenies (Haines 1994). It should be remembered, however, that in forest tree breeding the back-crossing programmes which are performed in order to minimise undesirable pleitropic effects of the transgenes (Jones & Cassells 1995, Oud et al. 1995), are not possible owing to the long generation intervals and the inbreeding depression (Williams & Savolainen 1996).

In Scots pine, the current methods for vegetative propagation are not applicable to operational forestry. The regeneration of plantlets through tissue culture by using organogenesis (Suprianto & Rohr 1994, Häggman et al. 1996) has been achieved, but at the moment the methods are suitable only for small scale research purposes, and somatic embryogenesis is still under development (Hohtola 1995, Keinonen-Mettälä et al. 1995). Rooting of Scots pine cuttings, on the other hand, is also difficult, especially if ortets are older seedlings or mature trees (as reviewed by Salonen 1990, Monteuuis & Barnéoud 1991). This is why one of the goals in the present study was to investigate whether a Ri-plasmid -mediated transformation could be used for improving the root induction in Scots pine cuttings (III), and thus also possibilities of utilizing transgenic trees in the future.

The advantageous effect of the *Agrobacterium*-treatment on the rooting of Scots pine fascicular shoots was demonstrated in the present work. However, the attemps to confirm the transformation of the root tissues formed after bacterial inoculations by using agropine assay, histochemical test for the β-glucuronidase expression, PCR analysis or root morphology comparisons failed (III). This indicates that the improved root formation was not accompanied by the stable integration and expression of foreign genes in Scots pine genome. The enhancement in root induction caused by agrobacteria could, nevertheless, be due to the transient expression of the T-DNA genes affecting the phytohormone balance in Scots pine cells. The same phenomenon has been suggested to explain the induction of untransformed roots after *A. rhizogenes* infections in chrysanthemum (van Wordragen et al. 1992). Alternatively, only a few cells could originally have been transformed by agrobacteria, but their auxin production was sufficient to induce the development of genetically normal roots. If transformation events, even transient ones, are involved in *Agrobacterium*-mediated root induction, the efficiency of the technique could be further improved by testing different bacterial strains as rooting agents, since remarkable host genotype - *Agrobacterium* strain interactions have been observed in other pine species (Bergmann & Stomp 1992, Huang & Tauer 1994).

Agrobacteria could also have promoted the root induction in the Scots pine cuttings independently of their transformation ability. Agrobacteria are acid-producing micro-organisms, which can increase the availability of soil minerals thus enhancing the nutrient uptake and growth of root systems, as shown also in the case of Scots pine (Leyval & Berthelin 1989). Moreover, agrobacteria may secrete growth regulator substances. Both *A. tumefaciens* and *A. rhizogenes* have cytokinin synthase genes in their Ti-/Ri-plasmid, outside the T-DNA (Powell et al. 1988, Regier et al. 1989), and *A. tumefaciens* is known to synthesize auxin encoded by both chromosomal and Ti-plasmid genes (Kutácek & Rovenská 1991, Costacurta & Vanderleyden 1995).

Whatever the actual mechanisms behind the *Agrobacterium*-mediated root induction in Scots pine cuttings, the observation that the roots formed by this techique are not transformed (III) is advantageous from the practical applications' point of view, since the release of foreign genes or chimeric plants into nature has raised conflicting views. In conclusion, of the vegetative propagation methods currently available for Scots pine, the rooting of fascicular shoots induced in young seedlings seems to be the best method for multiplication of transgenic material.

Transformation of Scots pine pollen, on the other hand, provides several different approaches for integrating transgenic tree material into the breeding programme. Explants for transformation, i.e. pollen grains, can be collected from mature elite trees, which have already proved their genetic characteristics in the progeny tests. Pollen transformation is, indeed, the best possibility to introduce foreign genes into tested material, taking into account that tissues derived from mature Scots pines cannot be regenerated through tissue culture. Moreover, the use of pollen as a target for genetic engineering allows the transformation of many genotypes - not only can the pollen donors be selected among the best elite trees, but also the female parents for the controlled pollinations. Even though all the genotypes are not equally compatible for transformation (VI), the collection, handling and testing of pollen samples from different individuals is a relatively fast and easy task. In other words, there are good possibilities to maintain genetic diversity while introducing novel traits. How effective the controlled pollinations with transgenic pollen are in the production of material expressing desired characteristics depends, of course, on the proportion of transgenic progenies. It is improbable that this technique could be used directly for producing seedlings for reforestation.

The pollen transformation method is also compatible with the vegetative propagation techniques, if the embryos or young seedlings born from the controlled pollinations with transgenic pollen are used as explants. Transgenic, mature or immature embryos could be cloned either via somatic embryogenesis, if a method effective enough can be developed for Scots pine, or through existing organogenic regeneration methods. One alternative is also the production of cuttings in a few years old transgenic seedlings. The organogenic regeneration method developed by Häggman and co-workers (1996) provides interesting opportunities both as regards breeding and investigations on the expression and inheritance of transgenes in Scots pine. The plantlets regenerated from the cotyledons of the germinated embryos by using this technique have shown mature characteristics, such as flowering at the age of three or four years. If also plantlets derived from transgenic embryos behaved in the same way, it would mean a unique possibility to raise a new generation of potentially transgenic Scots pines within a few years.

4. CONCLUSIONS

In the present study, the transient transformation of Scots pine was demonstrated. The best transformation frequencies, up to 55 %, were achieved when pollen grains were used as explants. Of the tested material, the pollen samples also proved to be the best targets for experiments aiming at stable transformation, and subsequent regeneration of transgenic Scots pines. If transgenic pollen is successfully used for controlled pollinations, the problems involved in artificial regeneration through tissue culture procedures can be avoided. Whether this approach will work in Scots pine, remains to be seen in the near future. The controlled pollinations with transformed pollen were performed in the present study, but owing to the fact that the development of Scots pine cones and seeds requires two growing seasons after the pollination, the seeds are not yet available. Since the transient expression levels in the Scots pine pollen were remarkably high, it seems realistic to expect that at least a few transformants will be recovered.

Of the tested transformation methods, the particle bombardment is more applicable for genetic engineering of Scots pine than the *Agrobacterium*-vectors. The chemical defence compounds of Scots pine are able to interfere with the infection process of agrobacteria, resulting in failure to transfer, integrate or express the T-DNA in host plant cells, even if the induction of the bacterial virulence genes has taken place. Nevertheless, agrobacteria could be utilized for improving the potential to propagate Scots pine vegetatively. Results of the present study showed an increase in the rooting frequency of Scots pine cuttings after the *Agrobacterium* treatment, but the roots remained untransformed. This finding is, however, advantageous from the practical applications' point of view, because the release of transgenic plants has raised conflicting views among the researchers and the public. A funtional vegetative propagation technique is, however, needed for the multiplication of transgenic trees expressing desired characteristics.

Gene transfer experiments performed in the present study resulted in much information on the factors affecting the transformation efficiency and transgene expression in Scots pine. It was evident that both the characteristics of the explants, i.e. genotype, age and physiological stage, and the transferable gene construct, especially promoter sequences, had remarkable influence on the success of transformation. These results emphasize the need for careful pre-screening of different factors before aiming at stable transformation and regeneration. Moreover, it should be realized that all the genotypes that are interesting or important from the breeding point of view, may not be compatible with the current transformation methods.

Genetic transformation proved to be an appropriate tool for studying gene expression in Scots pine. The present study revealed a yearly trend in the reporter gene activity under the control of the CaMV 35S promoter, indicating that endogenous mechanisms of the species are also involved in the regulation of foreign gene expression. This discovery agrees with the observations in other tree species, and gives

an idea of how the introduced genes would probably behave in transgenic Scots pines. An interesting observation in the present study was also the competence of the bud tissues derived from 50-year-old mature trees for transformation. This is the first time that transgene expression has been reported in the explants originating from such old gymnosperms. Possibilities to control maturation of trees would greatly benefit forest tree breeding, i.e. via rejuvenation of adult trees for vegetative propagation or via reduced generation intervals. The present results demonstrating the transient transformation of the bud tissues of older trees suggest that functioning of maturation-related genes could be studied by introducing them into meristems of both juvenile and mature Scots pines.

As regards the breeding aims of Scots pine, the most important single gene effects that could be introduced into the breeding material would be the ones related to improved timber quality. The Scots pine genotype E1101, "Kanerva pine", represents a natural mutant in which many desired characteristics, such as a narrow crown, short and thin branches, minimal tapering and abundance of foliage are combined with vigorous stem growth and a high harvest index. The "Kanerva phenotype" has been suggested to be caused by pleiotropic effects of a single dominant allele, but the gene has not been identified or isolated. The corresponding pendula gene from Norway spruce, on the other hand, has already been mapped. When the spruce pendula gene is available, it will be interesting to introduce it into Scots pine material and study whether it causes advantageous effects comparable to "Kanerva allele", and whether these are accompanied by a tortuous stem that is typical of the original "Kanerva phenotype". Other traits which may be genetically engineered in Scots pine include reproductive sterility, altered lignin content of wood, and potentially also resistance against pests and pathogens, if global warming causes drastic changes in the existing ecological balance.

Integration of genetic engineering into Scots pine breeding programmes could be achieved either through vegetative multiplication of transgenic trees or via pollen transformation and controlled pollinations. The current methods for vegetative propagation of Scots pine, i.e. rooting of cuttings, organogenesis, and somatic embryogenesis in tissue culture, are not applicable to operational forestry. Pollinations with transgenic pollen allow the use of several tested, mature genotypes as explant donors and female parents, which is an important aspect both from the breeding point of view, and as regards the maintenance of genetic diversity. It seems realistic, however, to assume that the pollen approach can not directly be used for producing seedlings for reforestation, but rather for raising interesting individuals and families for further selection and breeding.

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Differences in *Agrobacterium* infections in silver birch and Scots pine

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Summary

The aim of this study was to investigate the differences in infections caused by *Agrobacterium tumefaciens* in a conifer, Scots pine (*Pinus sylvestris*), and in a non-host deciduous species, silver birch (*Betula pendula*). All the *Agrobacterium tumefaciens* strains tested caused crown-gall formation in both tree species, but the infection rates varied remarkably. In Scots pine, the development of galls was rare, and slower than in silver birch. Inoculation into the base of the stem were the most successful in gall induction. Silver-birch galls were large, often surrounding the whole stem, in contrast to Scots pine galls, which were characterized by their small size and neck-like connection with the host plant. In silver birch, no other morphological changes could be seen. In Scots pine, abnormal phenotypes with proliferating short shoots above the galls were observed during the second and third growing season. The results indicate that, of the two non-host tree species, the deciduous one, silver birch, is more susceptible to an *A. tumefaciens* infection than the conifer, Scots pine. The matrix for *A. tumefaciens* infection in silver birch differs from that in Scots pine, since the terpene compounds of Scots pine seem either to kill the agrobacteria or to suppress their growth. The differences between the species could be partly caused by their difference in sensitivity to phytohormones. These features reflect evolutionary incompatibility between *A. tumefaciens* and a gymnosperm.

1 Introduction

Agrobacteria were isolated and described for the first time in 1907 by SMITH and TOWNSEND. At that time, the causal relationship between pathogenic *Agrobacterium* and crown-gall disease was also revealed. Phytopathogenic agrobacteria infect a very wide range of dicotyledonous plants, but are not known to cause diseases in monocotyledonous plants in nature. In the case of gymnosperms, there are only a few reports on naturally occurring crown galls: *Cupressus arizonica* (BROWN and EVANS 1933), *Juniperus sabina and Libocedrus decurrens* (SMITH 1935), *Picea glauca* (WHITE and MILLINGTON 1954), and *Sequoia sempervirens* (PHILLIPS and BURDEKIN 1982). Deciduous trees, especially fast growing species such as poplars and eucalypts, have been found to be more susceptible to *Agrobacterium* than conifers. For example, agrobacteria isolated from infected hybrid poplars (*Populus alba* × *grandidentata*) have been proven to have a wide host range, indicating a potential threat to plantations and natural stands (DOCHINGER 1969).

Phytopathogenic agrobacteria overwinter in infested soils in the form of saprophytes. They enter the roots or stems of host plants through fresh wounds. Once inside the tissue, the bacteria primarily occur intercellularly and cause diseases by transforming the host cells. Agrobacteria are able to transfer a part of their plasmid DNA, so called T-DNA, into plant cells. When T-DNA is integrated into plant genomic DNA and bacterial genes are expressed, the transformed cell starts to divide abnormally and to produce specific compounds called opines, which are utilized by agrobacteria (CLARE 1990).

Genetic-transformation experiments in forest trees have been made since the late 1980s. In experimental conditions, many of the forest-tree species are shown to be susceptible to bacterial infection, even if most of them are not natural hosts of *Agrobacterium*. This also holds true for the species in this study, silver birch and Scots pine. *Agrobacterium* mediated

gene transfer has been used successfully with deciduous tree species. The first report of a stably transformed forest tree, hybrid poplar (*Populus alba* × *grandidentata*) was published by FILLATTI et al. 1987. Since then, a fair amount of work has been carried out with poplars (PYTHOUD et al. 1987; DE BLOCK 1990; SHAN-PING et al. 1990; CHAREST et al. 1992; LEPLE et al. 1992), which, especially in nurseries, are also natural hosts of *A. tumefaciens*. At present, there has only been one report on a conifer, *Larix decidua*, stably transformed by *A. rhizogenes* (HUANG et al. 1991), but none on conifers transformed by *A. tumefaciens*.

The aim of this study was to investigate the differences in the infections caused by *Agrobacterium tumefaciens* in a conifer, Scots pine (*Pinus sylvestris* L.), and in a deciduous tree species, silver birch (*Betula pendula* Roth.). The host–pathogen relationships were examined from several points of view, including infection rates with different bacterial strains in different environments, crown-gall anatomy, and morphological changes in host plants, and integration of bacterial genes into the host genome.

2 Material and methods

Plant material

Silver-birch (*Betula pendula* Roth.) and Scots-pine (*Pinus sylvestris* L.) seedlings of various ages were used as research objects. Silver-birch seedlings were established with local (61 °48′N; 29 °17′E) seeds and grown under the normal greenhouse conditions. The seedlings were grown in horticultural peat in containers 6 cm in diameter and were fertilized twice a month during the growing season with 0.1–0.2% commercial Superex fertilizer (Kekkilä). During the growing season (April-September), the day and night temperatures in the greenhouse were kept above 15 and 10 °C, respectively. At the time of inoculation, potted silver-birch seedlings (n = 480) were 2 months old. Scots-pine seedlings were grown both under greenhouse and *in vitro* conditions. The potted greenhouse seedlings were established with seeds from Padasjoki (61 °25′N; 25 °E), and inoculated at 2, 4, 8, or 16 weeks old (n = 240 per age class). The *in vitro* seedlings established with local (61 °48′N; 29 °17′E) seeds, were grown in tissue-culture conditions as described by ARONEN and HÄGGMAN (1994), and inoculated at the age of 2 or 4 weeks (n = 450 per age class). The silver-birch seedlings were grown for one summer, but the Scots-pine material was cultured over several growing seasons.

Bacterial strains

The following *Agrobacterium tumefaciens* strains were used as inoculum source: A281, a wild-type agropine strain A281:pTiBo542 (HOOD et al. 1986), the same strain containing a supervirulent plasmid pTVK291 (KOMARI et al. 1986; JIN et al. 1987), and the same wild-type strain containing the pRT45 binary plasmid (RYAN et al. 1990), including a neomycin phosphotransferase (*npt*) reporter gene. To study factors affecting *vir*-gene induction and T-DNA methylation, a disarmed *A. tumefaciens* strain C58C1:pGV2260:GUS INT, including the β-glucuronidase (GUS) marker gene with an intron (VANCANNEYT et al. 1990) was used. All the strains were cultured on Luria Broth (LB) medium (MILLER 1972), solidified with 1.5% agar, containing the following antibiotics: kanamycin 50 mg/l for pTVK291, kanamycin 20 mg/l and tetracycline 20 mg/l for pRT45, and kanamycin 50 mg/l and rifampicin 30 mg/l for GV2260.

The effect of commercial terpenes on bacterial growth

The effect of pure commercial terpenes, limonene and α-pinene (Fluka) on bacterial growth, was tested by including either 12.5 mM filter-sterilized limonene or 12.5 mM α-pinene, or

both, into media after autoclaving. A single colony of each bacterial strain (A281, pTVK291, pRT45, and GV2260), taken from freshly growing cultures, was suspended in 1 ml H_2O, diluted 4×10^4 and 100 μl of this dilution was plated on three petri-dishes containing bacterial media both with and without terpenes. Growth of bacteria was scored by counting the colonies after 2 days of culture at 28 °C in the dark. Agrobacteria-free terpene media were also incubated as controls.

Inoculation experiments

Silver-birch seedlings grown in the greenhouse were inoculated by wounding the stem with a scalpel dipped in a bacterial suspension of strains A281, pTVK291 and pRT45. Bacterial strains were grown overnight in correspondingly modified LB solution (pH 5.6) containing 100 μM acetosyringone, and diluted to an absorbance of 1 ± 0.2 at 550 nm. Control seedlings were wounded with a scalpel dipped in the modified LB solution. Scots-pine seedlings, both under *in vitro* and greenhouse conditions, were inoculated by wounding the stem with a scalpel dipped in the bacterial colony from the modified LB media (pH 5.6) containing 100 μM acetosyringone. Silver birches and 16-week-old Scots pines were wounded both on the base and the upper part of the stem, while the 2-, 4- and 8-week-old Scots-pine seedlings were wounded on the stem below the cotyledons. After inoculation, all the greenhouse seedlings were wrapped in plastic bags and kept shaded for two days to maintain a high-humidity environment. After inoculation, the development of crown galls was observed every second week.

Vir-gene induction and T-DNA methylation in bacteria, in relation to their ability to infect Scots pine

Experiments were performed with disarmed *A. tumefaciens* strain GV2260. Efforts to affect the transformation efficiency of this strain were made by modifying bacterial-culture media prior to inoculation. The following media were used: 1. The basal LB medium for GV2260 containing kanamycin 50 mg/l and rifampicin 30 mg/l; 2. LB + 100 μM acetosyringone, 100 μM azacytidine and 10 mM D-glucose; 3. LB + 100 μM acetosyringone and 100 μM azacytidine; 4. LB + 100 μM acetosyringone and 10 mM D-glucose; 5. LB + 100 μM acetosyringone; 6. LB + 100 μM azacytidine and 10 mM D-glucose; 7. LB + 100 μM azacytidine; and 8. LB + 10 mM D-glucose. Each treatment included 80 2-week-old *in vitro* seedlings. Expression of the GUS reporter gene was followed histochemically (test conditions as given by ARONEN et al. 1994) by taking the inoculation sites from 16 individuals per treatment, 2, 5, 7, 14, and 30 days after the inoculation.

Testing of crown-gall material in silver birch

Several tests were performed to verify the presence of bacterial and marker genes in crown-gall tissues of silver-birch seedlings. Half of the seedlings in each treatment were randomly chosen for the tests. Crown galls were excised from seedlings, and the small ones (diameter <3 mm) were used for agropine assays. Bigger galls (diameter 3–20 mm) were divided in half; one half was used for agropine assays, and the other one to test the ability of tissue to grow on phytohormone-free culture medium.

The crown-gall halves were surface sterilized as follows: firstly for 30 min in 7.5% calcium-hypochlorite, then for 5 min in 70% ethanol, and then rinsed for 10 min in sterile H_2O. After presterilization, the gall halves were peeled and resterilized for 2 min in 70% ethanol, then for 20 min in 2% Ca-hypochlorite, and rinsed for 10 min in sterile H_2O. After sterilization, the gall tissues were sliced, and the 2–3 mm-thick tissue pieces were transferred

onto the phytohormone-free WPM media (LLOYD and MCCOWN 1980), solidified with 1% agar containing the antibioticum cefotaxime 500 mg/l, as well as kanamycin 50 mg/l in the case of pRT45 inoculation. Proliferating tissues were transferred onto fresh media once a month, and, after 6 months of culture, cefotaxime was excluded. Agropine assays were performed according to the published procedure (PARSONS et al. 1986), with minor modifications, and standards were prepared as described by PETIT et al. (1983).

Before DNA isolations and polymerase-chain reactions (PCR), the absence of agrobacteria in crown-gall tissues on phytohormone-free media was confirmed as illustrated in HOOD et al. (1990). DNA was isolated from gall tissues as described by DOYLE and DOYLE (1990), with the following modifications: tissue (150 mg) was homogenized in liquid nitrogen with a pestle and mortar and the frozen-tissue powder vortexed with 700 μl of 2 × CTAB buffer (60 °C) in a 2.2 ml Eppendorf tube, and kept at 60 °C for 1 h. Then, 1 ml of CIA (chloroform/isoamyl alcohol, 25:1) was added, mixed by vortexing and inverting the tubes. The samples were centrifuged at 8000 × *g* for 5 min. The top layer was transferred into a new tube, and the CIA extraction was repeated twice. DNA was precipitated by adding 1 volume of isopropanol into tubes that were allowed to rest for 30 min at room temperature. Samples were centrifuged at 11 600 × *g* for 15 min, supernatants were removed, and pellets washed with 70% ethanol. The DNA was dissolved in 100 μl of TE containing 50 μg/ml of Proteinase-K and 10 μg/ml of RNAase, and kept overnight at 37–40 °C. The DNA was reprecipitated by adding 25 μl of 1 M NaCl and 125 μl of cold isopropanol. After centrifugation, pellets were washed with 70% ethanol for 3 h, and the DNA finally dissolved in 20 μl of TE.

PCR reactions were performed to determine the presence of an *ipt*-gene of pTiBo542 T-DNA and the *npt*-gene in the DNA isolated from crown galls. Primers specific for the *ipt* sequence, GACTGCGATAGCTCTTGCCC and GAAACTCCTGAGCGATACCG, which border a 584 bp fragment from the *ipt* gene (nucleotides 751–1334, according to STRABALA et al. (1989)), and primers specific for the *npt* sequence, TGGGCACAACAGA CAATCGG and CAGCAATATCACGGGTAGCC, which border a 619 bp fragment of the *npt* gene (nucleotides 220–838 according to BECK et al. (1982)) were used for the PCR reaction. The reaction mixture contained 1 μl of template DNA, 100 μM dNPT's, 150 pM primers, and 1 U of Dynazyme DNA polymerase (Finnzymes) in 50 μl of manufacturer's buffer. The reaction mixtures were heated to 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min, with a final extension step of 72 °C for 7 min in a DNA Thermal Cycler 480 (Perkin–Elmer, Cetus). Amplified samples were electrophoresed on a 1.4% agarose gel, stained with ethidium bromide, and examined under UV-light.

Testing of crown-gall material in Scots pine

Crown galls formed on *in vitro* grown Scots-pine seedlings were tested for the presence of bacterial T-DNA and the *npt*-gene: the small galls were excised from stems and transferred onto callus-inducing MS media (ARONEN et al. 1994), supplemented with 100 mg/l arginine and 0.1% MES (2-[N-Morpholino]ethanesulfonic acid), containing the antibioticum carbenicillin 500 mg/l, and, in the case of pRT45 inoculation, also 50 mg/l kanamycin. After 1 month, the proliferating tissues were transferred on the same media without any growth regulators, and, after 3–4 months of culture, the carbenicillin was omitted. DNA isolations from gall tissues growing on phytohormone-free media, and PCR reactions, were the same as performed on silver-birch material.

Anatomical studies

The anatomical structure of crown-gall tissues was examined under a microscope (Zeiss Universal R). Inoculation sites with galls were fixed in FAA (formalin: acetic acid: 95%

ethanol, 10:5:85, v/v/v), embedded in paraffin, and stained with safranin-fast green (GERLACH 1984).

Statistical analyses

Comparisons between treatments in bacterial culture and silver-birch experiments were made by analysis of variance. Means were compared either by Tukey's test or by Student-Newman-Keuls multiple-range test.

3 Results

Infection rates

The ability of agrobacteria to infect silver birch and Scots pine differed remarkably. All the tested strains, A281, pTVK291, and pRT45, were able to cause gall formation in both tree species, but the infection rates varied according to the host-plant type.

In silver birch, gall development was fast (Fig. 1). First galls generally appeared on the inoculation sites within 2 weeks, and 6 weeks after the inoculation, 92% of the seedlings had galls. The point of inoculation had a significant effect on the gall induction, the base of the stem being more receptive for bacterial infection than the top of the stem ($p < 0.01$). The bacterial strain also influenced the infection rate. At 2 weeks after inoculation, the A281-strain had caused more tumours than the other strains ($p < 0.05$ for the base inoculations, $p < 0.01$ for the upper-stem inoculations), but later, the differences between the strains disappeared.

In the case of Scots pine, the overall frequency of gall formation was low when compared with silver birch. Gall formation depended on the host-plant age and on the environmental conditions (Fig. 2). In the first growing season, the infection rates in 2- and 4-week-old *in vitro* seedlings varied from 3 to 10%, but, in greenhouse seedlings of the same age, no gall formation could be observed. Under greenhouse conditions, the oldest seedlings (16-week-old) were the most susceptible ones, having infection rates of 5–22% when the inoculation was made at the base of the stem. Upper-stem inoculations were negative. The gall induction

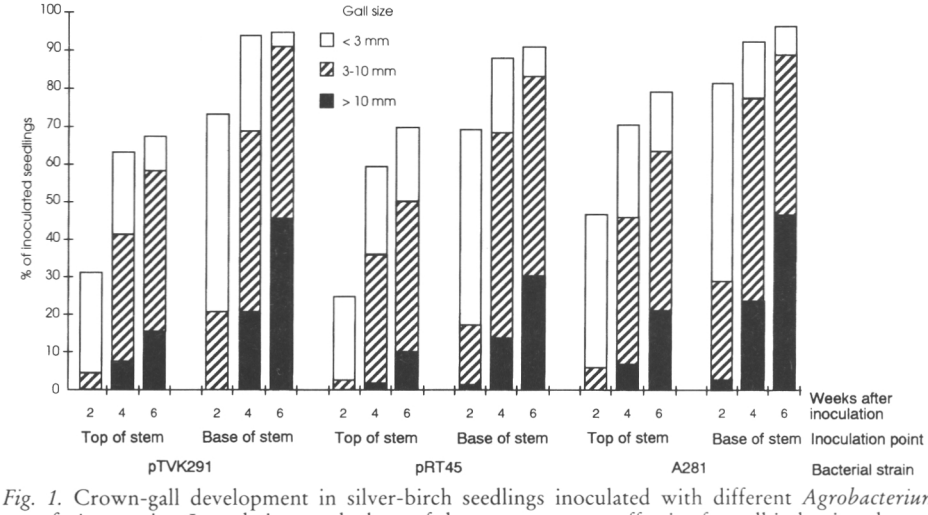

Fig. 1. Crown-gall development in silver-birch seedlings inoculated with different *Agrobacterium tumefaciens* strains. Inoculation on the base of the stem was more effective for gall induction than on the top of the stem ($p < 0.01$)

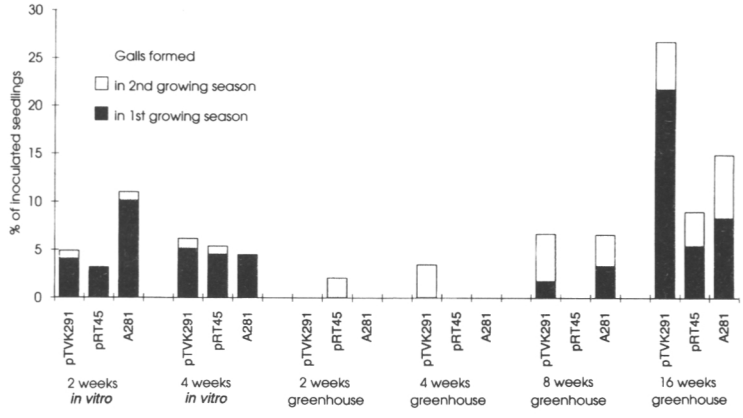

Fig. 2. Crown-gall development in Scots-pine seedlings of various ages under different environmental conditions. Gall formation induced by *A. tumefaciens* strains continued for two growing seasons

in Scots pine was slow. Under *in vitro* conditions, the first tumours were visible within approximately 4 weeks, but, in the greenhouse, the development of the galls often took several months. During the first cold season, 12% of the galls on the greenhouse seedlings dried and became detached. On the other hand, 2–7% of all the greenhouse seedlings produced new galls in the same inoculation sites at the beginning of the second growing season. There were no remarkable differences in the infection rate between the bacterial strains. However, one exception occurred: in the 16-week-old greenhouse seedlings, the supervirulent pTVK291 strain caused 3–4 times more galls than the A281 and pRT45 strain during the first growing season (Fig. 2). The control seedlings produced no tumourous tissues.

The effect of commercial terpenes on bacterial growth

Limonene and α-pinene were included in bacterial media to study their effect on the growth of agrobacteria. Limonene did not affect the bacterial growth, and the effect of α-pinene, either alone or in combination with limonene, varied depending on the strain (Table 1.). As regards the strains used for the inoculations, α-pinene decreased the number of colonies of strains A281 and pRT45, but, in strain pTVK291, which contains extra virulence genes, no decrease in colony number could be detected. The disarmed strain GV2260 increased its number of colonies in the presence of α-pinene. However, the size of the colonies of all strains tested was smaller in the presence of α-pinene than without this chemical.

Table 1. **The effect of commercial terpenes on the growth of agrobacteria. Number of colonies per plate ± SE. The means marked with the different letters within each strain differ significantly from each other ($p < 0.05$).**

Media	Bacterial strain			
	A281	pTVK291	pRT45	GV2260
Pinene (P)	107 ± 6^{b}	310 ± 32^{a}	0^{b}	869 ± 24^{a}
Limonene (L)	472 ± 34^{a}	371 ± 25^{a}	433 ± 48^{a}	793 ± 24^{ab}
P + L	153 ± 56^{b}	263 ± 70^{a}	0^{b}	890 ± 45^{a}
Control	442 ± 98^{a}	304 ± 22^{a}	418 ± 48^{a}	673 ± 57^{b}

Vir-gene induction and T-DNA methylation in bacteria, in relation to their ability to infect Scots pine

The effect of acetosyringone, azacytidine and monosaccharide glucose on the infection rate of agrobacteria in Scots pine was monitored for 1 month. The expression of GUS reporter gene could not be observed during the experiment. Agrobacteria were, however, found to grow on the inoculation sites, at every observation time. The same bacterial strain, GV2260, was simultaneously used in hybrid aspen (*Populus tremula* × *tremuloides* Michx), where the transformation was successful (data not shown).

Crown-gall anatomy and morphological changes in host plants

Crown galls formed on silver-birch seedlings were frequently large, exceeding 1 cm in diameter, and surrounded the stem at the point of inoculation (Fig. 3a). Often, part of the tumourous tissues had a tendency to turn brown and die towards the end of the growing season. In the *in vitro* seedlings of Scots pine, the average diameter of the galls varied from 1 to 3 mm, and the galls usually remained green (Fig. 3b). On greenhouse seedlings, the galls rarely exceeded this size before they became covered by bark (Fig. 3c).

In the silver-birch seedlings, no other phenotypic changes except the gall development could be found. In the first growing season, the Scots-pine seedlings also looked morphologically normal. However, during the following growing seasons, proliferation of short shoots from axillary buds above the inoculation site was observed for seedlings with galls (Fig. 3d, e). In the third growing season, 76% of remaining tumourous seedlings ($n = 17$) expressed this morphologically abnormal phenotype.

Crown-gall tissues in silver birch and Scots pine were compared anatomically. Silver-birch galls were characterized by an expansive sector of the initiation of the tissue proliferation (Fig. 4a). In Scots pine, the situation was in most cases just the opposite: the tumourous tissue proliferated from a few cells (Fig. 4b, d). In silver-birch galls, unorganized vascular cells were often found within tumourous tissue (Fig. 4c). This phenomenon was also observed in some Scots-pine samples (Fig. 4b).

Integration of bacterial genes in host genome

The integration of bacterial T-DNA genes into host-plant genomes was tested both on the level of DNA and the gene products (Table 2). Agropine production in silver-birch (Fig. 5a) was found, on average, in 96% of the gall samples. The presence of bacterial hormone genes in galls was tested by culturing them on phytohormone-free media. In the greenhouse material, fungal contaminations posed a severe problem. Despite stringent surface sterilization and peeling of galls, on average, 61% of material (107/175) showed contamination. The rest of the gall tissues, 68 samples, proliferated well on the phytohormone-free tissue-culture media (Table 2), and, 3 years later, 40 of them were still growing under these conditions. In *in vitro* Scots-pine galls, the percentage of proliferating samples varied from 8 to 33% when observations were made after 1 year of culture (Table 2). After 3 years of culture, three gall-tissue lines were still proliferating on phytohormone-free media.

Integration of the bacterial *ipt*-sequence into host-plant DNA was tested by PCR (Fig. 5b). In the case of pRT45 inoculations, *npt*-sequence integration was also studied. The presence of *ipt*-sequence was confirmed in 98% of the silver-birch samples. The *npt*-sequence was present in all pRT45 galls studied. No integration of these sequences was detected in Scots-pine galls (Table 2).

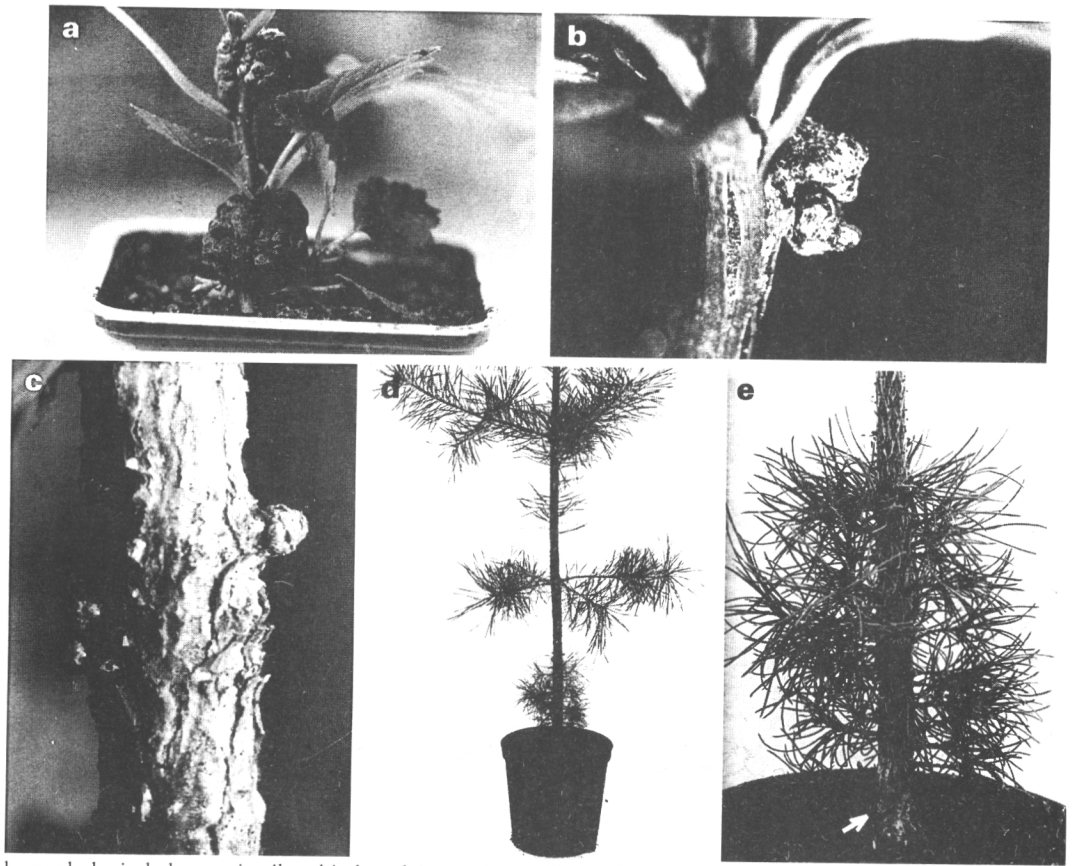

Fig. 3. Crown-galls and morphological changes in silver birch and Scots pine. a. Silver-birch seedling with crown galls 6 weeks after inoculation; b. Scots-pine seedling with crown gall grown *in vitro*; c. Crown galls formed on Scots-pine greenhouse seedling 5 months after inoculation; d, e. Abnormal shooty phenotype of Scots pine 3 years after inoculation. Arrow indicates the original inoculation site with the crown gall

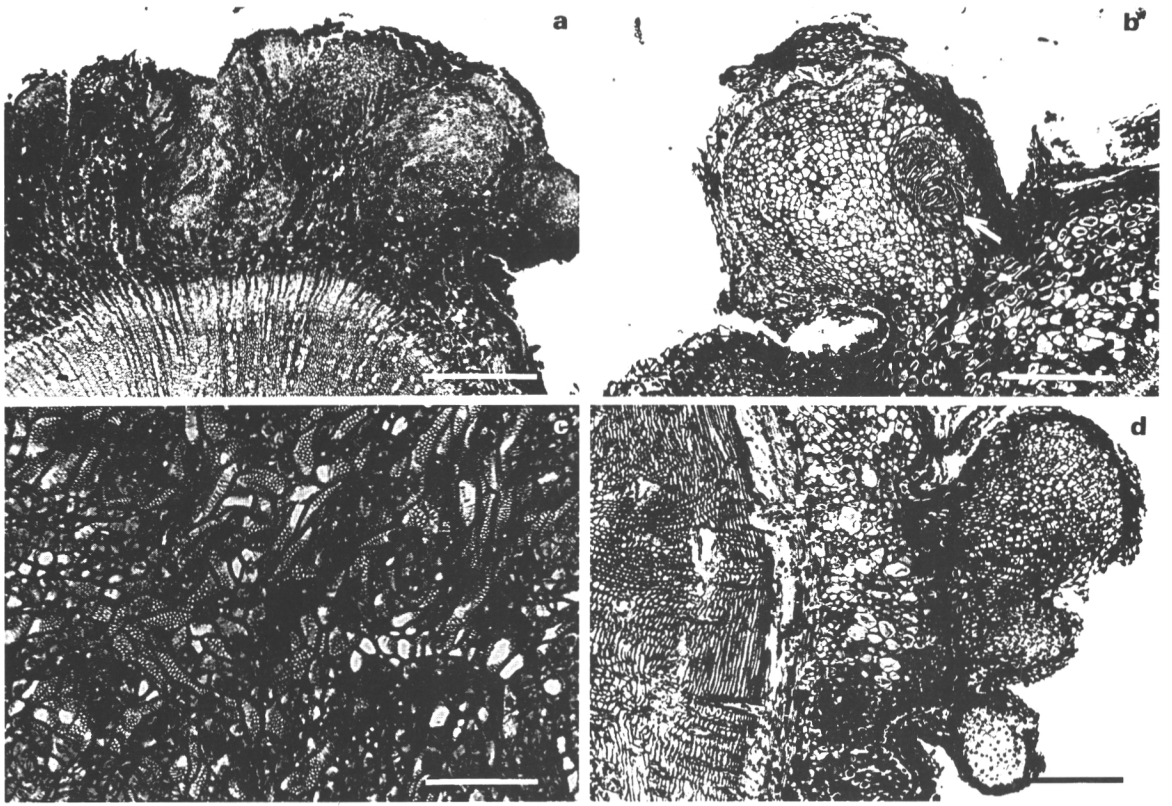

Fig. 4. Anatomy of crown-gall tissues in silver birch and Scots pine. a. Silver-birch gall arising from a wide area of wounded and infected tissue; b. Typical crown gall on Scots pine. Arrow shows abnormal vascular tissue within the gall; c. Unorganized vascular cells in the silver-birch gall tissue; d. Multiple galls formed in one inoculation site in Scots pine, longitudinal section. Bars in a, b and d represent 500 μm, and, in c, 100 μm

Table 2. **Integration of bacterial genes into the host genome according to different tests (ND = not determined)**

	Agropine production		Growth on phytohormone-free media		Presence of *ipt*-sequence		Presence of *npt*-sequence	
Bacterial strain	No. of tested galls	Positive	No. of tested galls	Positive[1]	No. of tested lines[2]	Positive	No. of tested lines[2]	Positive
Silver birch								
pTVK291	72	97%	23	100%	16	100%	ND	
pRT45	74	95%	28	100%	13	92%	10	100%
A281	71	96%	17	100%	12	100%	ND	
Scots pine *in vitro*								
pTVK291	ND		15	33%	6	0%	ND	
pRT45	ND		12	8%	3	0%	3	0%
A281	ND		22	32%	11	0%	ND	

[1] Observations made after 1 year of gall culture on phytohormone-free media
[2] DNA isolations from silver-birch material were made after 2 years of gall culture on phytohormone-free media, and from Scots-pine material after either 6 months or 2 years

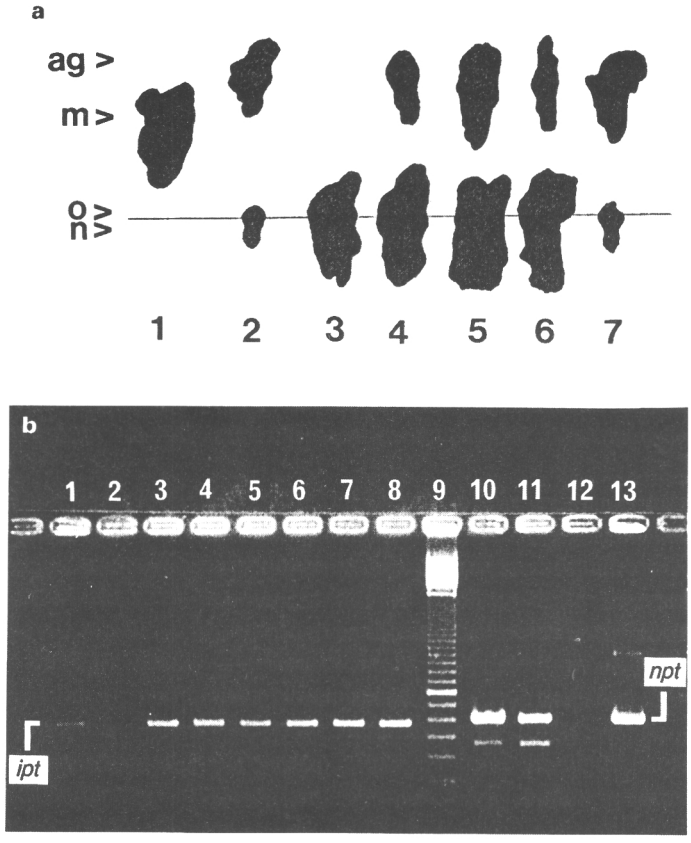

Fig. 5. Testing of crown-gall tissues in silver birch. a. Agropine assay: lane 1: mannopine standard; lanes 2 and 7: agropine and agropinic acid standards; lane 3: negative control — sample from an untransformed callus; lane 4: sample from a gall induced by pTVK291; lane 5: sample from a gall induced by pRT45; lane 6: sample from a gall induced by A281; ag = agropine, m = mannopine, o = origin of separation, n = neutral $AgNO_3$ positive compounds and agropinic acid; b. PCR products: lane 1: positive *ipt* control 584 bp, template DNA from agrobacteria; lanes 2 and 12: negative controls, template DNA from an untransformed callus; lane 3–4: template DNA from crown galls induced by pTVK291; lanes 5–6: template DNA from galls induced by pRT45; lanes 7–8: template DNA from galls induced by A281; lane 9: 100 bp ladder; lanes 10–11: template DNA from galls induced by pRT45; lane 13: positive *npt* control 619 bp, template DNA from agrobacteria

4 Discussion

The ability of *Agrobacterium* spp. to infect various tree species has been studied extensively. When seedlings are intentionally wounded and inoculated with agrobacteria, many deciduous and coniferous trees develop typical symptoms of crown gall and hairy root (DE CLEENE and DE LEY 1976; TEPFER 1989; STOMP et al. 1990; PORTER 1991; WORDRAGEN and DONS 1992). This is in agreement with the results of this study. Both silver birch and Scots pine were compatible with *A. tumefaciens*, although the infection rates varied.

A. tumefaciens strain A281 was chosen for the experiments due to its ability to infect conifers, such as several *Pinus* species, *Pseudotsuga menziesii* (LOOPSTRA et al. 1990; STOMP

et al. 1990; HUANG and TAUER 1994), *Picea abies* (HOOD et al. 1990), and *Larix decidua* (HUANG et al. 1993). The strain carrying extra virulence genes, pTVK291, has been shown to infect *Pseudotsuga menziesii* and several *Picea* species (ELLIS et al. 1989). All the strains in this study, A281, pTVK291, and pRT45, were highly virulent on silver birch, and, to some extent, on Scots pine. Genetic engineering of the strain A281 by incorporating either extra *vir*-genes or a binary plasmid pRT45, does not seem to affect its ability to infect these tree species.

Phytopathogenic agrobacteria are rhizosphere inhabitants, and cause crown-gall or hairy-root symptoms on roots or stems of the host plant near the soil line (AGRIOS 1988). For both silver birch and Scots pine, the position of inoculation had a significant effect on the infection rates: seedlings were more susceptible to agrobacteria on the base of the stem than on the top of the stem.

The effect of environmental conditions and host-plant age on the infection rate was investigated in Scots pine. Due to scarce gall induction, and the small size of galls, the success of the inoculations could be measured only as a binary response, 'gall' or 'no gall'. Unfortunately, statistical analyses of the Scots-pine inoculation data using the logit models turned out to be unsuccessful because of the extremely skewed distribution of the dependent variable. Under greenhouse conditions, however, the oldest (16-week-old), already woody seedlings, seemed to be most compatible with *Agrobacterium*. This observation agrees with the data from Norway spruce (HOOD et al. 1990), but contrasts with results in *Larix* (HUANG et al. 1993), *Picea* (ELLIS et al. 1989), and *Pinus* species (STOMP et al. 1990). On the other hand, *in vitro* grown 2- and 4-week-old Scots pines were superior in gall formation when compared with the greenhouse seedlings of the same age. Under *in vitro* conditions, the stems of the seedlings remained green throughout the experiment, suggesting that the succulence of the host-plant tissue does have a positive impact on the infection rate in Scots pine.

Scots-pine inoculation experiments in greenhouse and *in vitro* were performed with different seed sources because the seeds used for the greenhouse studies carried fungal contaminations that were not effectively killed by surface sterilization. Different seed sources can also partly explain the observed difference in the infection rates between the greenhouse and *in vitro* grown seedlings of the same age. There is both inter- and intra-specific variation in susceptibility of forest trees to *A. tumefaciens* (BERGMANN and STOMP 1992; CLAPHAM et al. 1990; NESME et al. 1990; RIEMENSCHNEIDER 1990; MACKAY et al. 1988). The variation between species and families indicates a possible genetic basis for selection of trees either susceptible or resistant to agrobacteria.

The growth rate and the anatomy of tumourous tissues in silver birch and in Scots pine were remarkably different. In silver birch, the crown galls were fastened to the seedlings with a wide base providing water and nutrients from the host plant to the fast-growing tissue. Later in the growing season, there was mainly unorganized vascular tissue in these galls, and parts of the galls were dying, probably due to deficiency in water and nutrient supply. Incomplete vascular systems, together with attacks of insects and micro-organisms, are considered the main reasons for the decay of tumourous tissues (AGRIOS 1988). In Scots pine, the crown galls were characterized by slow growth and occasional detachment, which can partly be explained by the anatomical features: a neck-like structure connecting gall to the plant, and absence of a functional vascular system.

In the greenhouse-grown Scots-pine seedlings, the development of visible crown galls often took several months, and occurred at the end of the growing season. New galls were also formed at the beginning of the second growing season. Comparable data on delayed gall formation have been achieved in *Pinus radiata*, galls emerging up to 32 weeks p.i. (BERGMANN and STOMP 1992). In grape plants inoculated with the strain A281, tumourigenesis was occasionally re-activated following necrogenesis in late autumn. This phenomenon was connected with elevated abscisic-acid (ABA) levels under reduced daylight conditions (PU and GOODMAN 1992). Endogenous ABA levels also increase in Scots pine

during the autumn (ODEN and DUNBERG 1984). It seems feasible that seasonal changes in the phytohormone balance could also affect the development of visible crown-gall symptoms in Scots pine. In silver birch, on the other hand, gall formation was constitutive. This may reflect the difference between deciduous and coniferous species in reactivity to phytohormones, which can be seen, for instance, as recalcitrance of conifers in tissue culture.

The formation of crown gall arises from the function of bacterial hormone genes in host plant cells. The hormone genes in the T-DNA of agrobacteria, like the *ipt* gene of pTiBo542, have constitutive promoters that are functional in eucaryotic cells (MORRIS et al. 1993). According to earlier studies, transferred foreign genes may be under the genetic control of the species. Seasonal changes in the expression of GUS gene introduced into Scots-pine buds were observed, despite the presence of a constitutive promoter (ARONEN et al. 1995). Similar types of genetic control could have been involved in the regulation of gene expression of transferred bacterial hormone genes in this study.

The infrequent crown-gall formation, and slow appearance of galls in Scots pine may also be dependent on virulence-(*vir*) gene induction and DNA methylation. It has been shown that methylation of transferred genes can take place both in *Agrobacterium* and in the host plant (PALMGREN et al. 1993). The induction of the *vir*-genes in agrobacteria is a prerequisite for T-DNA transfer. The *vir* genes are known to be induced by plant phenolic metabolites and sugars (SHIMODA et al. 1990; GODWIN et al. 1991). Phenolic inducers have also been found in non-host gymnosperms (MORRIS and MORRIS 1990). In this study, acetosyringone and D-glucose were included in media to enhance *vir*-gene induction, and azacytidine to prevent T-DNA methylation in agrobacteria. By using the GV2260 disarmed strain carrying the GUS reporter gene, the first transformation events were supposed to be easily detectable without interference of enhanced cell divisions caused by oncogenic strains. By monitoring the GUS expression, it would have been possible to follow the time schedule of transformation, as well as the competence of different cell types and cell layers of the pine shoots to *Agrobacterium* infection. None of the treatments, however, promoted transformation. It is still possible that transferred bacterial genes were inactivated in Scots-pine cells. Methylation and co-suppression involved in transgene silencing have been discussed recently by FINNEGAN and MCELROY (1994).

The matrix for *Agrobacterium* infection in silver birch differs from that in Scots pine. Essential oils consisting of different terpenes in conifers are antimicrobial (KARTNIG et al. 1991). According to BERNARD-DAGAN (1988), terpene biosynthesis is assumed to take place in the resin ducts found in the parenchymas of the whole plant. The differentiation of the resin duct proceeds very early from primary and secondary meristems, and terpene secretion starts at the early stages of differentiation. In addition, the large increase in the amounts of the terpenes α- and β-pinene are characteristic after a mechanical injury in pine (MARPEAU et al. 1989). In this study, α-pinene and limonene, two of the major components of essential oils in conifers (PARDOS et al. 1990; KARTNIG et al. 1991; CHALCHAT et al. 1994), were included in bacterial culture media to investigate their effect on bacterial growth. Based on the results, α-pinene in Scots pine seems to suppress the growth of the strains used for inoculations.

In silver-birch material, the integration of bacterial genes into host-plant genome was confirmed by several methods, but, in Scots pine, the results were contradictory. The Scots-pine galls did not grow well on tissue culture media in the absence of exogenous phytohormones. Agropine analyses were not possible due to the small size of the galls. The presence of the *ipt* sequence of the bacterial cytokinin gene could not be detected in gall tissues grown *in vitro*. However, the proliferation of the short shoots above the crown galls in the greenhouse seedlings suggests the effect of cytokinin-type phytohormones. This shooty phenotype is abnormal in Scots pine, although it can be induced by spraying exogenous cytokinin (SALONEN 1989).

In this study, the galls and the shooty phenotypes in Scots pine could be caused by cytokinins produced either in transformed plant cells or in agrobacteria. In the nopaline

strains, cytokinin genes (*tzs*) are present on the Ti-plasmid *vir*-region outside the T-DNA (AKIYOSHI et al. 1985; POWELL et al. 1988). The role of these *tzs* genes expressed in agrobacteria is to stimulate cell division at the wound site, thus contributing to the initiation of oncogenesis (MORRIS et al. 1993). HUANG et al. (1993) observed galls in European larch inoculated with disarmed nopaline strain, which they assumed resulted from the function of the *tzs* gene. There is, however, no evidence that the agropine strain A281 used in this study contains *tzs* gene. It is plausible that, in Scots pine, only a few cells are originally transformed, and the expression of the bacterial genes is not necessarily constitutive.

We conclude that, from the two non-host tree species used in this study, the deciduous one, silver birch, is more compatible with *Agrobacterium* infection than the conifer, Scots pine. The differences between the species could be partly caused by different sensitivity to phytohormones. Compared with silver birch, there may be specific functions in Scots pine that decrease the infection rates, such as production of suppressing terpenes, and probably also inactivation or season-dependent expression of transgenes. These features reflect evolutionary incompatibility between *Agrobacterium* and gymnosperms.

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Résumé

Différences d'infection par Agrobacterium entre Betula pendula et Pinus sylvestris

Le but du travail était d'étudier les différences d'infections provoquées par *Agrobacterium tumefaciens* chez un conifère (*Pinus sylvestris* L.) et chez un feuillu non-hôte (*Betula pendula* Roth.). Toutes les souches d'*Agrobacterium* testées ont induit la formation de galles chez les deux espèces, mais les taux d'infection étaient remarquablement différents. Chez le pin sylvestre, le développement des galles était rare et plus lent que chez le bouleau. L'inoculation à la base de la tige était plus efficace pour l'induction de galles. Les galles chez le bouleau étaient grosses, entourant souvent tout le tronc, alors que chez le pin elles étaient petites et reliées à l'hôte comme par une constriction. Chez le bouleau, aucune autre particularité morphologique n'a été observée. Chez le pin, on a observé des phénotypes anormaux avec la prolifération de rameaux courts au-dessus des galles, pendant la seconde et la troisième saison de végétation. La matrice bouleau était différente de la matrice pin dans la mesure où les composés terpéniques du pin sylvestre semblent tuer l'*Agrobacterium* ou inhiber sa croissance. Les résultats montrent que chez ces deux espèces non-hôte, la matrice feuillue est plus sensible à l'infection que la matrice résineuse. Les différences entre les deux espèces pourraient être partiellement dues à leurs différences de sensibilité aux phytohormones. Par rapport au bouleau, il peut y avoir des mécanismes spécifiques chez le pin sylvestre, qui réduisent le taux d'infection, comme la production de terpènes inhibiteurs. Ces faits reflètent l'incompatibilité évolutive entre *Agrobacterium* et un gymnosperme.

Zusammenfassung

Unterschiedliche Reaktion von Hängebirke und Kiefer auf künstliche Inokulation mit Agrobacterium

Das Ziel der vorliegenden Studie war es, die Auswirkungen künstlicher Inokulationen mit *Agrobacterium tumefaciens* auf zwei nicht zum Wirtskreis gehörende Baumarten, einer Konifere, der Kiefer (*Pinus sylvestris* L.), einerseits, und einer sommergrünen Baumart, der Hängebirke (*Betula pendula* Roth.), andererseits, miteinander zu vergleichen. Alle *A. tumefaciens* Stämme waren in der Lage, an beiden Baumarten Tumore zu verursachen, aber die Infektionsraten variierten stark. Auf der Kiefer war die Entstehung von Tumoren selten und langsamer als auf der Birke. Inokulation an der Stammbasis führte am häufigsten zu einer erfolgreichen Tumorinduktion. Die Tumore auf den Birken waren im

Gegensatz zu denjenigen auf der Kiefer groß und häufig stammumfassend. Die Tumore an der Kiefer waren klein und knollig und häufig nur mit einer dünnen, halsähnlichen Verbindung mit dem Wirt verbunden. Auf der Birke wurden neben den Tumoren keine morphologischen Veränderungen festgestellt. Dagegen kamen bei der Kiefer in der zweiten und dritten Vegetationsperiode Phänotypen mit aus Tumoren auswachsenden Kurztrieben vor. Die Resultate zeigten, daß von den beiden nicht zum Wirtskreis gehörenden Baumarten der sommergrüne Laubbaum gegenüber *A. tumefaciens* anfälliger ist als die immergrüne Konifere. Die beiden Wirte sind für eine Infektion durch *A. tumefaciens* unterschiedlich geeignet, insbesondere weil die Terpene der Kiefer offenbar imstande sind, die Agrobakterien abzutöten oder zumindest im Wachstum zu hemmen. Die Unterschiede in der Anfälligkeit der beiden Baumarten könnten zumindest teilweise durch Unterschiede in der Empfindlichkeit gegenüber Phytohormonen bedingt sein. Diese Befunde widerspiegeln eine evolutive Inkompatibilität zwischen *A. tumefaciens* und einer Gymnosperme.

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Interactions between *Agrobacterium tumefaciens* and coniferous defence compounds α-pinene and stilbene

By TUIJA S. ARONEN

Summary

The ability of *Agrobacterium* sp. to infect gymnosperms is generally poor, but as a gene transfer vector *Agrobacterium* would have several advantages. The present study characterises the interactions between coniferous defence compounds, a monoterpene α-pinene and a phenolic constituent trans-stilbene, and *Agrobacterium tumefaciens*. Of the chemical constituents studied, α-pinene proved to prevent the growth of agrobacteria in doses equal to the contents found in wounded trees. Either α-pinene or trans-stilbene had no inhibitory effect on the virulence gene induction in agrobacteria, but both compounds caused a reduction in gall formation frequency in susceptible woody model plants, i.e. birch seedlings, when applied after the *vir*-induction. This effect was smaller with the strain A281(pTVK291) containing extra copies of the *virA, virB, virG*, and *virC* genes than with the wild type strain A281. The efficiency of *Agrobacterium*-mediated transformation in a model gymnosperm, Scots pine, could not be enhanced by using the strains containing a constitutive mutant of *virG* gene or extra copies of *virA* and *virG*. The results suggest that the inefficiency of *Agrobacterium* sp. to infect many conifers is not caused by insufficient *vir*-gene induction but might be rather owing to interference by chemical defence compounds during the later stages of T-DNA-transformation.

Key words:

Agrobacterium tumefaciens, α-pinene, trans-stilbene, *vir*-gene induction, genetic transformation

1 Introduction

The ability of *Agrobacterium* sp. to infect gymnosperms is generally poor. There are only a few, often indecisive reports on naturally occuring crown galls (BROWN and EVANS 1933; SMITH 1935; WHITE and MILLINGTON 1954; PHILLIPS and BURDEKIN 1982), and also in artificial inoculations the infection rates are frequently low, even though many coniferous tree species are susceptible to *Agrobacterium* under experimental conditions (DINER and KARNOSKY 1987; ELLIS et al. 1989; HOOD et al. 1990; LOOPSTRA et al. 1990; STOMP et al. 1990; HUANG et al. 1991; HAN et al. 1994; HUANG and TAUER 1994; ARONEN and HÄGGMAN 1995). *Agrobacterium* as a vector system for genetic transformation has, however, several advantages. Transgene inactivation, for example, is often associated with multiple integrations of transgenes in plant genome. This phenomenon might be evaded most readily by using

Agrobacterium-mediated transformation, instead of direct gene transfer methods (FINNEGAN and MCELROY 1994). Moreover, *A. rhizogenes* can be used for inducing root formation in cuttings and in in vitro shoots, and this approach has been applied to several gymnosperms (MCAFEE et al. 1993; YIBRAH et al. 1996; ARONEN et al. 1996).

Phytopathogenic agrobacteria infect a host plant by transferring the part of their plasmid DNA, the T-DNA, into plant cells, where it integrates into the host genome. Subsequently, the symptoms of crown gall or hairy root develop owing to the expression of bacterial genes. The infection process, including nicking of the T-DNA borders, processing and transportation of the T-complex into the nucleus of the plant cell, as well as integration of bacterial genes into eukaryotic DNA, is primarily directed by the virulence (*vir*-) genes of the Ti-/Ri-plasmid (ZAMBRYSKI 1992). The induction of the *vir*-genes, which naturally occurs owing to acidic pH, and phenolic compounds and monosaccharides secreted by wounded plant cells, is a prerequisite for *Agrobacterium* infection (ANKENBAYER and NESTER 1990; SONG et al. 1991; WINANS 1991). Under the experimental conditions the *vir*-gene induction is usually assured by adding asetosyringone or some other *vir*-inducing agents (SHEIKHOLESLAM and WEEKS 1987; SHIMODA et al. 1990; GODWIN et al. 1991). A natural phenolic *vir*-inducer, coniferin, has been identified also from one gymnosperm, *Pseudotsuga menziesii* (MORRIS and MORRIS 1990).

Production of oleoresin, a mixture of cyclic monoterpenes and diterpene resin acids, is one of the major defence mechanisms of conifers against herbivore and pathogen attacks. As a primary response to wounding, a localized accumulation of oleoresin occurs owing to the disruption of resin ducts (LEWINSOHN et al. 1991; SJÖSTRÖM 1993). A secondary response involves the accelerated *de novo* biosynthesis of both monoterpenes and resin acids (GREF and ERICSSON 1985; MARPEAU et al. 1989; LEWINSOHN et al. 1991; FUNK et al. 1994). Monoterpenes have been shown to inhibit the growth of several bacteria (KARTNIG et al. 1991; HIMEJIMA et al. 1992), including some *Agrobacterium* strains (ARONEN and HÄGGMAN 1995). Phenolic constituents are another group of defence compounds produced by gymnosperms. Stilbenes, i.e. derivatives of 1,2-diphenylethylene, for example, are known to have wide algicidal, fungicidal, and bactericidal effects (FRYKHOLM 1945; SHEERS 1971; SJÖSTRÖM 1993). In Scots pine wounding has also been shown to induce the biosynthesis of stilbenes (FLIEGMANN et al. 1992).

The aim of the present study was to investigate the interactions between coniferous defence compounds, a monoterpene α-pinene and a phenolic constituent stilbene, and *Agrobacterium tumefaciens*. The effects of these compounds on *vir*-gene induction, as well as on the later functions during the bacterial infection, were studied. Furthermore, the infection ability of genetically engineered agrobacteria carrying either extra copies of the *vir*-genes or a mutant gene leading to constitutive *vir*-gene induction was tested in a model gymnosperm, Scots pine.

2 Materials and methods

Bacterial strains

The bacterial strains used in the experiments included a wild type *A. tumefaciens* strain A281 carrying the Ti-plasmid Bo542 (HOOD et al. 1986), and the same strain containing one of the following plasmids in addition to the pTiBo542: pTVK291 with extra copies of the *virA, virB, virG,* and *virC* genes (KOMARI et al. 1986; JIN et al. 1987), pSY203 carrying a wild type

virG together with *virB* promoter-ß-galactosidase reporter gene fusion (*virB::lacZ)*, or pSY204 carrying a constitutive mutant of *virG* (N54D) together with the *virB::lacZ* fusion (JIN et al. 1993). All the bacterial strains were cultured either in liquid Luria Broth (LB) medium (MILLER 1972) or on LB medium solidified with 1.5 % agar. The antibiotic kanamycin at the concentration of 50 mg/l was added for the pTVK291, and carbenicillin 100 mg/l and tetracycline 5 mg/l for the pSY203 and pSY204.

Plant material

Two-month old seedlings of a susceptible non-host tree species, silver birch (*Betula pendula* Roth.), were used as a model system in the experiments dealing with the accumulation of avirulent bacterial clones and the progress of *Agrobacterium* infection after *vir*-gene induction. The birch seedlings were established with local (61°48'N; 29°17'E) seeds, and grown in containers 4 cm in diameter under normal greenhouse conditions, as described by ARONEN and HÄGGMAN (1995). For testing the infection ability of genetically engineered bacterial strains, two-week-old *in vitro* seedlings of a gymnosperm, Scots pine (*Pinus sylvestris* L.), were used. Also the pine seedlings were established with local seeds, and grown in tissue culture conditions, as described by ARONEN and HÄGGMAN (1994).

The effect of acetosyringone, α-pinene and stilbene on the growth of A281

The effect of pH, acetosyringone (Aldrich, stock solution 100 mM in dimethyl sulfoxide, DMSO), (-)α-pinene (Fluka, > 97 %), and trans-stilbene (Sigma, stock solution 250 mM in DMSO) on the growth of *A. tumefaciens* strain A281 was tested by growing the bacteria in the following liquid media: LB at pH 7.0, LB at pH 7.0 with the equal amount of DMSO as required for stilbene addition at a maximum (0.8 %, v/v), LB at pH 5.6 with the same amount of DMSO as required for acetosyringone addition at a maximum (0.1 %, v/v), LB at pH 5.6 with 20, 60 or 100 µM acetosyringone, LB at pH 7.0 with 5, 10 or 20 mM pinene, LB at pH 7.0 with 0.5, 1 or 2 mM stilbene, and LB at pH 7.0 with both pinene and stilbene at concentrations of 5 mM and 0.5 mM, 10 mM and 1 mM, or 20 mM and 2 mM, respectively. Cultures of 25 ml were initiated with 250 µl of the stationary-phase inoculum which was adjusted to optical density at 600 nm (OD_{600}) of 0.20 ±0.01 with fresh medium, containing 2 x 10^8 CFU/ml. Cultures were then incubated by shaking (160 rpm), at 28°C in the dark, and OD_{600} was measured every three hours.

The effect of α-pinene and stilbene on the accumulation of avirulent Agrobacterium clones

The bacterial strains A281 and A281(pTVK291) were first grown in liquid LB media, then adjusted to OD_{600} value of 0.20 ±0.01, and plated as a 1:100 000 dilution on the corresponding solid media. The media used for the bacterial cultures were LB at pH 7.0 with 0.4 % (v/v) DMSO, LB at pH 5.6 with 60 µM acetosyringone, LB at pH 7.0 with 10 mM pinene, LB at pH 7.0 with 1 mM stilbene, and LB at pH 7.0 with 10 mM pinene together with 1 mM stilbene. Single colonies from bacterial plates were picked up after three days of culture at 28°C, and the two-month-old birch seedlings were inoculated by wounding the stem with a scalpel dipped in a bacterial colony. The number of inoculated seedlings varied from 52 to 58 per treatment. After inoculation the seedlings were kept shaded and under continuous mist

(relative humidity being 90 %) for two days. The formation of crown galls at the inoculation sites was observed two weeks and one month after the bacterial treatment.

Vir-gene induction experiments

Bacterial strains A281(pSY203) and A281(pSY204) were cultured in the following liquid media: LB at pH 5.6 containing either the same amount of DMSO as required for acetosyringone and stilbene addition (0.46 %, v/v), 60 µM acetosyringone, 60 µM acetosyringone together with 10 mM pinene, 60 µM acetosyringone together with 1 mM stilbene, or 60 µM acetosyringone together with 10 mM pinene and 1mM stilbene; or in another series of experiments: LB at pH 5.6 containing either the same amount of DMSO as required for stilbene addition, 60 µM acetosyringone, 10 mM pinene, 1 mM stilbene, or 10 mM pinene together with 1 mM stilbene. Cultures of 13 ml were initiated with 130 µl of the stationary-phase inoculum, adjusted to OD_{600} of 0.20 ±0.01 and containing 0.7-2 x 10^8 CFU/ml. Cultures were grown to OD_{600} of 0.6-0.7 in the case of A281(pSY203) and to OD_{600} of 0.3-0.4 in the case of A281(pSY204), and the activity of ß-galactosidase was measured according to MILLER (1972). Measurements were made in three individual cultures per medium, and the experiments were repeated twice.

The effect of α-pinene and stilbene on Agrobacterium infection after vir-induction

Bacterial strains A281 and A281(pTVK291) were grown for three days on solid LB media containing 60 µM acetosyringone, and single colonies were used for inoculating the two-month-old birch seedlings by wounding the stem with a scalpel dipped in a colony. After inoculation, the inoculation sites of the seedlings were treated twice a day by spraying them gently with an aqueous solution containing either 10 mm pinene, 1 mm stilbene or both. Control seedlings were not sprayed. The spraying was started either immediately after the inoculation or two days later. The number of seedlings used per treatment varied from 50 to 56. In all treatments, the seedlings were kept shaded and under continuous mist for the first two days after the inoculation. The formation of crown galls at the inoculation sites was scored two weeks and one month after the bacterial treatment.

In vitro inoculations of Scots pine

Bacterial strains A281, A281(pTVK291) and A281(pSY204) were grown on solid LB media with or without 60 µM acetosyringone for three days, and single colonies were used for inoculating the *in vitro* Scots pine seedlings. Stems of the seedlings were wounded with a scalpel dipped in a bacterial colony under aseptic conditions, and the development of the crown galls was observed two months later. Forty-seven to fifty seedlings were used per treatment, and the experiment was performed twice: in May in the spring time and in September in autumn.

Statistical analysis

Comparisons between treatments in the experiments on bacterial growth and *vir*-gene induction were made by analysis of variance, and the means were compared either by Tukey's test or by Student-Newman-Keuls multiple range test. The results of the birch and pine inoculations were analysed by fitting logistic regression models on the binary data ("gall" or

"no gall"), and by evaluating them with the goodness-of-fit χ^2 tests (BMDPLR procedure, ENGELMAN 1988). The odds ratios received for gall formation in the different treatments were calculated with 95 % confidence intervals.

3 Results

The effect of acetosyringone, α-pinene and stilbene on the growth of A281

The growth curves of A281 in the LB media including different concentrations of acetosyringone, pinene and stilbene are represented in Fig.1. Decrease of pH from 7.0 to 5.6 retarded the growth of agrobacteria slightly, but at the lower pH there were no differences in growth due to acetosyringone addition (Fig. 1A). Pinene inhibited bacterial growth at the highest concentration (20 mM) (Fig. 1B), but none of the tested stilbene concentrations had any effect on the growth at all (Fig. 1C). When both pinene and stilbene were applied, the growth of agrobacteria was totally inhibited at concentrations 20 mM and 2mM, and also the lower doses, 5-10 mM pinene together with 0.5-1 mM stilbene, caused small but significant growth reduction (Fig. 1D).

The effect of α-pinene and stilbene on the accumulation of avirulent Agrobacterium clones

Accumulation of avirulent clones in the strains A281 and A281(pTVK291) was studied by following gall formation in birch seedlings. The seedlings were inoculated with bacteria, which were grown prior to inoculation on the media containing either acetosyringone, pinene or stilbene. Gall formation frequencies are shown in Table 1. No significant associations between gall development and the culture conditions of the inoculum or the bacterial strains could be detected.

Table 1. **Percentages of birch seedlings with crown gall development after *Agrobacterium* inoculations by using strains grown on different media containing either acetosyringone (AS), α-pinene (P) or stilbene (S).**

	Bacterial strain			
	A281		A281(pTVK291)	
Culture medium	2 weeks	1 month	2 weeks	1 month
LB, pH 7.0	67	89	63	89
LB, pH 5.6 & 60 μM AS	69	94	50	89
LB, pH 7.0 & 10 mM P	60	95	49	95
LB, pH 7.0 & 1 mM S	60	90	64	87
LB, pH 7.0 & 10 mM P & 1 mM S	78	80	45	87

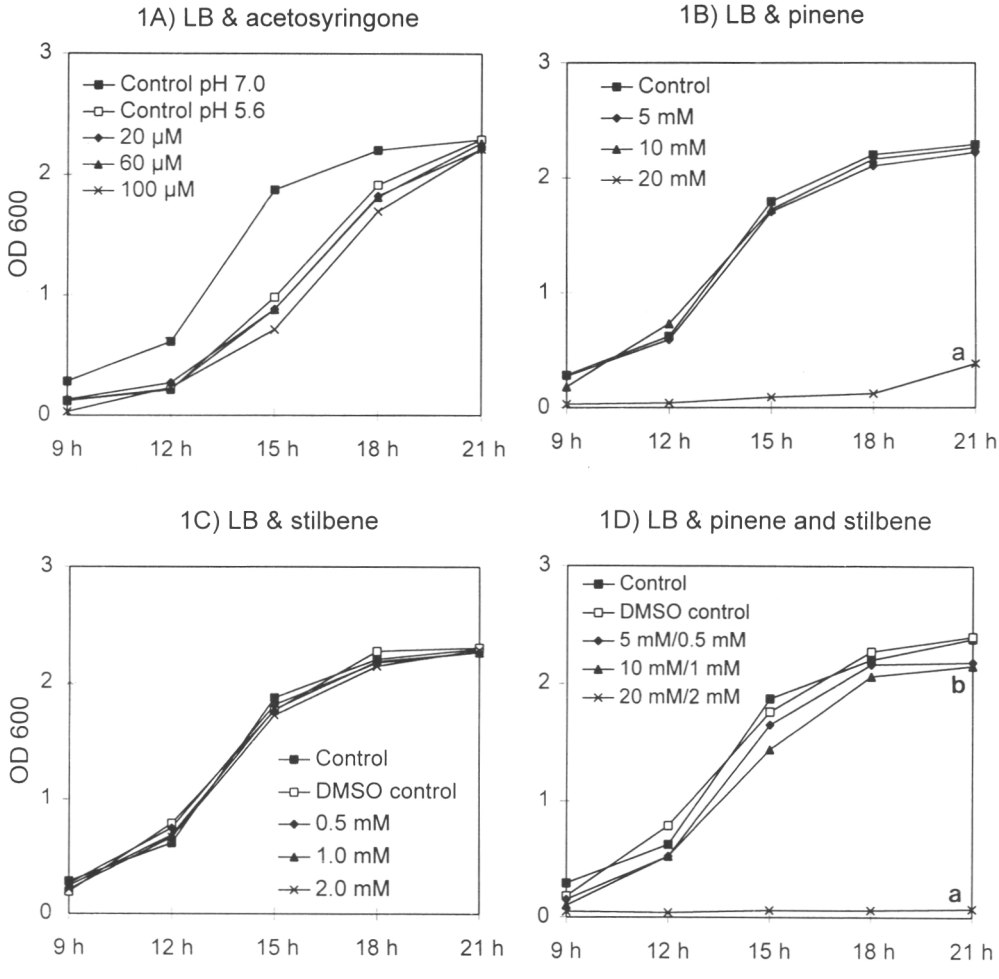

Fig. 1. The growth of the *A. tumefaciens* strain A281 in the media containing varying concentrations of acetosyringone, α-pinene, stilbene, or α-pinene together with stilbene. With the concentrations marked by (a) the growth of agrobacteria was significantly retarded after 21 h of culture compared with other treatments ($p < 0.01$). With the concentrations marked by (b) the growth was significantly better than in the ones marked by (a) after 21 h of culture, but worse than in the control treatments ($p < 0.05$).

Vir-gene induction experiments

Two series of experiments were performed to determine the effect of pinene and stilbene on *vir*-gene induction in agrobacteria. The inhibitory effect of these compounds was investigated by applying them together with acetosyringone, and the inducing effect was studied by applying them as such. As seen in Fig. 2, the addition of acetosyringone into the culture media increased the *vir*-induction both in A281(pSY203) and A281(pSY204), although the *lacZ*-activity in A281(pSY204) was rather high even without it. Pinene and stilbene did not repress the *lacZ*-activity induced by acetosyringone. On the contrary, they enhanced it when they were both applied together with acetosyringone (Fig. 2A, 2B). On the other hand, without asetosyringone the compounds had no *vir*-gene inducing effect (Fig. 2C, 2D).

Application of acetosyringone influenced the growth of the bacterial strains A281(pSY203) and A281(pSY204). The bacteria were generally cultured for 25 to 26 hours to the OD_{600} of 0.6-0.7 or 0.3-0.4, respectively. In the absence of acetosyringone the strain A281(pSY203) grew more slowly than in the media containing acetosyringone; it took 5 to 6 hours more before the cultures achieved the desired OD_{600} value. In the strain A281(pSY204) there were no such big differences, even though the presence of acetosyringone in the media retarded the growth of the cultures to the desired OD_{600} value by one or two hours.

The effect of α-pinene and stilbene on Agrobacterium infection after vir-induction

The percentages of birch seedlings forming galls at the inoculation sites after pinene and stilbene sprayings and control treatments are shown in Table 2. When the data were analysed by fitting a logistic regression model, it was seen that both the spraying treatments and the bacterial strains had a significant effect on gall formation, whereas the starting time of spraying did not influence gall development (Table 3). As described by the logistic model, the application of pinene, stilbene or both decrease the probability for gall formation compared with the control treatment. The bacterial strains react differently. In the strain A281(pTVK291) the probability for gall formation is higher than in the wild type strain A281, reflecting the fact that the chemical treatments had only a minor effect on gall development when it was induced by A281(pTVK291).

In vitro inoculations of Scots pine

The percentages of in vitro Scots pine seedlings forming galls after the *Agrobacterium* inoculations were low. Most of the wounding sites became necrotic, and sometimes an emerging resin droplet could be seen. In the spring replication, 2.1 % of the seedlings inoculated with A281(pSY204) grown without acetosyringone, and 4.2 % of the seedlings inoculated with the same strain induced by acetosyringone, formed a gall at the inoculation site. In the case of the strain A281(pTVK291), 4.0 % of the inoculated seedlings developed a gall when the inoculum was cultured without acetosyringone. Unfortunately, the seedlings inoculated with A281(pTVK291) induced by acetosyringone were contaminated by fungi, and comparisons with this treatment are impossible. The seedlings inoculated with A281 formed no galls at all. In the autumn replication, the gall formation was even more rare. Only the treatments with A281 and A281(pSY204), both inoculums induced by acetosyringone, resulted in gall formation in 2.0 % of the seedlings. There were no associations between gall development and different treatments of the experiment.

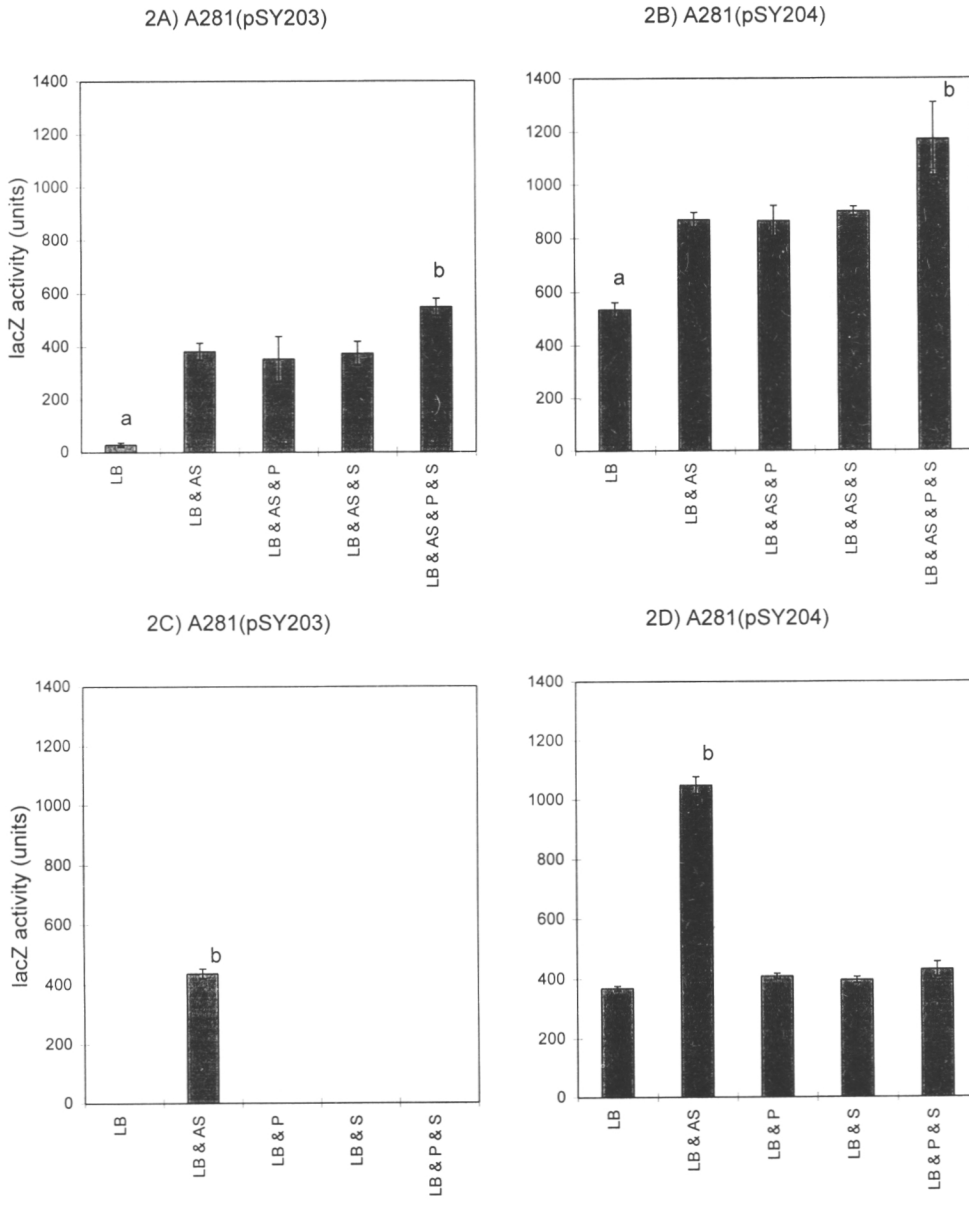

Fig. 2. The activity of ß-galactosidase (*lacZ*) reporter gene in the strains A281(pSY203) and A281(pSY204) under the influence of 60 µM acetosyringone (AS), 10 mM α-pinene (P), 1 mM stilbene (S), or 10 mM α-pinene together with 1 mM stilbene, in the LB medium. In the treatments marked by (a) the *lacZ* activity was significantly smaller than in other treatments ($p < 0.01$), and in the treatments marked by (b) it was significantly higher than in other treatments ($p < 0.05$ in 2A-B, $p < 0.01$ in 2C-D).

Table 2. **Percentages of birch seedlings with crown gall development after *Agrobacterium* inoculations and different spraying treatments.**

	Bacterial strain			
	A281		A281(pTVK291)	
Spraying treatment	2 weeks	1 month	2 weeks	1 month
Control	84	92	53	79
Started 2 days after bacterial inoculations				
- with 10 mM α-pinene	50	65	47	71
- with 1 mM stilbene	38	43	42	78
- with 10 mM α-pinene and 1 mM stilbene	47	47	31	70
Started immediately after bacterial inoculations				
- with 10 mM α-pinene	46	66	42	79
- with 1 mM stilbene	16	41	27	67
- with 10 mM α-pinene and 1 mM stilbene	28	56	45	70

Table 3. **Multivariate associations between crown gall development, spraying treatments, and *Agrobacterium* strains used for inoculations. The logistic regression model generated was *log (p/1-p) = 1.44 - 0.954s_1 - 1.541s_2 - 1.386s_3 + 0.713b*, where the design variables used for the spraying treatments are s_1, s_2, and s_3, and the desing variable used for the bacterial strain is *b*. The starting time for spraying was not included in the model as a variable due to its insignificant nature. The goodness-of-fit χ^2 test with the p-value of 0.025 showed that the model fits the data well.**

Variable	p Value	Odds ratio (95 % confidence interval)
Spraying treatment	< 0.0001	
Control		1
α-pinene		0.385 (0.205 to 0.723)
Stilbene		0.214 (0.115 to 0.398)
α-pinene & stilbene		0.250 (0.134 to 0.465)
Starting time for spraying	0.9774	
Bacterial strain	< 0.0001	
A281		1
A281(pTVK291)		2.04 (1.48 to 2.81)

4 Discussion

Gymnosperm defence systems against pathogens and predators are complicated including both structural protection and biosynthesis of secondary defence compounds. Because agrobacteria usually infect host plants through wounds both in nature and in genetic transformation applications, this study emphasizes the interactions between *Agrobacterium* and coniferous defence compounds.

Based on biosynthetic criteria, the plant secondary products can be divided into three principal groups: terpenes, phenolics and nitrogen-containing compounds (TAIZ and ZEIGER 1991). A monoterpene, α-pinene, and a phenolic constitutent, stilbene, were chosen for study for several reasons. Pinene is one of the most common monoterpenes in oleoresin of the gymnosperms (CHUNG 1981; VON RUDLOFF and GRANAT 1982; DELORME and LIEUTIER 1990; SJÖSTRÖM 1993; CHALCHAT et al. 1994; NERG et al. 1994; KLEPZIG et al. 1995; VALTEROVA et al. 1995). Both mechanical wounding (MARPEAU et al. 1989; LEWINSOHN et al. 1991; KLEPZIG et al. 1995) and pathogen or insect attacks (DELORME and LIEUTIER 1990; KLEPZIG et al. 1995; VALTEROVA et al. 1995) cause an increase in the α-pinene content in many conifers. Essential coniferous oils, and also purified single monoterpenes have antimicrobial activities mainly against fungi and Gram-positive bacteria (KARTNIG et al. 1991; HIMEJIMA et al. 1992), and α-pinene has been found to inhibit *in vitro* growth of some Gram-negative *Agrobacterium* strains as well (ARONEN and HÄGGMAN 1995).

Stilbenes, on the other hand, are a wide-spread group of phenolic compounds in conifers, mainly located in heartwood and bark, but also present as traces in sapwood. A typical member of the stilbene group is pinosylvin (SJÖSTRÖM 1993), a trans-3,5-dihydroxystilbene, which has strong inhibiting and bactericidal effects against many bacterial species (FRYKHOLM 1945). This compound has been patented for controlling undesirable algae and micro-organisms in aqueous environments (SHEERS 1971). According to FLIEGMANN and co-workers (1992) stilbene, i.e. pinosylvin biosynthesis, is in *Pinus sylvestris* a reaction to stress conditions, such as wounding. Because pinosylvin is not commercially available, trans-stilbene was used in the present study instead.

The concentrations of α-pinene and trans-stilbene, used in the present work for studying their effects on the *vir*-gene induction and other aspects of *Agrobacterium* infections, were determined based on the culture experiments of the strain A281. The idea was to study the interactions between the defence compounds and *Agrobacterium vir*-gene function under conditions not deleterious for bacteria. The selected concentrations, 10 mM α-pinene (approximately 0.14 %, w/w) and 1.0 mM trans-stilbene (approximately 0.02 %, w/w), had no inhibitory effects on the growth of the A281 when used alone, and only a small reducing effect when used combined. The α-pinene content in cortical tissues of maritime pine has been determined both before and after wounding. Before wounding it varied from 0.03 to 0.05 %, and after wounding from 0.13 to 0.3 %, depending on the season (MARPEAU et al. 1989). According to LINDSTEDT (1949a, -b,- c, -d, -e) the content of pinosylvin and its monomethyl ether in the heartwood of different pine species (*Pinus contorta, P. Jeffreyi, P. montana, P. ponderosa, P. radiata)* varies between 0.014 % and 0.41 %. The highest values, up to one percent , were found in *P. sylvestris*. On the other hand, very small concentrations of pinosylvin, from 1×10^{-9} to 2×10^{-5}, have previously been reported to be effective against microbes (FRYKHOLM 1945; SHEERS 1971). It is worth noticing that the contents of α-pinene and stilbene measured in natural environments, in which they occur together with a huge amount of other constitutents, are higher than those used in the present work. This indicates

that chemical defence systems of gymnosperms are often able to kill invaders, such as agrobacteria, effectively.

Application of α-pinene, trans-stilbene or acetosyringone during the culture of bacterial inoculum caused no accumulation of avirulent *Agrobacterium* clones in the present study. FORTIN and co-workers (1992) reported that under the influence of acetosyringone avirulent clones were produced in the cultures of nopaline type *Agrobacterium* strains, but this phenomenon was not observed in the present study with the agropine type strain A281. Conversely, growth inhibition of agrobacteria treated with acetosyringone under acid culture conditions was also found in the strain A281, as already described by FORTIN and co-workers (1992) in the case of nopaline type strains.

The coniferous defence compounds studied, α-pinene and trans-stilbene, had no inhibitory or inducing effects on *vir*-gene induction in *Agrobacterium*. The treatment in which both of these compounds were applied together with acetosyringone was the only exception, resulting in enhanced *vir*-induction. Previously, a phenolic *vir*-gene inducer, coniferin (a coniferyl alcohol β-glucoside), has been isolated from the pinaceous gymnosperm *Pseudotsuga menziesii* (MORRIS and MORRIS 1990). Also other coniferyl alcohol β-glycosides have been shown to have *vir*-inducing activities (DELAY et al. 1994).

Even though α-pinene and trans-stilbene were not able to inhibit *vir*-induction in the present work, they significantly affected the development of *Agrobacterium* infections afterwards. When the compounds were applied as gentle sprays on the inoculation sites of the susceptible host plants, the gall formation frequency was significantly reduced. The *vir*-induction in this experiment was assured by culturing the bacterial inoculum under the influence of acetosyringone. The results suggest that the chemical defence of gymnosperms is functional during the later stages of *Agrobacterium* infection when the induction of virulence genes has already taken place. The defence compounds may either interfere with the functions of the *vir*-gene products during T-DNA processing, transportation and integration or they simply prevent the division of transformed host plant cells.

With the strain A281(pTVK291) containing extra copies of the *virA, virB, virG* and *virC* genes the inhibition of gall formation caused by the spraying treatments was not as big as with the strain A281. The VirG protein is a transcriptional activator for the *vir*-genes being activated by VirA-mediated phosphorylation (WINANS 1991; ZAMBRYSKI 1992). The products of the *virB* locus are proposed to form a complex pore structure or a T-DNA export channel on the bacterial membrane (ZAMBRYSKI 1992; THORSTENSON et al. 1993), and VirC is believed to enhance T-DNA border nicking and T-strand production (TORO et al. 1988; ZAMBRYSKI 1992). Thus it seems feasible that extra copies of the *virB* and *virC* in the strain A281(pTVK291) could partly compensate for the interfering influence of α-pinene and trans-stilbene on the transformation events, assuming that owing to higher copy number there are more VirB and VirC proteins present in the A281(pTVK291) than in the wild type strain A281.

Constitutive expression of the virulence genes or multiple copies of the *virG* have been demonstrated to enhance the efficiency of *Agrobacterium*-mediated transformation in several plant species, such as celery, carrot, rice, tobacco and cotton (LIU et al. 1992; HANSEN et al. 1994). In the present study, no such effects could be found in the model gymnosperm, Scots pine. This agrees with the idea that failures in *Agrobacterium* infections in many conifers are not due to insufficient induction of the virulence genes, but rather to interference by chemical defence compounds during the later stages of T-DNA transformation. In fact, resin droplets consisting of toxic terpenoids are often visible at the wounding sites, not only creating a chemical barrier to invaders but also sealing the wound effectively.

The present study characterises the interactions between coniferous defence compounds, α-pinene and trans-stilbene, and *Agrobacterium tumefaciens*. Of the chemical constituents studied, α-pinene proved to prevent the growth of agrobacteria when high doses were supplied. Either α-pinene or trans-stilbene had no inhibitory effect on the induction of the *Agrobacterium* virulence genes, but both compounds caused a reduction in gall formation frequency when applied after the *vir*-induction. The efficiency of *Agrobacterium*-mediated transformation in the model gymnosperm, Scots pine, could not be enhanced by using the strains possessing either the constitutive mutant of *virG* or extra copies of *virA* and *virG*. The results suggest that inefficiency of *Agrobacterium* sp. to infect many conifers is not caused by insufficient *vir*-gene induction but might be rather owing to failures to accomplish later stages of infection, i.e. T-DNA processing, transportation and integration to host plant genome. It seems probable that the chemical defence compounds, such as terpenoids and phenolic constituents, may interfere with the functions of *vir*-gene products thus inhibiting the transformation events.

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III

ROOTING OF SCOTS PINE FASCICULAR SHOOTS BY *AGROBACTERIUM RHIZOGENES*

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ABSTRACT

The advantageous effect of *Agrobacterium rhizogenes* on root induction in Scots pine cuttings of fascicular bud origin was demonstrated. Two lots of cuttings, a spring and a late summer lot, were first incubated for 20 h in 0.5 mM IBA solution, then dipped in bacterial colonies of strains A4, A4::GUSint or R1600, and then planted. In the summer lot the *Agrobacterium* treatment enhanced root formation, which was on an average 24.5% (±5.1), and 16.2% (±2.9) in IBA treatment alone. The genotypic variation in rooting ability was remarkable. In the best genotypes more than 80% of the fascicular shoots rooted, while the best results in the IBA treatment were around 30–40%. The chromosomal matrix of the A4Ri-plasmid seemed to affect the results, indicating that functions encoded by the bacterial chromosome are also involved. The agropine assays, the histochemical tests for ß-glucuronidase expression, PCR tests, and observations on root morphology show that the roots formed after *Agrobacterium* treatments are untransformed. When considering the practical applications of this rooting method, normal roots instead of transformed ones will be preferred due to the conflicting views on the release of foreign genes or chimeric plants into nature.

Key words: *Pinus sylvestris*, fascicular shoots, vegetative propagation, *Agrobacterium rhizogenes*, DNA transfer

INTRODUCTION

Vegetative propagation has many uses in forestry, such as conservation of genotypes, multiplication of genotypes for specific purposes, *e.g.* seed orchards, evaluation of genotypes through clonal testing, and capture of maximum genetic gain when used for regeneration in operational planting programs (ZOBEL & TALBERT 1984). Rooted cuttings are one of the most commonly used methods for vegetative propagation. Also some coniferous tree species, such as sugi (*Cryptomeria japonica* D. Don) in Japan, radiata pine (*Pinus radiata* D. Don) in Australia and New Zealand, and Norway spruce (*Picea abies* (L.) Karst.) in France, are propagated on a commercial scale by rooting cuttings (TALBERT *et al.* 1993).

Vegetative propagation of Scots pine (*Pinus sylvestris* L.) has been investigated by using either long shoots (BALLENGER & HUANG 1984, BOEIJINK & VAN BROEKHUIZEN 1974, MAYNARD & BASSUK 1987, MONTEUUIS & PAGES 1987, STRUVE & GERHOLD 1985) or needle fascicles as such (MONTEUUIS & PAGES 1987, YLI-VAKKURI & PELKONEN 1976), or after pruning the branches (BOEIJINK & VAN BROEKHUIZEN 1974, HILSON & DANCIK 1978) or by using chemical spraying treatment of stock plants (BORNMAN 1984, MAYNARD 1986, SALONEN 1990, WHITEHILL & SCHWABE 1975). In these studies the rooting percentages of cuttings have varied between 0 and 100%, depending on several factors, such as the stock plant age and growth regulator treatments. The success of rooting in Scots pine cuttings has been rather low, and so far this propagation method has not been applied to practical scale forestry.

Agrobacterium rhizogenes is a common soil inhabitant belonging to the *Rhizobiaceae* family. Phytopathogenic strains of *A. rhizogenes* cause hairy root formation in the infection sites in the host plants. Agrobacteria are able to transfer part of their Ri-plasmid DNA, the so called T-DNA, into plant cells, where it integrates into the plant genome. The disease symptoms are due to the expression of bacterial genes affecting the phytohormone balance in the plant cells at the infection site (CLARE 1990, GELVIN 1990, TEPFER 1989).

The root induction ability of *A. rhizogenes* has been used successfully for rooting cuttings of hybrid poplars (*Populus deltoides* × *nigra, P. nigra* × *maximowiczii*) in *in vitro*-conditions (CHAREST *et al.* 1992), apple cuttings *in vivo* and *in vitro* (LAMBERT and TEPFER 1991), and olive, apple, almond and pistachio shoots *in vitro* (RUGINI 1992). The root formation in these cases was caused by genetic transformation. There are also

reports on other tree species, such as hazelnut (*Corylus avellana* L.) (BASSIL *et al.* 1991), tamarack (*Larix laricina* K.Koch), jack pine (*Pinus banksiana* Lamb.) and western white pine (*Pinus monticola* Dougl.) (MCAFEE *et al.* 1993), in which rooting of the cuttings or the *in vitro* shoots has been improved by using *A. rhizogenes*. In these cases, however, the increased rooting could not be ascribed to DNA transfer, but the presence of agrobacteria in the rooting medium appeared to be beneficial.

The aim of the present work was to investigate if genetic transformation by *A. rhizogenes* could be used for inducing root formation in Scots pine cuttings. This approach was compared with the commonly used growth regulator treatment, and a range of Scots pine genotypes were evaluated for their competence for *Agrobacterium* transformation.

MATERIAL AND METHODS

Production of fascicular shoots

The method used was based on cytokinin spraying treatments, which stimulate development of fascicular buds (KOSSUTH 1978, WHITEHILL & SCHWABE 1975). Two-year-old seedlings of Scots pine originating from controlled crossings of elite trees from southern Finland were used as stock plants. During the summer 1993 normally over-wintered seedlings were grown in the greenhouse under natural light, and they were treated eight times, twice a week, with foliar applications of 0.5 mM benzylaminopurine (BAP, Sigma). BAP dissolved in 1M NaOH was applied as an aqueous solution containing 0.004% (v/v) Tween 20 as a surfactant. Spraying started when the height growth of the leading shoots was almost finished, and new annual shoots were sprayed to run-off with a manual pump sprayer. Within 4–8 weeks development of fascicular buds could be seen in needle fascicles.

After a normal autumn the stock plants were subjected to another growing season between December 1993 and February 1994, by providing a 16-hour photoperiod (about 200µE $m^{-2}s^{-1}$) and by maintaining temperature at 22 °C in daytime and at 17 °C at nights. During this period the fascicular buds elongated into fascicular shoots (Fig.1). The plants were then given 8 weeks of short-day and cold treatment. The photoperiod was shortened to 7 hours and light intensity was half of the original. The temperature was gradually decreased from 22 °C to 3 °C, and for the last four weeks the seedlings were kept at 3 °C. Fascicular shoots, 2–5 cm of length, were taken from the stock plants in March, directly after the cold treatment (the spring lot) and in June, after the second growing season of the stock plants (the summer lot). Thus the cuttings in the summer lot had elongated for two growing seasons. The spring lot contained 698 cuttings from 12 different genotypes, the summer lot 596 cuttings from 22 different genotypes.

The dry matter content was estimated in the cuttings in the two lots. Fascicular shoots (5–9) were harvested, weighed and dried at 105 °C for 24 hours. The dry matter content, calculated as a percentage of fresh weight, was 32–35% for the cuttings in the spring lot and 26–35% for the ones in the summer lot.

Bacterial strains

Bacterial strains used for the rooting experiments were a wild-type *Agrobacterium rhizogenes* strain A4 (MOORE *et al.* 1979), a genetically engineered version of the A4 containing the ß-glucuronidase reporter gene with an intron (A4::GUSint) (VANCANNEYT *et al.* 1990), and an *A. tumefaciens* strain C58 carrying the Ri-plasmid A4b together with the extra copies of the virulence genes in the pTVK291 plasmid, called R1600 (PYTHOUD *et al.* 1987). The R1600 strain was cultured on Luria Broth (LB) medium (MILLER 1972), solidified with 1.5% agar, including the antibioticum kanamycin 50 $mg.l^{-1}$, and the A4 strains were cultured on MYA medium (TEPFER & CASSE-DELBART 1987), also solidified with 1.5% agar, including kanamycin 50 $mg.l^{-1}$ in the case of the strain A4::GUSint.

Rooting experiments

Two different lots of Scots pine fascicular shoots were treated with *Agrobacterium* strains carrying Ri-plasmids. In the spring lot one third of the cuttings were treated with the strain R1600, one third with the wild-type strain A4, and one third were controls, not treated with *Agrobacterium*. The number of cuttings of each genotype per treatment varied from 8 to 50. In the summer lot two thirds of the cuttings were treated with the strain A4::GUSint, and one third were kept as controls.The number of cuttings of each genotype was between 8 and 26 in the A4::GUSint inoculations.

All the cuttings were first incubated for 20 hours in 0.5 mM indole-3-butyric acid (IBA, Fluka) solution, in the dark. After the growth regulator incubation, a 1 mm piece was excised from the base of the cuttings to provide a fresh wound for agrobacteria. The control cuttings were also excised as described, and planted in a soil mixture of horticultural peat, bark humus, and perlite (5:3:2). In the bacterial treatments the bases of the cuttings were dipped in bacterial colonies grown for three days on the appropriate agar medium including 100 µM acetosyringone. After bacterial inoculation the cuttings were planted in the same way as the controls.

Figure 1 Production of fascicular shoots in Scots pine. Cytokinin spraying of the two-year-old Scots pine seedling (A) causes the proliferation of the fascicular shoots (B), which have been used as cuttings in the present study

The planted cuttings were grown in greenhouse, in 7 cm diameter pots under continuous mist (the relative humidity being 90%), and during the rooting period the greenhouse benches were warmed from beneath to be approximately 5 °C warmer than the surrounding space, which was between 15–18 °C. After the rooting period (from 6 to 24 weeks) when the mist was turned off, the relative humidity was 60%, and the temperature in the greenhouse was kept above 15 °C. Root formation was examined 6, 10, 16, and 24 weeks after the treatments, and the rooted cuttings were transplanted into new containers. After transplantation the cuttings were fertilized with 0.2% commercial Superex fertilizer (Kekkilä) once a month during the growing season. In the first growing season, during and after the rooting period, the cutting plants of fascicular bud origin were sprayed once a week with a fungicide (0.1% Ronilan or 0.3% Tirama, Kemira), and in the second growing season the attacks of aphids were controlled three times by 0.1% Roxion (Kemira) sprays. The transplanted cutting plants were over-wintered for six months at 1–6 °C. At the beginning and at the end of the second growing season the elongation growth of the plants was measured. At the end of the season the growth habit and the root systems of the cutting plants were also examined.

Histochemical ß-glucuronidase test

Root tips (n = 82) from the cutting plants, rooted after the A4::GUSint treatment, were tested histochemically for the expression of the ß-glucuronidase (GUS) gene as described by JEFFERSON (1987), with minor modifications: the staining solution contained 0.1 M sodium phosphate buffer (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.1% Triton X-100. Samples were kept in test solution in the dark at 37 °C for 24 h, and the presence of GUS expressing cells or cell clusters was examined under a stereomicroscope.

Opine assay

Freshly formed roots of the one-year-old rooted cuttings (n = 150) were rinsed in tap water, and used for agropine assays, which were performed as described by PARSONS *et al.* (1986), with minor modifications. Agropine and mannopine standards for the assays were prepared according to the previously published protocol (PETIT *et al.* 1983).

PCR test for the presence of the GUSint sequence or remaining agrobacteria in root tissues

Genomic DNA was isolated from the roots of 76 plants originating from fascicular shoots and treated with A4::GUSint as described by DOYLE and DOYLE (1990), with minor modifications according to ARONEN and HÄGGMAN (1995). Fresh roots were excised from the two-year-old plants, rinsed in tap water, and used as material for the DNA extraction.

Polymerase chain reactions (PCR) were performed to determine the presence of the intron containing ß-glucuronidase reporter gene introduced into the *A.*

rhizogenes strain A4::GUSint. Primers specific for the GUSint sequence, ACGTCCTGTAGAAACCCCAA and CCCGCTTCGAAACCAATGCC (BLAKE *et al.* 1991), which border a 1286 bp fragment of the GUSint gene (nucleotides 23–1120 according to JEFFERSON *et al.* 1986) were used for the reactions. The reaction mixture contained 1 µl of template DNA, 100 µM dNTP's, 150 pM primers, and 1 U of Dynazyme DNA polymerase (Finnzymes) in 50 µl of manufacturer's buffer. The reaction mixtures were heated at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min, with a final extension step of 72 °C for 7 min in a DNA Thermal Cycler 480 (Perkin-Elmer, Cetus). Amplified samples were electrophoresed on a 2.0% agarose gel, stained with ethidium bromide, and examined under UV-light.

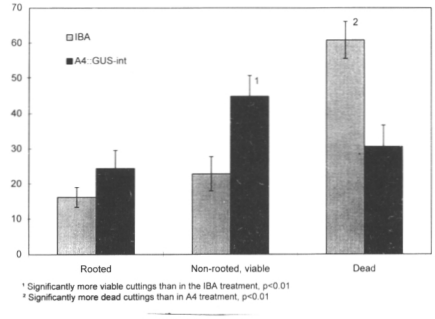

Figure 2 The effect of *Agrobacterium rhizogenes* strain A4::GUSint on rooting and viability of Scots pine cuttings in the summer lot. Pooled data (SE) is based on 596 fascicular shoots treated with 0.5 mM indole-3-butyric acid (IBA) and agrobacteria

Microscopical observations

The anatomical changes in the base of fascicular shoots were examined in microscopical sections. The bases were excised six months after the auxin and *Agrobacterium* -treatment, fixed in FAA (formalin: acetic acid: 95% ethanol, 10:5:85, v/v/v), embedded in paraffin, and stained with safranin-fast green (GERLACH 1984).

Statistical analysis

Comparisons between treatments were carried out by analysis of variance. Means were compared either by Tukey's test or by Student-Newman-Keuls multiple-range test. For correlation analysis, the Pearson's product-moment correlation *r* was used.

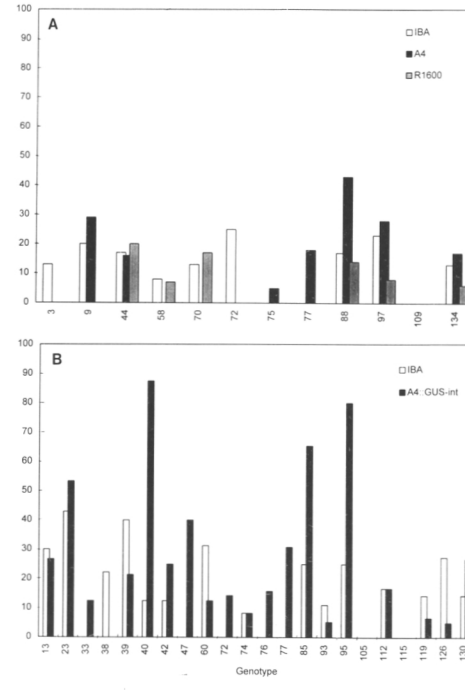

Figure 3 The effect of genotype on the rooting response with and without *Agrobacterium* treatment. Data of the genotypes included in the spring lot is presented in (A) (n=698) and data of the summer lot in (B) (*n* = 596)

RESULTS

Rooting of the fascicular shoots

In the spring lot, rooting percentages for the A4, R1600 and control treatments were 13.4 (±SE 4.1), 5.9 (±2.1), and 12.2 (±2.5), respectively. There were no significant differences between the means. In the summer lot, the treatment with *A. rhizogenes* strain A4::GUSint enhanced root formation, which was on an average 24.5% (±5.1) in the bacterial treatment, and 16.2% (±2.9) in the control treatment. Also the amount of non-rooted, viable cuttings was doubled, 44.9% (±5.9), when compared with the control, 22.9% (±4.9) (Fig. 2). In both lots, genotypic variation was remarkable (Fig. 3), and no correlation could be found between the rooting percentages in bacterial and control treatments. In the best genotypes, more than 80% of the fascicular shoots rooted after A4::GUSint treatment, while the best results achieved in the control treatment varied around 30–40% (Fig. 3B).

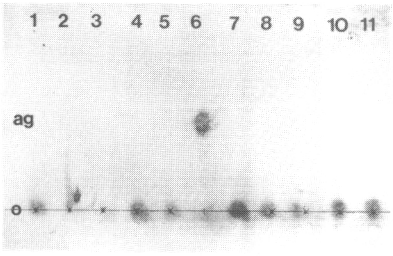

Figure 4 Agropine assay of the roots of the Scots pine cuttings. Lane 6: an agropine standard; lanes 1–5 and 7–11: root samples of ten cuttings; ag = agropine, o = origin of separation, in which neutral $AgNO_3$ positive compounds are visible

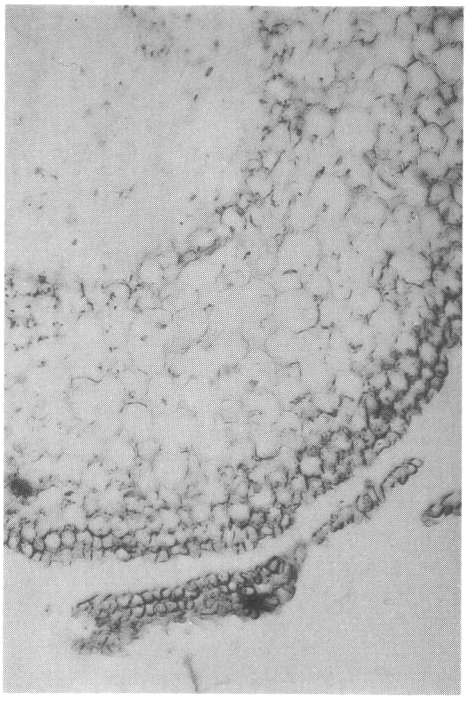

Figure 5 A cross section of a cutting root tested histochemically for -glucuronidase expression. A few blue-stained cells were found in superficial cell layers detached from the root

Opine analyses and histochemical tests for reporter gene expression in the roots

The roots of one-year-old cutting plants were tested for the presence of agropine, but it could not be detected in any of the tested 150 roots (Fig. 4). When the root tips of the plants from the A4::GUSint treatment were tested histochemically for the expression of ß-glucuronidase reporter gene, a few light-blue stained cells could be found in some samples. These cells were, however, not located in the inner part of the root tissue, but in the superficial cell layers often detached from the root (Fig. 5).

PCR test for the presence of GUSint sequence or remaining agrobacteria

Amplification of the GUSint fragment of 1286 bp could not be detected in any of the 76 DNA samples tested (Fig 6). Only some faint bands representing non-specific amplification products were seen in some of the samples. Positive controls using the *A. rhizogenes* strain A4::GUSint as a template produced a strong and clear amplification product of expected size.

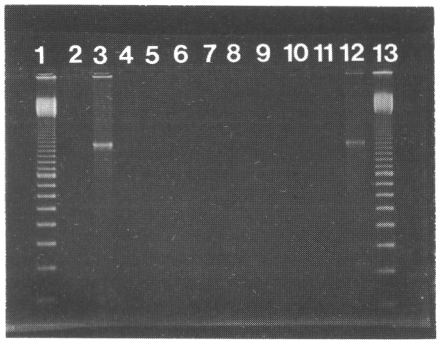

Figure 6 The PCR amplification of the GUSint fragment (1286 bp) by using the DNA samples isolated from the roots of Scots pine cuttings as a template. Lanes 1 and 13: a 100 bp ladder; lanes 3 and 12: a positive control using the DNA of the *A. rhizogenes* strain A4::GUSint as a template; lane 2: a negative control using water as a template; and lanes 4–11: amplification results with the DNA of root samples of Scots pine cuttings.

Microscopical observations

Different kinds of anatomical changes were observed in the basal areas of the stems of the fascicular shoots after the rooting treatments. Some of the cuttings did not respond in any visible way (Fig.7A), and these cuttings usually died within a few months after treatment. Other cuttings did not form roots, but stayed green and viable through the whole first growing season, and were characterized either by callus formation (Fig.7B) and / or axillary bud proliferation (Fig.7C). In the rooted cuttings (Fig.7D) proliferation of a single root was

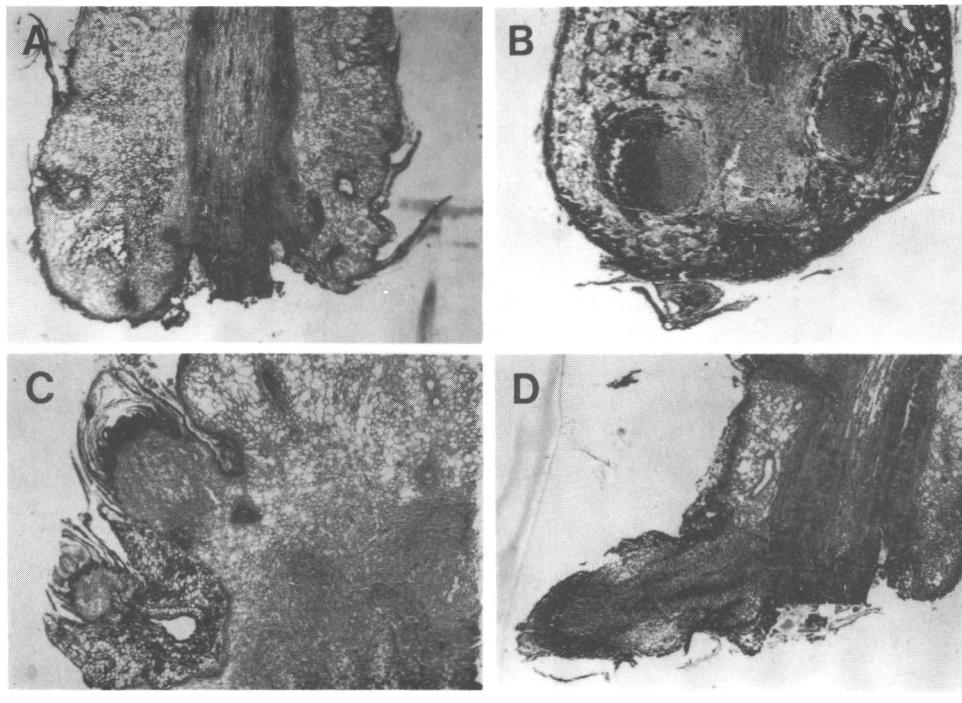

Figure 7 Anatomical changes in the basal areas of the cytokinin-induced fascicular shoots of Scots pine after *Agrobacterium rhizogenes* treatments. The cuttings without root formation could be divided into two categories: the ones with no visible changes in basal area (A), and the ones characterized by callus production (B) and / or axillary bud proliferation (C). In the rooted cuttings (D) usually formation of a single root was observed

usually observed. Typical symptoms of hairy roots, such as numerous roots with short internodes and hairy characteristics, could not be detected.

Growth measurements

During the first growing season the elongation growth of the cutting plants was more or less retarded. The mean height of the rooted cuttings after the first cold period was 1.4 (± SE 0.07) cm. At the beginning of the second growing season the elongation growth of the cuttings was more pronounced (Fig. 8A), and at the end of the season the mean height of the cuttings was 6.3 (±0.25) cm. There were no differences in the growth rate between cuttings rooted after bacterial treatments and the control treatment. The growth habit and the root systems of the cuttings were also examined at the end of the second growing season. Approximately 40% of the cuttings were orthotropic, 51% were slightly plagiotrophic, and 9% were badly plagiotrophic, growing at an angle of 45 degrees or less to the horizontal plane (Fig. 8B). No significant differences could be seen in growth habit when comparing cuttings from the bacterial and control treatments. A majority of the cuttings had well developed root systems with one strong root and highly ramified lateral roots.

DISCUSSION

Rooting of Scots pine vegetative propagules has often proved difficult regardless of the type of the material used, *e.g.* long shoots (BALLENGER & HUANG 1984, BOEIJINK & VAN BROEKHUIZEN 1974, STRUVE & GERHOLD 1985), fascicular shoots (MAYNARD 1986), hypocotyl and epicotyl cuttings (GRÖNROOS & VON ARNOLD 1988, FLYGH *et al.* 1993), or adventitious shoots (HÄGGMAN *et al.* 1995). In the present study we demonstrate the positive effect of the *Agrobacterium rhizogenes* treatment on the rooting of fascicular shoots in Scots pine.

The ability of Scots pine to produce adventitious roots will diminish rapidly as the cutting donor ages: only 3.3% of cuttings rooted when the donor plant was 8 years old, compared with a rooting percentage of 43.4 for 3-year-old and 70% for 3.5-month-old seedlings (MONTEUUIS & PAGES 1987). Fascicular shoots were

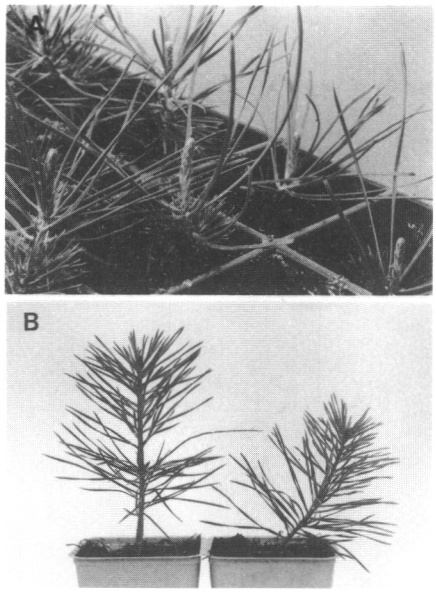

Figure 8 Rooted cuttings of fascicular bud origin in the greenhouse. Vigorous growth started in the beginning of the second growing season (A). Both orthotropic and plagiotropic growth habits were observed (B)

chosen as vegetative propagules for the present study, because cytokinin spraying of the ortets makes it possible to produce high numbers of cuttings in young seedlings (SALONEN 1990). In addition to the age, the genotype of the donor plant has been reported to affect the rooting capacity of cuttings (BERG 1980, MONTEUUIS & PAGES 1987, SALONEN 1990). This phenomenon was also very remarkable in the present study.

External factors, such as conditioning and treatments of the ortets, and the excision time of the ramets can also have an important impact on root formation (reviewed by MONTEUUIS & BARNÉOUD 1991). In the present study, the summer lot cuttings rooted better than the spring lot, which may be due to differences in the developmental stage of the propagules. Fascicular shoots in the spring lot were comparable with the natural shoots at the beginning of the growing season, while the cuttings in the summer lot were collected at the time when their growth had already stopped. The best rooting results are generally obtained either during early spring or during early autumn (HANSEN & ERNSTSEN 1982, MONTEUUIS & PAGES 1987). On the other hand, the cuttings in the spring and summer lots in the present work were derived from different genotypes, which, of course, may also have an effect on rooting percentages.

Fascicular shoots responded to the bacterial and growth regulator treatments by producing either differentiated roots or undifferentiated callus tissue. In some cases, proliferation of axillary buds was also seen. This may be an after-effect of the cytokinin sprays of ortets. Over-stimulation with BAP during *in vitro* culture is supposed to lead to abnormalities. Some plantlets of *Alnus glutinosa*, for example, produced an excessive number of buds low on the trunk a few months after they had been planted in the field (EVERS *et al.* 1988). Cuttings with callus formation around the base of the stem often survived for several months after planting. As seen in tissue culture, callus tissue is effective in taking water and nutrients from surrounding medium (*e.g.* AITCHISON *et al.* 1977). In the present study, callus proliferation has probably maintained the housekeeping metabolism of the cuttings and thus enhanced their survival.

The growth of the fascicular shoots during the first growing season was more or less retarded, probably due to root formation. This growth retardation may also be considered an after-effect of the growth regulator treatments of the stock plants. In loblolly pine (*Pinus taeda* L.), plantlets exposed *in vitro* to BAP had a poor initial growth under greenhouse conditions compared to seedlings (TIMMIS & RITCHIE 1988). The plagiotrophic growth habit of some cutting plants could probably have been avoided by supporting the fascicular shoots with a stick in vertical position during the initial growth period.

The *Agrobacterium rhizogenes* strain A4 was chosen due to its good infection ability in several plant families (PORTER 1991), as shown also in woody plants (HUANG *et al.* 1993, LAMBERT & TEPFER 1992, PHELEP *et al.* 1991). The genetically engineered version of A4 containing the GUSint gene was included to enable easy monitoring of the transgene integration and expression in the roots of the cuttings. The *A. tumefaciens* strain R1600 carrying the A4Ri-plasmid, on the other hand, was used to study the effect of the chromosomal background on the root induction ability of the Ri-plasmid. When the results from the bacterial treatments in the spring experiment were examined, the strain A4 doubled root formation compared with the strain R1600. Since the Scots pine cuttings in the present study were derived from the same donors and all the environmental conditions as well as the Ri-plasmids were alike, the difference seems to be due to the chromosomal matrix.

The results of the agropine assays, the histochemical tests for ß-glucuronidase reporter gene expression, PCR tests, as well as the observations on the root morphology suggest that the roots formed in the Scots pine cuttings after the *Agrobacterium* treatments were not stably transformed. Negative results in agropine assays do not necessarily indicate the absence of the

whole T-DNA in root tissues, because TL- and TR-segments of T-DNA are transferred independently (TEPFER 1989). On the other hand, MAGNUSSEN Íet al. (1994) reported that the histochemical test for ß-glucuronidase expression was functional in the roots of *Pinus contorta* induced by *A. rhizogenes.* Negative results of the same histochemical test in the present study suggest that the roots are untransformed.

The enhancement in root induction caused by agrobacteria could, nevertheless, be due to the transient expression of the T-DNA genes in Scots pine cells. In chrysanthemum, a low efficiency gene transfer and transient expression of the TR-DNA auxin synthase genes after *A. rhizogenes* infection can mediate the induction of untransformed roots without stable integration of the T-DNA into the host plant genome (VAN WORDRAGEN *et al.* 1992). There are also reports on tree species, such as *Pinus monticola, P. banksiana* and *Larix laricina* (MCAFEE *et al.* 1993) and *Corylus avellana* (BASSIL *et al.* 1991), in which *A. rhizogenes* has improved rooting, but with no evidence of transformation. The other possibility is that a few cells have originally been transformed and are producing auxin due to the presence of the T-DNA genes, and normal roots can develop under the influence of transported auxin.

Agrobacteria may promote root induction, not only by transforming the host plant cells, but also by modifying the rhizosphere. LEYVAL & BERTHELIN (1989) have shown that interactions with rhizospheric micro-organisms are important for plant growth and nutrition. *Agrobacterium* sp. are acid-producing micro-organisms, which can increase the availability of soil mineral elements thus enhancing nutrient uptake and growth of the Scots pine root system (LEYVAL & BERTHELIN 1989). Besides affecting the nutrient balance in the rhizosphere, agrobacteria can secrete growth regulator substances. Both *A. tumefaciens* and *A. rhizogenes* strains have been shown to contain cytokinin synthase genes in their Ti- and Ri-plasmids, outside the T-DNA region (POWELL *et al.* 1988, REGIER *et al.* 1989). Moreover, KUTÁČEK and ROVENSKÁ (1991) have found that *A. tumefaciens* can synthesize auxin encoded by both the chromosomal and Ti-plasmid genes. In the present study, the chromosomal background of A4Ri-plasmid seemed to have an effect on rooting response. This observation suggests that the functions encoded by the chromosomal genes of agrobacteria are also involved.

The negative amplification results in the PCR tests indicate that the root tissues of the cutting plants do not contain the GUSint gene integrated into the host genome or remaining agrobacteria. After transforming the host plant, agrobacteria usually live in the intercellular spaces or on the surface of the transformed tissue, by using specific compounds, opines, secreted by transformed cells as a source of carbon, nitrogen and energy (AGRIOS 1988, CLARE 1990). DNA amplification by PCR is a very sensitive method for detecting micro-organisms in plant tissues (SCHAFF *et al.* 1992), and a remaining *A. rhizogenes* population in root tissues should have caused amplification of the GUSint fragment. It is, of course, still possible that root tissues contain agrobacteria, but without any selection pressure the strain A4::GUSint may have lost the GUSint plasmid. As for practical application small numbers of remaining agrobacteria in the rhizosphere of the cutting plants are hardly hazardous, because agrobacteria do occur also in forest soils (BELL & RAMEY 1991).

The genotypic variation in the rooting ability of the Scots pine fascicular shoots was remarkable. Since the existence of the host-pathogen specificity between tree genotype and *Agrobacterium* strain has been demonstrated for other pine species, such as *Pinus taeda* (HUANG & TAUER 1994), and *P. radiata* (BERGMANN & STOMP 1994), the root induction in Scots pine might be improved further by testing different *A. rhizogenes* strains as rooting agents. Alternatively, agrobacteria could be genetically engineered to be more infectious, *e. g.* by incorporating a mutant gene leading to constitutive virulence gene induction, as suggested by HANSEN *et al.* (1994).

In the present study, we have shown the advantageous influence of *Agrobacterium rhizogenes* on root induction in Scots pine cuttings of fascicular bud origin, with no evidence of transformation. As for the practical applications of this rooting method, normal roots instead of transformed ones will be preferred, due to the conflicting views on the release of foreign genes or chimeric plants into nature. This is especially important in long-living forest trees, which grow in natural ecosystems in contrast to agricultural crops.

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IV

Transient β-glucuronidase expression in Scots pine tissues derived from mature trees

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Vegetative buds and bud-derived calli and suspension cells from 5- to 50-year-old Scots pines (*Pinus sylvestris* L.) were used as targets for biolistic transformation. The gene construct used in the experiments was 35S CaMV – β-glucuronidase (GUS). The highest average level of transient GUS expression was found in suspension cells: 1229 ± 359 (mean ± SE) expressing cells per million. Transient expression was found in 35 of 44 (79%) tree genotypes studied. The expression level in buds and in calli was low: one or two spots per expressing bud. Growth-regulator pretreatment (BAP and 2,4-D) increased the number of GUS-expressing buds significantly. The high transient expression level in suspensions and the high percentage of GUS-expressing genotypes suggest that mature Scots pine tissues have potential for further transformation and genetic regulation studies.

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Des bourgeons végétatifs et des cellules en suspension et des cals issus de bourgeons végétatifs de pin sylvestre (*Pinus sylvestris* L.), ont été utilisés comme matériel cible pour la transformation par la méthode de biolistique. La construction génétique utilisée porte le gène de la β-glucuronidase (GUS) sous le contrôle du promoteur 35S CaMV. L'expression transitoire optimale du gène GUS a été obtenue avec des cellules en suspension où 1229 ± 359 (moyenne ± ET) cellules positives pouvaient être observées sur un total d'un million. L'expression transitoire du gène rapporteur GUS a été détectée chez 35 des 44 (79%) génotypes étudiés. De faibles niveaux d'expression ont cependant été observés avec les bourgeons et les cals, avec un ou deux îlots positifs par bourgeon. Cependant un traitement avec des régulateurs de croissance (BAP et 2,4-D), augmente de façon significative le niveau d'expression GUS. La forte expression transitoire observée avec les suspensions cellulaires et le fort pourcentage de génotype exprimant le gène GUS, suggèrent que des tissus matures de pin sylvestre possèdent un bon potentiel pour des études ultérieures de transformation et de régulation génétique.

[Traduit par la Rédaction]

Introduction

During the last few years, gene transfer experiments by particle acceleration have been performed with several coniferous species (Duchesne and Charest 1991; Ellis et al. 1991; Goldfarb et al. 1991; Stomp et al. 1991; Loopstra et al. 1992; Newton et al. 1992; Robertson et al. 1992). The particle-acceleration method does not require the protoplast preparation usually needed for electroporation, and host specificity problems of *Agrobacterium*-mediated gene transfer are avoided (Potrykus 1991).

In most cases, transformation experiments with different coniferous species have been done with embryogenic material, such as whole embryos (Ellis et al. 1991; Robertson et al. 1992), cotyledons (Goldfarb et al. 1991; Stomp et al. 1991), calli, or suspension cells (Duchesne and Charest 1991; Newton et al. 1992). Also in Scots pine (*Pinus sylvestris* L.), transient expression has been achieved in cotyledons (Aronen and Häggman 1992; Häggman and Aronen 1992). Embryogenic material is a potential target not only for gene transfer, but often also for vegetative propagation and regeneration of conifers.

Older trees are recalcitrant to vegetative propagation owing to presumably irreversible changes in gene expression. To study maturation processes or floral development, mature tree tissues are needed. Transient gene expression has been achieved in differentiating xylem of 7- to 8-year-old loblolly pines (*Pinus taeda* L.) (Loopstra et al. 1992). Because of the long rotation time and generation cycle of most coniferous species, 7- to 8-year-old trees are still regarded as juveniles (Zobel and Talbert 1984). Microprojectile bombardment experiments have not been performed on tissues taken from mature coniferous trees. A gene-transfer system is needed to study genetic regulation of traits expressed in mature Scots pines. The objective of the present work was to study the competence of pine buds, calli, and suspension cells derived from 5- to 50-year-old trees to express DNA introduced by microprojectile bombardment.

Materials and methods

Plant material and culture conditions

Vegetative buds (including both apical and axillary buds), bud-derived calli, and suspension cultures of Scots pine were used as target tissues and organs.

Vegetative buds were collected from 5- and 15-year-old pines growing in Punkaharju, Finland, (61°48′N, 29°17′E) in late August, late September, and early December 1992. Buds were also collected from 50-year-old trees in Punkaharju in August and December. Samples from the 50-year-old pines were from the same trees in both collections, but the samples from the younger trees were taken from different individuals in each of the three collections. A limited number of buds were obtained from the younger trees. Cryopreserved buds from 25-year-old pines growing

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in Oulu, Finland (65°N, 25°30′E) (Kuoksa and Hohtola 1991), from April 1990 (n = 40) and 1992 (n = 50) were also used.

Buds were surface sterilized in 3% calcium hypochlorite for 20 min and rinsed four times in sterile distilled water, and the bud scales were removed aseptically. Explants consisted of apical meristems with approximately 3 mm of basal tissue, which were kept on modified MS medium (Murashige and Skoog 1962; see below) for 1 day before bombardment. Callus cultures were established from cryopreserved bud material from April 1992. Suspension cultures were initiated from 6-week-old calli derived from 25-year-old pines.

Callus growth was supported by a modified MS medium (macro- and micro-nutrients, and vitamins, according to Hohtola 1988) supplemented with 1.3 μM BAP, 4.5 μM 2,4-D, 0.55 mM *myo*-inositol, and 87 mM sucrose. Media were solidified with 0.06% agar and 0.2% Gelrite.

Suspension cultures were maintained in the same modified MS medium with three different hormone combinations: (*i*) cytokinin-free medium with 4.5 μM 2,4-D; (*ii*) low-level cytokinin medium with 0.2 μM BAP and 4.5 μM 2,4-D; and (*iii*) moderate-level cytokinin medium with 1.3 μM BAP and 4.5 μM 2,4-D. The suspension medium was replaced once a week. Suspension cultures were kept on an orbital shaker at 23°C in a 16 h light : 8 h dark cycle.

Viability and density of suspension cultures

The viability of each suspension culture used for bombardment was determined by fluorescein diacetate staining (Kartha et al. 1988). Cell density was determined with light microscopy using a 0.0025-mm^2 blood cell chamber.

Particle bombardment

The plasmid DNA pBI221.1 (Jefferson 1987) includes the CaMV 35S promoter – β-glucuronidase (GUS) gene. Plasmid DNA was prepared by the alkali lysis method followed by phenol–chloroform extraction (Sambrook et al. 1989).

Plasmid DNA (5 μg) was precipitated on gold particles (1 or 1.6 μm) by mixing 50 μL containing 1.5 mg of particles in sterile water, 5 μL DNA, 50 μL 2.5 M $CaCl_2$, and 20 μL 0.1 M spermidine. This microcarrier suspension was vortexed for 3 min, and microcentrifuged at 8000 × g for 10 s. The supernatant was removed and the gold microcarriers were washed with 250 μL of absolute ethanol and resuspended in 60 μL of absolute ethanol. Ten microlitres of microcarrier suspension was dried on a macrocarrier immediately before bombardment. Bud material was bombarded with both 1- and 1.6-μm gold particles; calli and suspensions, with 1.6-μm particles.

DuPont's Biolistic® PDS-1000/He device was used for bombardment. The DNA on macrocarriers was delivered using 650-psi (1 psi = 6.895 kPa) helium pressure for suspensions, 1100 or 1550 psi for buds, and 1550 psi for calli under 28-in. Hg (1 in. Hg = 3.386 kPa) vacuum. The different bombardment pressures used for different types of material were based on preliminary tests (data not shown). The target material was located 9.5 cm from the DNA delivery source.

For each bombardment, a 0.75-mL suspension was collected on a GN-4 filter (Gelman Sciences). The filter was kept on top of 10 pieces of filter paper during the bombardment. After shooting, the filter with suspension cells was transferred to modified MS medium to prevent dehydration of the cells, until all the filters were bombarded.

β-glucuronidase assay

The bombarded bud and callus specimens were incubated on modified MS media at 23°C in a 16 h light : 8 h dark cycle for 24 h. The bombarded suspension cells from each filter were resuspended in 1 mL of suspension medium and cultured in multiwell tissue culture plates on an orbital shaker for 48 h.

The histochemical GUS staining was performed according to Jefferson (1987), with minor modifications. The GUS-staining solution for the samples included 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.1 M sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0,1% Triton X-100. Before scoring, the buds and calli were kept in staining solution for 24 h at 37°C. The suspensions were kept on an orbital shaker for 24 h at room temperature in the dark.

Experimental designs

The effect of the tree age (5, 15, and 50 years old) and the collection time (August and December) on transient GUS expression in bud apices was studied. In each age-class, buds were collected from three trees. In August and December 40 and 60 buds per tree, respectively, were used in the experiment, 10 buds per bombardment. Half of the bombardments were performed with 1100-psi pressure, and the rest with 1550-psi pressure. With both pressures, 1.0-μm gold particles were used.

The effect of auxin and cytokinin pretreatments on the level of transient expression was tested. Twigs from three 15-year-old trees collected in late September were kept in growth-regulator solution with 28.5 μM IAA, 22.2 μM BAP, or 9.1 μM TDZ, or in tap water for 1 week before bud excision. Fifty buds per tree were used in the experiment, five buds per bombardment. The experiment was repeated twice. In another experiment, the bud explants were collected from eight 5-year-old trees in December. Twenty buds per tree, five buds per bombardment, were bombarded both 1 day after preparation and after 1-week pretreatment on modified MS medium (as above). All the bombardments in the growth-regulator experiments were performed with 1550-psi pressure and 1.6-μm gold particles.

The effect of the size of gold particles (1.0 or 1.6 μm) was tested by combining the data from the experimental designs described above. The samples from 5-year-old trees bombarded 1 day after preparation both in August and in December were included, together with bud samples from three additional 5-year-old trees collected in August and bombarded with 1.6-μm gold particles.

Cryopreserved buds (n = 90) from 25-year-old pines were bombarded with 1550-psi pressure and 1.6-μm gold particles, five buds per bombardment. Calli (n = 75), each derived from a cryopreserved bud, were also used for bombardment experiments, five calli per bombardment. The callus experiment was repeated three times, and every callus was divided into three pieces for three replicates.

Samples of suspension cultures in three different solutions with varying cytokinin content (see above) were bombarded after 2, 4, and 6 days of subculture. On every sampling day, 10 samples from each suspension culture were bombarded, and the experiment was repeated twice.

Statistical analysis

Statistical comparisons among treatments were made using analysis of variance. Where significant differences were found, means were compared either by Tukey's test or by Student–Newman–Keuls multiple range test or pairwise t-test with Bonferroni's significance levels. Nonparametric Kruskal–Wallis tests were used when the data were not normally distributed (BMDP Statistical Software Inc. 1988).

Results

Bud explants

Transient GUS expression was found in 35 of 44 (79%) tree genotypes used in the study. The typical number of transient expression events was one or two spots per bud apex. The maximum value in all of the experiments was eight spots per bud apex. In most cases, expression was found in short shoot primordia and cataphylls (Fig. 1A) and in meristems of axillary primordia. Spots were observed in meristems of apical buds on only a few occasions. Depending on the treatment (tree age, collection time, bombardment pressure, etc.), the percentage of GUS-expressing buds

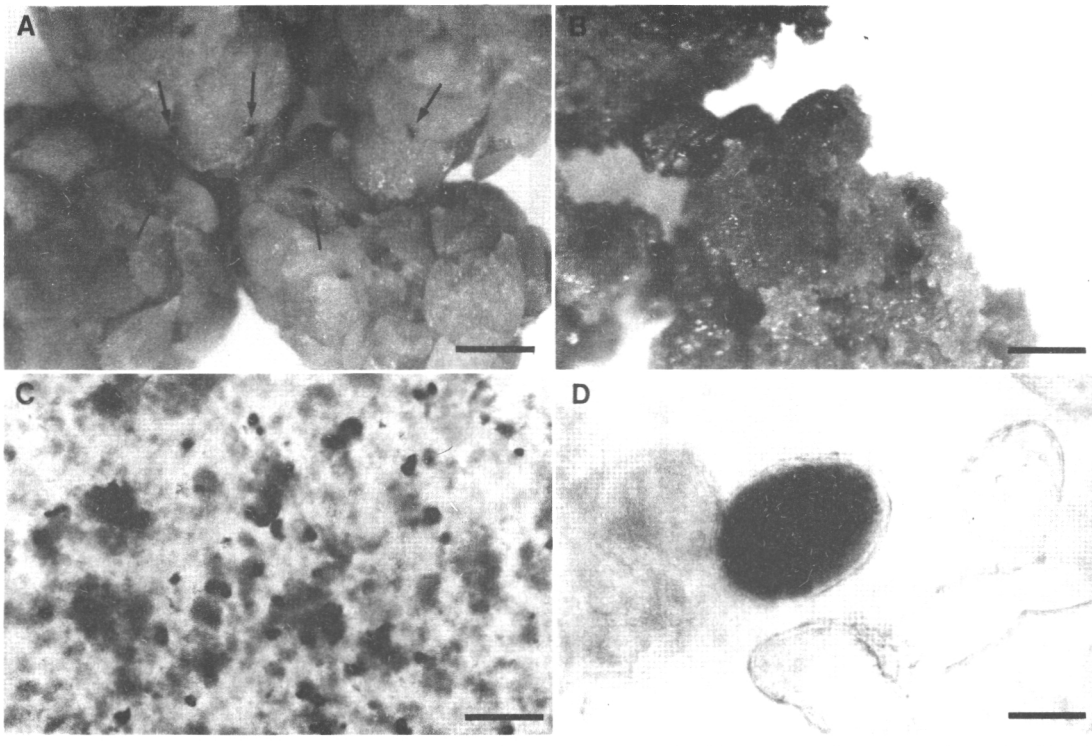

FIG. 1. Transient GUS expression after histochemical staining in material derived from mature Scots pines. (A) Buds. (B) Calli. (C) Suspension culture. (D) Suspension cell. Scale bars in (A), (B), and (C) represent 1 mm and scale bar in (D) 100 μm.

TABLE 1. Percentage of GUS-expressing buds collected from 5-, 15-, and 50-year-old Scots pines in August and December

Month	Pressure (psi helium)	Age of the sample trees			Mean
		5 years	15 years	50 years	
August	1100	5.0 (6)	1.7 (6)	0.0 (6)	2.2 (18)
	1550	11.8 (6)	1.7 (6)	0.0 (6)	4.5 (18)
	Mean	8.4[a] (12)	1.7 (12)	0.0 (12)	3.4 (36)
	SE	3.2	1.1	0.0	1.3
December	1100	10.0 (9)	20.0 (9)	5.6 (9)	11.8 (27)
	1550	5.0 (9)	16.7 (9)	7.8 (9)	10.0 (27)
	Mean	7.8 (18)	18.3 (18)	6.7 (18)	10.9[b] (54)
	SE	2.2	5.8	1.8	2.2

NOTE: Numbers in parentheses are the numbers of bombardments.
[a]Significantly greater than the mean of 15- and 50-year-old trees of August collections ($p < 0.05$).
[b]Significantly greater than the mean of August collections ($p = 0.0044$**; Bonferroni probability).

ranged from 0 to 26%. The best results (26%) were achieved by bombarding (1550-psi pressure, 1.6-μm gold particles) pretreated material from 5-year-old trees collected in December.

Bud apices from late August and early December from 5-, 15-, and 50-year-old trees were used as targets (Table 1). In December, the number of bud apices expressing the GUS gene was significantly higher than in August. In the August samples, juvenile material from 5-year-old trees was slightly more reactive to foreign DNA than buds from older trees, but in the December samples there were no significant differences between the age groups.

There were no significant differences between the two tested pressures (1100 and 1500 psi; Table 1). The average percentage of GUS expression in buds bombarded with 1.6-μm particles (12.2 ± 3.2%; mean ± SE) was not significantly different from the expression in buds bombarded with 1-μm particles (8.0 ± 1.8%).

The growth-regulator pretreatments of twigs had no significant effect on the level of transient GUS expression in buds. The average proportion of expressing buds was 3% ($n = 1200$), and the average proportion of bombardments with expressing buds was 13% ($n = 240$). The treatment of

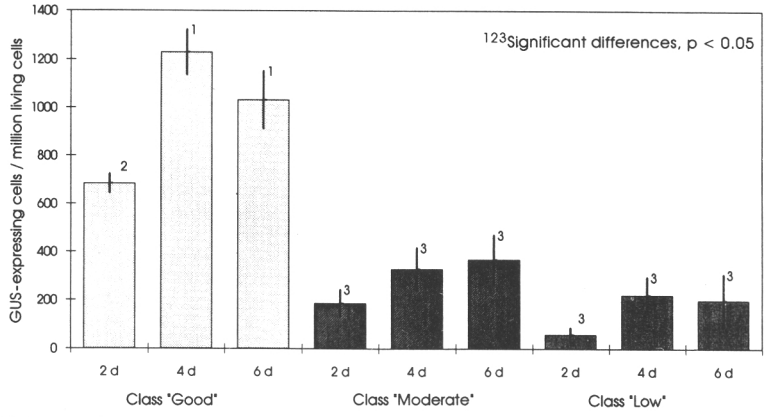

FIG. 2. Transient GUS expression in suspension cultures of different condition classes bombarded after 2, 4, and 6 days of subculture; mean values (±SE) are from 15–25 shots (n = 180).

TABLE 2. Distribution of bombarded samples from suspensions grown in different culture solutions within condition classes according to cell viability and cell density

Condition class (viable cells/mL, $\times 10^6$)	Class mean		No. of bombarded samples in different culture solutions		
	Viability (%)	Total cell density/mL ($\times 10^6$)	Cytokinin-free	Low cytokinin	Moderate cytokinin
Good (>0.4)	71	1.10	0	30	15
Moderate (0.1–0.4)	48	0.52	0	30	45
Low (<0.1)	6	0.21	60	0	0

excised scale-free bud apices on tissue culture media (1.3 μM BAP and 4.5 μM 2,4-D) for 1 week before bombardment enhanced the number of GUS-expressing buds significantly (p = 0.0135). The average percentage of GUS-expressing buds increased from 11 ± 3.5%, when the bombardment was done 1 day after bud preparation, to 26 ± 6.1% when the bombardment was done 1 week after preparation.

Cryopreserved bud explants

In the cryopreserved buds (n = 90) collected in April 1990 and 1992 from 25-year-old pines, the average percentage of expressing buds was 5.3% and the expression level was one or two spots.

Bud-derived calli

Transient GUS expression could be found in 64% of the bombarded samples (Fig.1B) and the level of expression was two spots per sample on average, with each sample containing five callus pieces. The callus pieces disintegrated during bombardment and histochemical testing; thus, the number of spots could not be counted per individual callus piece.

Suspension cultures

For statistical analysis, the suspensions used for bombardment were sorted into three condition classes according to their viability and cell density (Table 2), which reflect the overall health of the culture. Transient expression of GUS was readily achieved in the bombarded suspension cultures (Fig. 1C). In the best sample, 2255 cells per 1×10^6 living cells (0.23%) expressed GUS.

The condition of the suspension had a significant effect on the level of transient expression (Fig. 2). The suspensions in good condition (class I) resulted in 3–10 times more expression than the other classes. There was also a significant difference between culture solutions: low (0.2 μM BAP) or moderate (1.3 μM BAP) cytokinin content enhanced transient expression in suspension cells (Fig. 3). The timing of bombardment had also a significant effect (p = 0.0167) on the level of transient expression. When bombardment was performed after 4 or 6 days of subculture, the expression level was higher than after 2 days of subculture (p = 0.05).

Discussion

Gene transfer techniques provide good tools with which to study genetic regulation in plants (Smart et al. 1991; Smigocki 1991). In conifers, these techniques have not been applied to adult material. This is partly because young material is easier to propagate in vitro than older material (Häggman and Stomp 1990). The characteristics of juvenile and mature trees are in most cases poorly correlated (Lambeth 1980). This means that the mature phenotype is of great importance in tree breeding. Studies of genetic regulation of important mature traits, such as flowering, competence for rejuvenation, and ageing, have to be done with mature tree tissues.

The CaMV 35S promoter was chosen for these experiments because no tissue-specific promoters for this type of tissue were available and because it has previously been shown to be expressed in coniferous tissues (Ellis et al. 1991). Gold particles were used instead of tungsten particles to avoid tungsten trauma, which reduces the efficiency of biolistic transformation (Russell et al. 1992). According

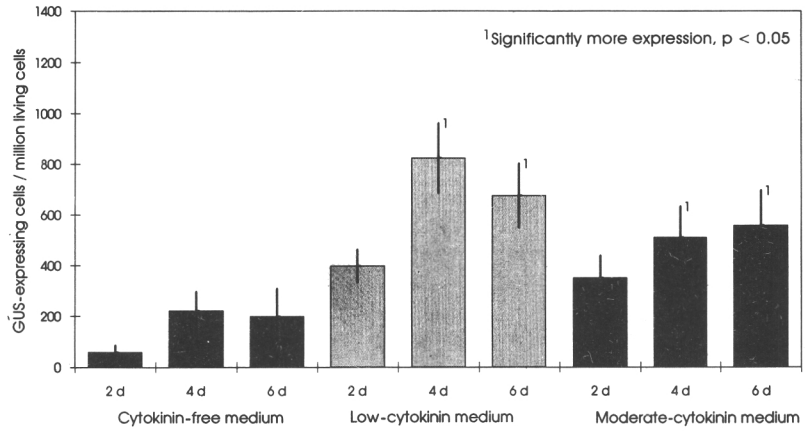

FIG. 3. Transient GUS expression in suspensions cultured in different solutions and bombarded after 2, 4, and 6 days of subculture; mean values (±SE) are from 20 shots (n = 180).

to Yamashita et al. (1991), more than 90% of GUS-expressing cells after particle bombardment receive the foreign gene in their nucleus. In the present study, the combination of the 35S CaMV promoter coupled with gold microcarriers insured expression in the recalcitrant conifer material.

The apices of vegetative buds from mature trees were chosen for starting material because they offer the potential for genetic regulation studies, and they provide meristematic regions, which are good targets for transformation (Potrykus 1991). In the case of mature trees, bud tissues are also potential explant material for in vitro propagation and regeneration purposes (Monteuuis 1991).

With bud material, we observed transient expression in 79% of all genotypes studied. The expression level was, however, on the average between one and two spots per bud apex, and the average percentage of buds expressing GUS was under 20%. In most cases, the expression was observed in short-shoot primordia or in cataphylls, regardless of pretreatments. The low level of GUS expression could be related to the season. Most of the buds were from early autumn or winter, and collections during this time have previously been shown to be unsuitable for micropropagation (Bonga 1981; Hohtola 1988). In the present study, there were significant differences between early autumn and winter material with the number of expressing buds being higher in winter material than in autumn material.

The expression was also relatively low in the cryopreserved spring buds. Eighty to 90% of the buds produced calli from the same cryopreserved lots, indicating that many of the bud cells were alive and capable of expressing foreign DNA. The competence of spring buds for transformation is being studied in ongoing experiments.

Growth-regulator treatments were performed to enhance mitotic activity in buds. Increased mitotic activity has been shown to enhance transient GUS expression in tobacco (Iida et al. 1991). GUS expression was increased in Scots pine when the bud apices were kept for 1 week on the tissue culture media with growth regulators prior to bombardment. The difference observed was attributed to the fact that more buds showed GUS expression after the treatment, rather than more expression per individual bud. This increase following the tissue-culture treatment could be due to the recovery of bud tissues from preparation stress. The 1-week hormone treatment of twigs with buds gave no significant improvement, which may have been due to lack of uptake of the growth regulators by the twigs.

Calli derived from cryopreserved spring buds were used in the bombardment experiments because callus proliferation has been shown to be highest from explants collected during the spring (Hohtola 1988). Spring callus material seemed to be slightly better than bud material from either autumn or winter.

The clone of suspension cultures that had the best viability and growth was used in transformation experiments. Transient expression was readily achieved, and the material appears to be suitable for further study of gene expression in Scots pine. Through the use of this method, one can achieve high transient expression levels, 2255 events per 1×10^6 living cells, compared with some crop plants, 1000 per 1×10^6 cells (reviewed by Sanford 1990), or to Monterey pine (*Pinus radiata* D. Don), one per 1×10^6 cells (Campbell et al. 1992).

The condition of the suspension cultures was related to the amount of GUS expression observed. Growth regulators have also been shown to be important for transient expression (Goldfarb et al. 1991; Newton et al. 1992), and similar results were observed in the present study. Cytokinin in the media contributed to the condition of the suspension culture, and bombardment 4–6 days after subculture was better than that of 2 days. The interval between subculturing to fresh media and bombardment have been important also in other studies (Vasil et al. 1991), and it is correlated with the maximum growth period of suspensions (Lulsdorf et al. 1992).

The present results of transient expression indicate that it is possible to transfer foreign DNA into organs and tissues derived from mature Scots pines. From a forest-genetics point of view, these results give the opportunity to study genetic regulation of traits expressed in mature Scots pines. The benefit for tree-breeding programs could be transformation of mature trees, which have readily exemplified attributes to enhance improvement. This, of course, depends on the regenerability of the transformed tissues. Bud tissues are appropriate owing to the high percentage of

GUS-expressing tree genotypes, but suspensions exhibit the highest amount of transient expression. The amount of stable expression as well as the effect of different promoters remain to be determined in future experiments.

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Seasonal changes in the transient expression of a 35S CaMV-GUS gene construct introduced into Scots pine buds

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Summary Seasonal changes in the transient expression of ß-glucuronidase gene (GUS) driven by a constitutive 35S CaMV-promoter in Scots pine (*Pinus sylvestris* L.) buds were studied by the microprojectile DNA-delivery method. Buds were collected from 5-, 15- and 50-year-old trees. In buds from all age groups the amount of transient expression was dependent on the season; the highest values were found in March, and values were lowest both at the beginning and at the end of the growing season. Pretreatment with growth regulators increased both the amount of transient GUS expression and arginine decarboxylase (ADC) activity in buds indicating an increase in metabolic activity. These results confirm that the genetic transformation technique can be used to study seasonally dependent regulation in mature Scots pine tissues.

Keywords: Pinus sylvestris, gene regulation, genetic transformation, microprojectile bombardment.

Introduction

Scots pine (*Pinus sylvestris* L.) is a major source of timber in Nordic countries. Its long rotation time and ability to tolerate severe climatic conditions suggests that the species has high genetic adaptability. During the last few years new environmental factors, including air pollutants, greenhouse gases and their interactions, have imposed additional pressure on the genetic organization and physiology of the species.

Newly developed genetic transformation technologies have facilitated studies of genetic regulation in several plant species (Ryan et al. 1990, Smart et al. 1991, Smigocki 1991), including tree species (Loopstra et al. 1992, Newton et al. 1992). However, transformation studies on trees have only been made on young tissues, such as whole embryos (Robertson et al. 1992, Ellis et al. 1993), cotyledons (Goldfarb et al. 1991, Stomp et al. 1991), calli or suspension cells (Duchesne and Charest 1991, Newton et al. 1992). Because trees have long life spans, a knowledge of the genetic regulation of mature tissues is of major importance. At present, nothing is known about the stability or expression of the transferred genes in adult tissue.

Direct gene transfer is the most effective method of studying genetic regulation in conifers. Recently, gene transfer experiments by microprojectile bombardment have been performed with several coniferous species (Duchesne and Charest 1991, Ellis et al. 1991, Goldfarb et al. 1991, Stomp et al. 1991, Loopstra et al. 1992, Newton et al. 1992, Robertson et al. 1992, Ellis et al. 1993). Some preliminary results have also been presented for Scots pine (Aronen and Häggman 1992, Häggman and Aronen 1992).

Scots pine is an excellent species to study genetic regulation in mature tree tissues because the physiology of adult trees has been studied extensively. Long-term studies on the metabolism of Scots pine and seasonal changes at the cellular level are well documented (Kupila-Ahvenniemi et al. 1978, Kupila-Ahvenniemi and Hohtola 1979, 1980, Hohtola et al. 1984, Häggman et al. 1985). Because gene expression of pine buds varies with season (Häggman 1986, Nuotio et al. 1990), we studied foreign gene expression during different seasons. We determined whether foreign genes under a constitutive promoter are expressed in a similar way to native genes, and we also assessed the effects of growth regulators on the amount of transient expression. Metabolic activity was measured by assaying arginine decarboxylase activity.

Material and methods

Plant material and culture conditions

Vegetative parts of apical and axillary buds of Scots pine were used as target material in the transformation experiments. Twigs were collected from 5-, 15- and 50-year-old trees growing in Punkaharju (61°48′ N, 29°17′ E). Buds were taken from five to 15 twigs per tree of three to six trees in August 1992, December 1992, March 1993 and May 1993. Because of the small size and small number of buds present on the 5- and 15-year-old trees, only one collection could be made from each tree. In the case of 50-year-old trees, collections were made from the same trees each time.

The buds were surface sterilized in 3% calcium hypochlorite for 20 min and rinsed four times in sterile distilled water. After sterilization, the bud scales were removed aseptically. Explants consisted of apical meristems plus the surrounding tissue with needle primordia and cataphylls.

Explants were placed on modified MS medium (Murashige and Skoog 1962) containing macro- and micronutrients and

vitamins according to Hohtola (1988), supplemented with 1.3 µM benzylaminopurine (BAP), 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.55 mM myoinositol and 87 mM sucrose. Media were solidified with 0.7% agar.

The effects of auxin and cytokinin pretreatments on the amount of transient expression were tested in March and May. Before bombardment, bud explants from 15-year-old trees were pretreated for 1 week on modified MS medium (Hohtola 1988) supplemented with 4.5 µM 2,4-D and 0.13 (low), 1.33 (moderate) or 4.44 µM (high) BAP. In total, 900 buds were treated.

Particle bombardment

The plasmid DNA was pBI 221 including CaMV 35S promoter-ß-glucuronidase gene. Plasmid DNA was prepared by the alkali lysis method followed by phenol-chloroform extraction (Sambrook et al. 1989).

Plasmid DNA (5 µg) was precipitated onto gold particles 1.6 µm in diameter. This was done by mixing a 50 µl batch of 1.5 mg gold particles in sterile water, 5 µl DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. This microcarrier suspension was vortexed for 3 min and centrifuged at 8000 *g* for 10 s. The supernatant was removed, and the microcarriers were washed with 250 µl of absolute ethanol and resuspended in 60 µl of absolute ethanol. Ten µl of microcarrier suspension was dried on a macrocarrier immediately before bombardment.

For bombardment, the Biolistic® particle delivery system PDS-1000/He device (DuPont, Wilmington, DE) was used. The DNA on the macrocarrier was delivered under conditions of 1550 psi He pressure and 710 mm of Hg vacuum. The target material was located 9.5 cm from the DNA delivery source.

Target tissues, consisting of five bud apices, were placed on modified MS medium so that the apex faced the DNA source. Ten replicates were made per treatment. The data presented here are based on 2850 bombarded bud apices and 570 shots.

ß-Glucuronidase assay

The bombarded buds were placed on tissue culture media at 23 °C in a 16-h photoperiod. After 24 h, histochemical GUS-staining was performed according to Jefferson (1987) with minor modifications. The GUS-staining solution contained 0.1 M sodium phosphate buffer (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 0.1% Triton X-100. Samples were kept in test solution in the dark for 24 h at 37 °C, after which GUS expressing cells and cell clusters were counted under a stereomicroscope.

Arginine decarboxylase assay

In May, arginine decarboxylase activity (ADC) was measured in shoot tips, both immediately after bud collection and after 1 week on modified MS medium containing 4.5 µM 2,4-D and 4.44 µM BAP. Buds were excised from branches of three 15-year-old trees at the day of branch collection, except in the case of Tree 9. The branches of Tree 9 were kept overnight at 2 °C because it was impossible to prepare all of the samples on the same day. Samples consisting of 30 bud apices were ground in liquid nitrogen and homogenized with mortar and pestle in 2 ml of extraction buffer (0.05 M Tris-HCl, pH 8.5, 0.5 mM pyridoxal-5-phosphate, 0.1 mM Na_2-ethylenediamine-tetra-acetate and 5.0 mM dithiothreitol). The homogenate was centrifuged at 20,000 *g* for 20 min and the supernatant was used for enzyme assays. Enzyme activities were determined by measuring the amount of $^{14}CO_2$ released from L-(U-^{14}C)-arginine (specific activity 318 mCi $mmol^{-1}$) according to Robie and Minocha (1989). Radioactivity was determined with a Wallac 1410 liquid scintillation spectrometer.

Paraffin preparates

After bombardment and GUS-staining, the bud samples with GUS expressing cells were fixed in FAA (formalin/acetic acid/95% ethanol, 10/5/85 v/v/v), dehydrated in a series of ethanol and embedded in paraffin according to Gerlach (1984). Photographs were taken with an Olympus CK2 microscope.

Statistical analysis

Statistical comparisons among treatments were made by analysis of variance, and means were compared by Tukeys test or by Student-Newman-Keuls multiple range test. Nonparametric Kruskal-Wallis test followed by multiple mean comparisons was used when the data were not normally distributed. For correlation analysis, nonparametric Spearman rank correlation was used (BMDP 1988).

Results

Transient GUS expression was found in Scots pine buds collected from 5-, 15- and 50-year-old trees throughout the different seasons. The average amount of transient expression was one to two GUS expressing cells per expressing bud. The maximum value was 18 GUS expressing cells or cell clusters per bud. The percentage of expressing buds varied between 0 and 92%. In most cases the expression was found in the cataphylls of axillary primordia (Figure 1).

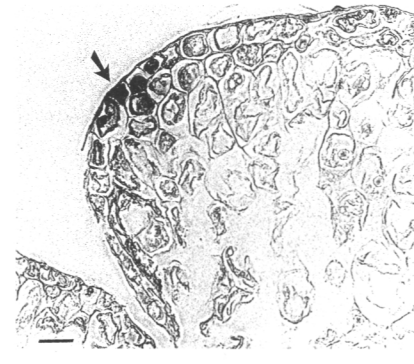

Figure 1. Transient GUS expression (arrow) in cataphylls of a Scots pine shoot apex. Bar = 0.1 mm.

Significant variation was found in the amount of transient GUS expression in bud samples collected in different seasons ($P < 0.0001$ for pooled data including all age groups). The highest amount of expression was observed in March, and the lowest amounts occurred in May and August (Figure 2C). There was no significant difference in transient GUS expression between bud samples collected in March and December, but the bud samples collected in March and December exhibited significantly more expression than the bud samples collected in May ($P = 0.05$). The bud samples collected in December had a higher amount of transient GUS expression than the bud samples collected in August ($P = 0.05$).

In the 50-year-old Scots pines, individual variation between trees did not mask the comparison between seasons because the same trees were sampled at each collection (Figure 2A). All of the 50-year-old trees showed the same seasonal trend in the amount of transient GUS expression, and the highest amount of expression was observed in March. The average values of transient GUS expression in the 5-year-old trees were also highest in March (Figure 2B). Although 18% of the buds from the 15-year-old trees expressed GUS in December compared with 12% in March (Figure 2B), the difference between December and March was not significant because variation among the individual 15-year-old trees was high. In December, individual 15-year-old trees had 3, 45 and 7% of buds expressing GUS, whereas in March the values were 22, 2 and 12%.

In March, bud tissue from the 5-year-old trees was more competent for foreign DNA than bud tissue from the 15- and 50-year-old trees ($P < 0.001$). The average number of GUS expressing cells per bud apex was highest in material collected from 5-year-old trees, 0.35 ± 0.76 SD (23% of buds expressing GUS, $n = 150$). In 15-year-old trees, the corresponding value was 0.15 ± 0.43 (12%, $n = 150$) and in 50-year-old trees the value was 0.14 ± 0.40 (12%, $n = 150$).

Pretreatment with 0.13, 1.3 or 4.44 µM BAP in the presence of 4.5 µM 2,4-D for 1 week before bombardment significantly enhanced the number of GUS expressing cells per bud ($P < 0.0001$), and the increase was greater in the 1.3 and 4.44 µM BAP treatments than in the 0.13 µM BAP treatment ($P = 0.05$, Figure 3A). Pretreatment with growth regulators also enhanced the percentage of expressing buds ($P < 0.0001$). In March, the frequency of expressing buds increased from 12% when the bombardment was done 1 day after bud pretreatment to 21% when the bombardment was done 1 week after pretreatment. In May, the increase was larger, from 1.3 to 58%. All of the growth regulator pretreatments enhanced the frequency of expressing buds when compared with the control treatment ($P = 0.05$), but they did not differ from each other (Figure 3B).

When the bud material was treated for 1 week on modified MS medium containing 4.5 µM 2,4-D + 4.44 µM BAP, the average amount of ADC activity increased about threefold ($P = 0.01$), and the average number of GUS expressing cells or cell clusters per bud increased 50-fold ($P = 0.01$, Table 1). In Tree 9, the amount of ADC activity did not increase in response to the growth regulator pretreatment, perhaps because activity of this enzyme was high before the pretreatment. The branches of this tree were kept overnight in a cold room before sample preparation. There was a significant ($P < 0.0001$) positive correlation (Spearman's $r = 0.6091$) between ADC activity and the number of GUS expressing cells per bud.

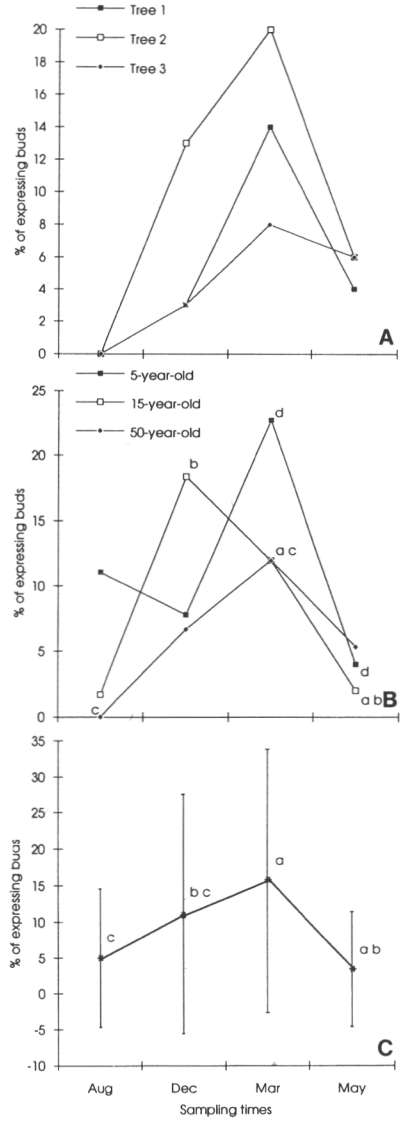

Figure 2. (A) Seasonal changes in transient GUS expression in the buds of three 50-year-old Scots pine trees. Data based on 600 buds. (B) Seasonal changes in transient GUS expression in the buds of 5-, 15- and 50-year-old Scots pine trees. Data points marked with the same letter differ significantly from each other at $P = 0.05$. Data based on 1950 buds. (C) Pooled data of seasonal changes in transient GUS expression of Scots pine buds. Data points marked with the same letter differ significantly from each other at $P = 0.05$. Data (± SD) based on 1950 buds.

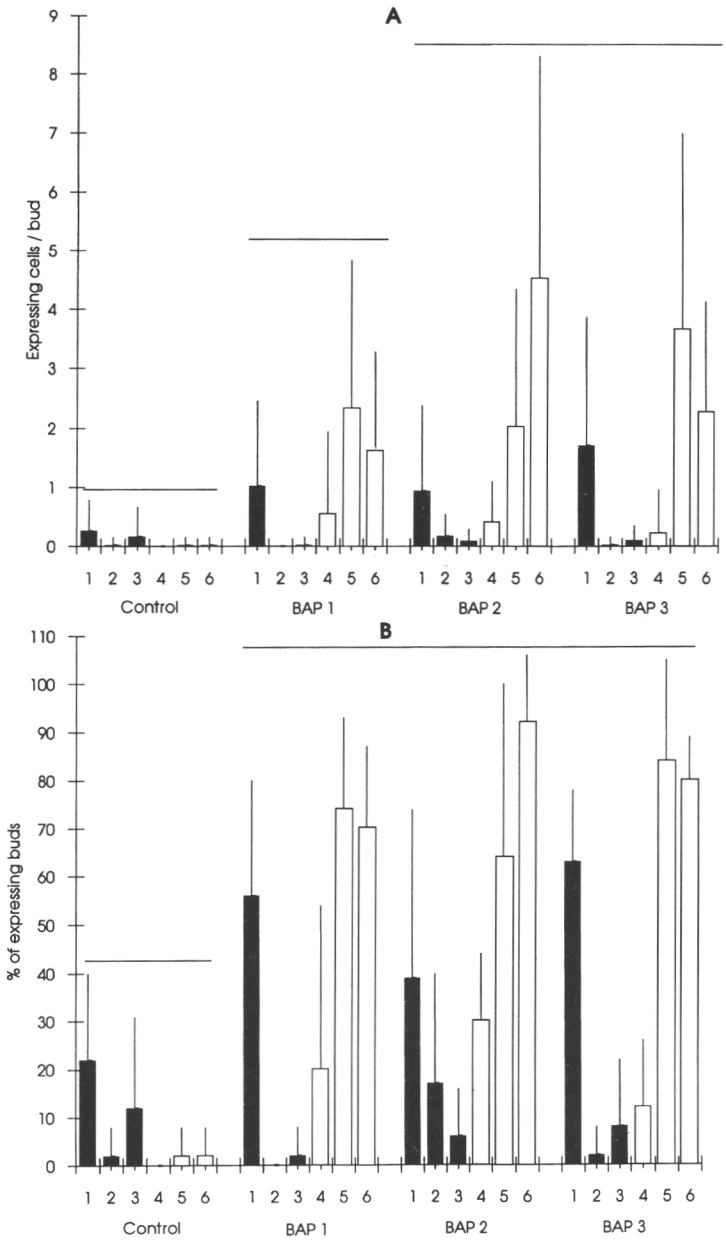

Figure 3. (A) Number of GUS expressing cells per bud in the 15-year-old Scots pine trees after 1 week of pretreatment with BAP and 4.5 μM 2,4-D in March (Trees 1, 2 and 3) and May (Trees 4, 5 and 6). Different cytokinin concentrations: BAP 1 = 0.13 μM, BAP 2 = 1.3 μM, and BAP 3 = 4.44 μM. Control buds were bombarded 1 day after preparation. Groups marked with different horizontal lines differ significantly from each other at $P = 0.05$. Data (± SD) based on 900 buds. (B) Percentage of GUS expressing buds in 15-year-old Scots pine trees after 1 week of pretreatment with growth regulators BAP and 4.5 μM 2,4-D in March (Trees 1, 2 and 3) and May (Trees 4, 5 and 6). Different cytokinin concentrations: BAP 1 = 0.13 μM, BAP 2 = 1.3 μM, and BAP 3 = 4.44 μM. Control buds were bombarded 1 day after preparation. Groups marked with different horizontal lines differ significantly from each other at $P = 0.05$. Data (± SD) based on 900 buds.

Discussion

Season had a marked effect on the amount of transient GUS expression in Scots pine buds. A marked seasonal variation in overall gene expression in Scots pine bud tissues has been observed in earlier studies (Nuotio et al. 1990). Similarly, Ziegler and Kandler (1980) and Hutchison and coworkers (1988) reported season dependent gene expression in other conifer species. The receptivity of bud tissues for gene insertion may change as a result of altered physical and biological status of the target tissue in different seasons. In the present study, however, the activity of foreign gene expression resembled that of native genes. This is the first time that foreign gene expression has been shown to be dependent on the season, which implies that the transferred foreign genes are under the control of the genetic system of the species despite the pres-

Table 1. The average amount (cpm g_{FW}^{-1}, ± SD) of ADC activity and the average number of GUS expressing cells per bud (± SD) in Scots pine buds collected in May. Measurements were made at the time of collection and after a 1-week pretreatment with 4.44 µM BAP + 4.5 µM 2,4-D.

Tree no.	Untreated buds		Pretreated buds		Increase (%)	
	ADC	GUS	ADC	GUS	ADC	GUS
7	439 ± 129	0.06 ± 0.31	1739 ± 747	2.96 ± 2.65	400	4900
8	129 ± 44	0.02 ± 0.14	2460 ± 796	1.70 ± 1.93	1900	8500
9	1349 ± 1054	0.04 ± 0.28	1468 ± 926	1.39 ± 1.61	110	3500
Mean	639 ± 775a[1]	0.04 ± 0.26b	1889 ± 787a	2.01 ± 2.20b	300	5000

1 The letters a and b indicate significant differences at $P = 0.01$.

ence of a constitutive promoter.

The highest degree of transient GUS expression was found in March, which according to Kupila-Ahvenniemi's model of pine bud metabolism (1985) represents the transition phase between reduced wintertime activity and renewed metabolic activity. The frequency of transient GUS expression was also relatively high in December. According to Kupila-Ahvenniemi's model (1985), this period corresponds with the transition phase between the periods of growth and differentiation and reduced wintertime activity. Typically in both December and March, values of transient expression varied between different aged trees and between individual trees. At the cellular level, several functions are activated during the transition phase in March, and the size of nucleoli (Kupila-Ahvenniemi and Hohtola 1980), the amount of ribosomes (Häggman 1987), the *in vitro* translation capacity of ribosomes (Kupila-Ahvenniemi et al. 1987) and the regenerative response of pine buds are all increased (Hohtola 1988). These changes, which coincide with the period of maximum transient GUS expression, indicate that the ribosomal machinery is ready for active gene expression.

Pretreatment with growth regulators increased the amount of transient GUS expression significantly. Enhanced expression resulting from growth regulator pretreatment may be due to stimulation of cell division. The effect of growth regulator pretreatment was more prominent in May than in March, whereas transient GUS expression in untreated material was higher in March than in May. This difference could be the result of a general inability of bud cells to react to cytokinin at the end of the winter period. In May, on the other hand, when the endogenous cytokinin supply is lower than in March (Häggman 1991) and the elongation of buds has already started, the cytokinin pretreatment had a significant effect. In Finland, the accumulation of endogenous zeatin in Scots pine bud tissues in March (Häggman 1991) precedes the commencement of growth which starts with first mitosis in April (Kupila-Ahvenniemi and Hohtola 1980). Similarly, Quamaruddin and coworkers (1990) reported that cytokinin activity in pine buds decreased after a maximum in late March or early April. Growth regulators are also important for transient GUS expression in other coniferous species (Goldfarb et al. 1991, Newton et al. 1992).

In many dividing plant tissues, the concentration of free polyamines is high and is correlated with the activity of the biosynthetic enzymes arginine decarboxylase and ornithine decarboxylase (see Evans and Malmberg 1989). In the present study, pretreatment with growth regulators caused significant increases in both GUS expression and ADC activity. This result is in agreement with previous studies in which cytokinin and 2,4-D caused an increase in ADC activity in several plant species (Palavan et al. 1984, Smith 1985, Koetje et al. 1993). Iida and coworkers (1991) have shown that an increase in mitotic frequency enhances the amount of transient GUS expression.

We found that variation in the amount of transient expression of the GUS gene driven by constitutive 35S CaMV-promoter in Scots pine buds paralleled the variation in the native gene expression, indicating that the inserted foreign gene is under the control of the genetic system of the species. This phenomenon was observed in buds of trees from all age groups studied. These results confirm that this genetic transformation technique can be used to study season-dependent regulation in mature Scots pine tissues.

Acknowledgments

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VI

Manuscript

Transformation of Norway spruce and Scots pine pollen by particle bombardment

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Abstract

The aim of the present work was to transform Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) pollen in order to study the possibilities of obtaining transgenic progenies. The effects of different pollen origins and regulative sequences on the β-glucuronidase (GUS) expression in pollen were significant. The highest GUS expression in pine (55 ±7.7 %) was achieved with polyubiquitin promoter and in spruce (44 ± 12%) by using an abscisic acid inducible promoter. Our results indicate that high levels of transient foreign gene expression can be achieved in pine and spruce pollen by using regulatory sequences proper for the target material and its physiological stage. Bombarded pollen was used for controlled pollinations in the greenhouse, after which 21 % of the pine cones and 56 % of the spruce cones developed normally.

Introduction

Genetic transformation studies on gymnosperms have been carried out extensively during the 1990's. Most of the work has been done by using particle bombardment, and transient expression of foreign genes has been reported in several species. Explant material used in these studies has usually been derived from embryos or young seedlings to ensure better regeneration potential (Clapham et al. 1995; Bommineni et al. 1994; Walter et al. 1994; Charest et al. 1993; Duchesne et al. 1993; Campbell et al. 1992; Robertson et al. 1992; Duchesne and Charest 1991; Ellis et al. 1991; Goldfarb et al. 1991; Stomp et al. 1991). Stably transformed plantlets have, however, been regenerated only in two cases: in European larch by *Agrobacterium*-mediated transformation (Huang et al. 1991), and in white spruce by particle bombardment (Ellis et al. 1993). Difficulties in tissue culture are one reason for unsuccessful regeneration of transformed cells or tissues in many conifers. Pollen, on the other hand, is a natural vector for gene transfer. Consequently, regeneration problems could be avoided by using transformed pollen in controlled pollinations.

Pollen transformation by using particle bombardment has mostly been investigated in herbaceous species, such as lily, peony, tobacco, and tomato (Miyoshi et al. 1995; Nishihara et al. 1993; Twell et al. 1991; Twell et al. 1989). Moreover, production of trans-genic plantlets through pollen transformation has been successful in tobacco (van der Leede-Plegt et al. 1995; Stoger et al. 1995). Recently, also gymnosperm pollen has been used as a target for genetic transformation. Transient expression of reporter genes has been achieved in Norway spruce, maritime pine (Martinussen et al. 1995; Martinussen et al. 1994), white spruce (Li et al. 1994), lodgepole pine, yellow cypress, western hemlock, jack pine, and black spruce (Hay et al. 1994). In these studies the main effort has been focused on optimizing the transformation method, and no attempts have been made to use the transformed pollen for fertilization and subsequent recovery of transgenic seeds.

Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst) are the most common conifers in the Nordic countries. The traditional breeding programs have been focused on these species, and during the last few years also biotechnological studies have been performed. In Norway spruce, somatic embryogenesis as a regeneration method works well (Becvar et al. 1989; von Arnold and Hakman 1988; Hakman et al. 1985), while in Scots pine the method is still under development (Hohtola 1995). Genetic transformation of Scots pine (Aronen and Häggman 1995; Aronen et al. 1995; Aronen et al. 1994) and Norway spruce (Clapham et al. 1995; Yibrah et al. 1994; Newton et al. 1992; Robertson et al. 1992) has been studied, but the regeneration of transformed plantlets has not yet been achieved. Thus, in both species a transformation method independent of in vitro regeneration techniques will be favoured.

The aim of the present work was to transform both Scots pine and Norway spruce pollen, and to use the transformed pollen in the controlled pollinations in order to study the possibilities of obtaining transgenic progenies. Also different regulative sequences, pollen sources (tree genotypes), and antibiotic selection were evaluated as regards transformation efficiency.

Materials and Methods

Pollen collections and storage

Pollen of Scots pine (*Pinus sylvestris* L.) was obtained from 10 plus trees grafted in a clone bank at Punkaharju (61°48'N; 29°17'E). Five of the trees, E2012, E2133, E2574, E2575, and E3131 originate from the southern part of Finland, and the other five, K44, K189, K658, K670, and K829 from the central part of Finland.

Pollen of Norway spruce (*Picea abies* (L.) Karst.) was obtained from 5 plus trees grafted in a clone bank at Punkaharju (61°48'N; 29°17'E). Four of the trees, E9, E236, E253, and E1205 originates from the southern part of Finland and the fifth tree U2301 originates from Själland, Denmark.

Pollen was collected by isolating developing microsporangiate strobili with paper bags before pollen shedding. Pollen shed in the isolation bags was harvested by using a vacuum cleaner. After drying the pollen was stored at -20 °C in sealed bottles.

In vitro conditions for pollen germination

For in vitro germination tests 100 mg of dry pollen, without surface sterilization, was suspended in 5 ml liquid medium. The in vitro germination medium (pH 5.6) consisted of 1.62 mM H_3BO_3 , 1.27 mM $Ca(NO_3)_2$ x 4 H_2O, 0.81 mM $MgSO_4$ x 7 H_2O, 2.20 mM KH_2PO_4, and 0.3 M sucrose. This is a modification of the medium first developed by Brewbaker and Kwack (1963), and later on refined by Muren et al. (1979). Pollen suspension cultures in 50 ml Erlenmeyer flasks were kept on an orbital shaker (Infors AG, 180 rpm) at +28°C in dark for 24 or 48 hours. The germination capability of pollen was also tested on the same media solidified with 1 % Agar. Samples of 30 µl from the original pollen suspensions (100 mg dry pollen suspended in 5 ml medium) were spread over 1.5 ml solidified media in 24-well plate and incubated at +28°C in dark for 24 hours.

Preparation of pollen for microprojectile bombardment

For microprojectile bombardment 100 mg of pollen was suspended in 5 ml of in vitro germination medium. One ml of well mixed suspension was pipetted on a GN-4 filter or on its support pad (Gelman Sciences) 2.5 cm in diameter. During pipetting the filter was kept on top of ten pieces of filter paper and for microprojectile bombardment the pollen filter was transferred on the solidified in vitro germination medium. Pollen samples were bombarded immediately after the sample preparation.

Pollen lots collected from all the ten Scots pine and five Norway spruce genotypes were tested for their competence for transformation. In the other experiments of the present study the pollen lots from the Scots pine E2575 and the Norway spruce U2301 were used as targets.

Gene constructs used in transformation experiments

The gene constructs used in the experiments are listed in Table 1. All plasmid DNAs were prepared by the alkali lysis method followed by phenol-chloroform extraction (Sambrook et al. 1989). Plasmid DNA of 5 µg was precipitated onto gold particles 1 µm in size according to Aronen and co-workers (1994). The construct used for optimizing the bombardment parameters, for testing the differences among pollen sources, and for producing transformed pollen for the controlled pollinations was pBI221.

Microprojectile bombardment

DuPont's Biolistic^R PDS-1000/He device was used for bombardment. When the bombardment parameters were optimized the following helium pressures were tested: 400 psi, 650 psi, 900 psi, and 1300 psi. The pollen samples were bombarded once or twice. In further experiments 900 psi pressure was used for Scots pine pollen, 1300 psi pressure for Norway spruce pollen, and the pollen samples were bombarded twice. The target material was located 9.5 cm from the DNA delivery source and the Hg

Table 1. Gene constructs used in pollen transformation experiments.

Plasmid	Regulative sequences and reporter genes	Reference
pBI221.1	35S-GUS	Jefferson 1987
pRT99	35S-GUS and 35S-NPT	Töpfer et al.1988
pBI410	35S-AMV-GUS:NPT	Datla et al. 1991 Charest et al. 1993
pBI426	35S-35S-AMV-GUS:NPT	Datla et al. 1991 Charest et al.1993 Bommineni et al.1994
pBM113	EmP-GUS	Marcotte et al. 1988 Quatrano et al. 1991
pCGUΔ0	UbB1-GUS and 35S-NPT	Binet et al. 1991a Binet et al. 1991b

Abbreviations: 35S = the 35S promoter of the cauliflower mosaic virus; GUS = β-glucuronidase; NPT = neomycin phosphotransferase; AMV = the enhancer element of the alfa-alfa mosaic virus; EmP = the abscisic acid inducible promoter of the wheat EM gene; UbB1 = the promoter of the sunflower polyubiquitin gene UbB1

vacuum used was 28 inch. In all the experiments ten pollen samples were bombarded per treatment.

The pollen samples were resuspended immediately after bombardment in 1 ml of in vitro germination medium and incubated in 24-well tissue culture plates +28°C in dark for 24 hours. In the experiments which were planned to follow the expression of β-glucuronidase during pollen germination, the pollen samples were incubated for three days.

Histochemical β-glucuronidase assay

After 24 h incubation, 100 µl of each pollen suspension and equal amount of 2x test solution including 2 x substrate was pipetted in the 96-well plate for the histochemical GUS-test. The test was performed according to Jefferson (1987), and the test solution used was a modification according to Aronen and co-workers (1994). Before scoring, the pollen samples were kept in the test solution at 37°C in dark for 24 h.

Diffusion of a soluble intermediate of the final blue-coloured product, dichloro-dibromoindigo (Stomp 1992) from the staining solution into germinating unbombarded pollen grains during the 24-hour histochemical GUS assay was also tested. Pollen samples from the Norway spruce U2301 and Scots pine E2575, germinated for 24 hours, were mixed with leaf discs of GUS-positive tobacco, and they were tested histochemically for GUS expression as described above.

Scoring of pollen density, pollen germination, and GUS-expression

Pollen grain density was determined with an Olympus CK2 microscope using a 0.0025 mm^2 blood cell chamber. Pollen densities as well as pollen germination percentages were determined by counting 400 -500 pollen grains per tree genotype in 8 different cell chamber areas. A pollen grain was regarded as germinated when the tube length exceeded the pollen diameter. In transformation experiments germination percentage and β-glucuronidase (GUS) expression were determined by counting at least 200 pollen grains per bombarded sample.

Kanamycin selection

The effect of antibioticum kanamycin at the concentrations 0-400 mg/l on the germination ability of Norway spruce and Scots pine pollen was tested by germinating pollen samples for 48 h in the liquid medium as described above. Furthermore, pollen samples bombarded with the constructs pRT99, pBI410 and pBI426 were germinated in the media with and without kanamycin (175 mg/l for Scots pine or 300 mg/l for Norway spruce) in order to study the possibility of enriching the proportion of transformed pollen grains in the samples.

Controlled pollinations

The two potted grafts of plus trees of Scots pine (E 97 and K 675) and two of Norway spruce (E4262 and E4350) were control pollinated in greenhouse. The microsporangiate strobili of the grafts were removed

before pollen shedding. The megasporangiate strobili of the grafts were not isolated because flowering of both species in the greenhouse took place several days earlier than in nature. A modification of the liquid pollination technique (Sweet et al. 1992 and 1993) was used. When the female strobili were emerging from their scales they were first sprayed with water including 0.5 % Triton X-100 to decrease the surface tension and immediately after that pollinated by spreading the strobilus with pollen suspension with 0.25 ‰ Triton X-100. The pollen used for pollinations was bombarded and resuspended as described, and the controlled pollinations were performed within two hours after the particle bombardment. In Norway spruce 16 and in Scots pine 237 strobili were pollinated. The controlled pollinations were repeated twice after two and four days from the first pollination.

Statistical analyses

Statistical comparisons among treatments were made by Analysis of Variance, and means were compared by Tukeys test or by Student-Newman-Keuls multiple range test.

Results

In vitro germination of pollen

When 100 mg of dry pollen was suspended in 5 ml of the germination medium, the pollen densities in Scots pine and Norway spruce were on an average 12.5 (±0.6 SE) x 10^5 and 4.5 (±0.3) x 10^5 pollen grains per ml, respectively. The germination ability varied among the pollen lots in both species. The average germination percentages are shown in Table 2. The liquid germination medium proved to be better than the solified one (Table 2).

Optimization of bombardment parameters

The bombardment pressures tested, 400 psi, 650 psi, 900 psi, and 1300 psi, did not significantly affect the amount of transient GUS expression in Norway spruce and Scots pine pollen. When the pollen samples were bombarded twice, better results were achieved than with single bombardments (Fig. 1).

Table 2. In vitro germination percentages (± SE) of Norway spruce and Scots pine pollen on the solified medium and in the liquid medium.

Species	Genotype	Medium	
		Solidified	Liquid
Norway spruce	E9	79.5 (±2.5)	76.7 (±2.2)
	E236	85.7 (±3.2)	81.4 (±2.2)
	E253	65.8 (±2.1)	71.8 (±2.4)
	E1205	74.5 (±3.2)	80.6 (±3.8)
	U2301	81.9 (±2.0)	82.8 (±1.1)
	Mean	77.5 (±1.5)	78.6 (±1.2)
Scots pine	K44	57.8 (±3.2)	51.1 (±3.6)
	K189	42.1 (±1.8)	53.1 (±2.4)
	K658	48.7 (±3.4)	64.0 (±3.0)
	K670	58.6 (±2.9)	52.6 (±2.7)
	K829	53.4 (±3.7)	54.8 (±3.5)
	E2012	53.4 (±3.8)	53.7 (±4.0)
	E2133	42.9 (±3.6)	47.5 (±2.8)
	E2574	45.0 (±2.7)	59.3 (±2.7)
	E2575	53.1 (±4.59	68.2 (±3.8)
	E3131	61.9 (±3.0)	59.0 (±2.1)
	Mean	52.9 (±1.2)	57.4 (±1.1)[a]

[a] Significantly higher germination percentage than on the solidified medium, $p<0.001$

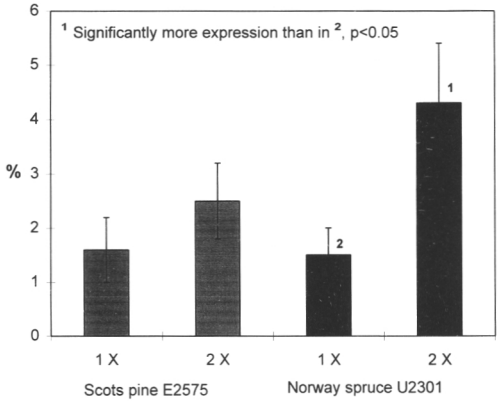

Fig. 1. The effect of bombardment times on the GUS expression in Scots pine and Norway spruce pollen bombarded with the pBI221. The results are presented as GUS-expressing pollen grains as a mean percentage (±SE) of the germinated ones.

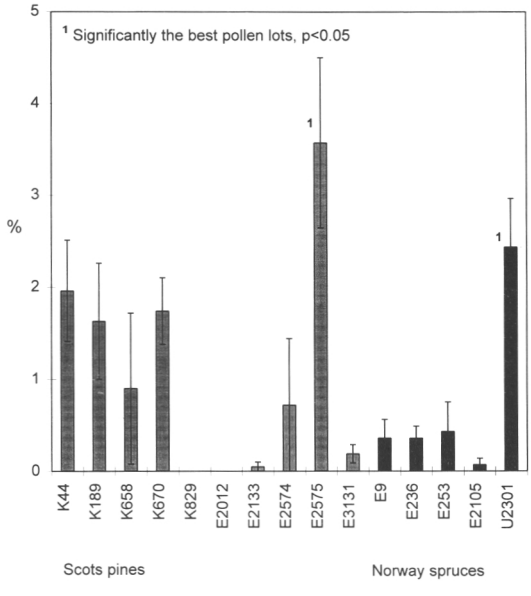

Fig. 2. Competence of different pollen sources (tree genotypes) of Scots pine and Norway spruce for transformation with the pBI221. The results are presented as GUS-expressing pollen grains as a mean percentage (±SE) of the germinated ones.

Competence of different pollen sources for transformation

Significant differences in the competence for transformation were found among the pollen lots collected from different plus tree genotypes both in Norway spruce and Scots pine (Fig. 2). The best pollen lots, the Norway spruce U2301 and the Scots pine E2575, were selected for further experiments.

Transient GUS expression during pollen germination

Most of the GUS expressing pollen grains germinated normally, but there were also some non-germinated, GUS-expressing ones. In Norway spruce, the amount of these pollen grains in different experiments varied from 0.3 to 1.4 % out of the total population, and in Scots pine from 0.2 to 2.5 %, respectively. When the proportion of the GUS-expressing pollen grains was very high in Scots pine, as in the case of some gene constructs, a significant reduction ($p<0.01$) in the germination percentage could be observed. In pollen bombarded with pBM113 or pCGUΔ0 only 54 (±2.4) % of the pollen grains germinated within 24 h, while the germination percentage in the same pollen bombarded with other gene constructs was 77 (±1.1). In Norway spruce, the same phenomenon was not observed. When the germinated GUS-expressing pollen grains in both species were examined, it was observed that various parts of the pollen grain were stained blue. The majority of the expressing pollen grains had a blue pollen tube, and approximately one third of the expressing pollen grains were totally blue.

The GUS-expressing pollen grains as a percentage of germinated grains was followed during the first three days of the in vitro germination. In Scots pine, the germination percentage increased but the percentage of GUS-expressing pollen grains remained relatively stable. In contrast, the amount of GUS expression decreased rapidly in Norway spruce (Fig. 3).

Effect of different regulatory sequences on GUS expression

The percentage of the GUS expressing pollen grains in both species varied significantly according to the gene constructs used. In Scots pine, the highest GUS expression, 55 (±7.7) % of the germinated pollen grains, was achieved with the pCGUΔ0 (Fig. 4A, 5B). In Norway spruce, the best gene construct was pBM113 generating GUS expression in 44 (±12) % of the germinated pollen grains (Fig. 4B, 5C).

In the pollen samples bombarded with pCGUΔ0 or pBM113, the test solution in the histochemical GUS assay was dark blue in most cases (Fig. 5A). The same was true when unbombarded pollen grains were tested together with the leaf discs of GUS positive tobacco. Diffusion of the soluble intermediate of the dichloro-dibromoindigo from the test solution into germinating unbombarded pollen grains during the 24-hour histochemical GUS assay caused background staining of 11 (± 1.1) % in Scots pine, and 13 (±0.9) % in Norway spruce.

Kanamycin selection

The antibiotic kanamycin effectively inhibited germination of Norway spruce and Scots pine pollen.

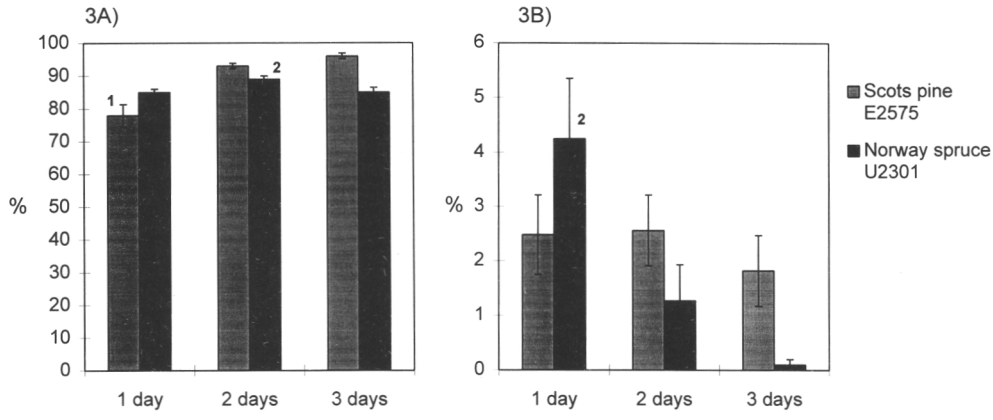

Fig. 3. Transient GUS expression during pollen germination in Scots pine and Norway spruce samples bombarded with the pBI221. A) Germination percentages after one, two, and three days of in vitro germination. B) GUS-expressing pollen grains as a mean percentage (±SE) of the germinated ones after one, two, and three days of in vitro germination. The germination percentage marked with **1** was significantly ($p<0.01$) lower than after two or three days of germination. Results marked with **2** were significantly higher ($p<0.05$) than in other time points tested.

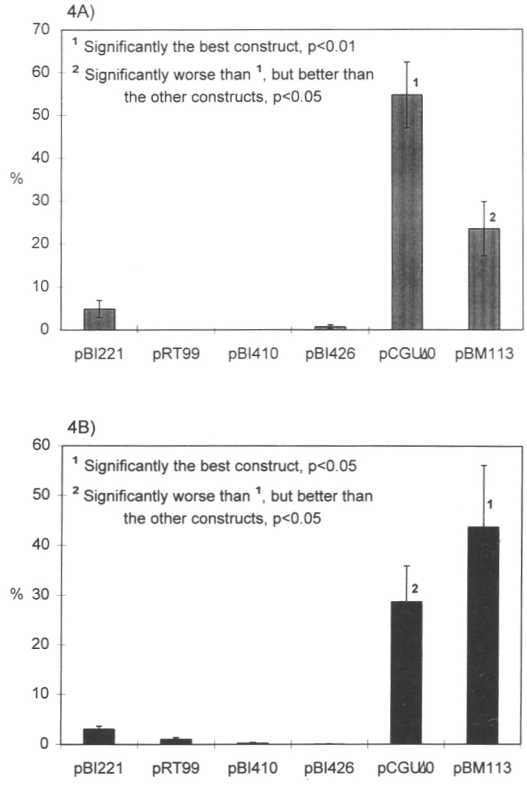

Fig. 4. The effect of different regulatory sequences on the GUS expression in pollen of A) Scots pine and B) Norway spruce. The results are presented as GUS-expressing pollen grains as a mean percentage (±SE) of the germinated ones.

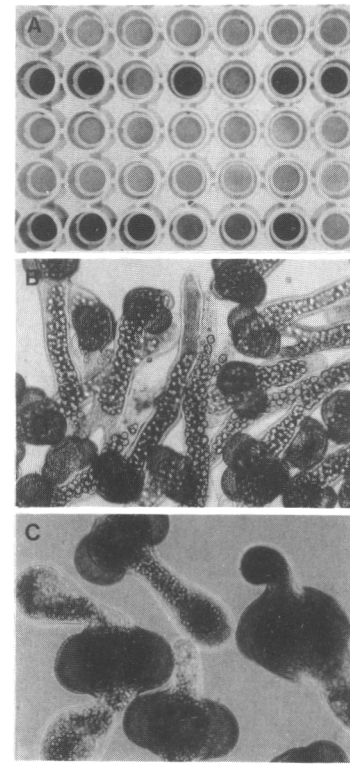

Fig. 5. Transient GUS expression in the bombarded pollen samples after the histochemical GUS assay. A) Pollen samples in 96-well-plate bombarded with different gene constructs. Lanes showing dark blue colour correspond the constructs pCGUΔ0 and pBM113. B) Scots pine pollen grains bombarded with the pCGUΔ0, and germinated for two days. C) Norway spruce pollen grains bombarded with the pBM113, and germinated for one day.

The suitable kanamycin concentration for selection of transformed pollen grains in Norway spruce is 250 mg/l, and in Scots pine 175 mg/l (Fig. 6). Unfortunately, the attempts to enrich the proportion of transformed pollen grains by using kanamycin selection failed. There were no differences in the amount of GUS-expressing pollen grains between the germination media with and without kanamycin in both species. Thus further experiments were performed without kanamycin selection.

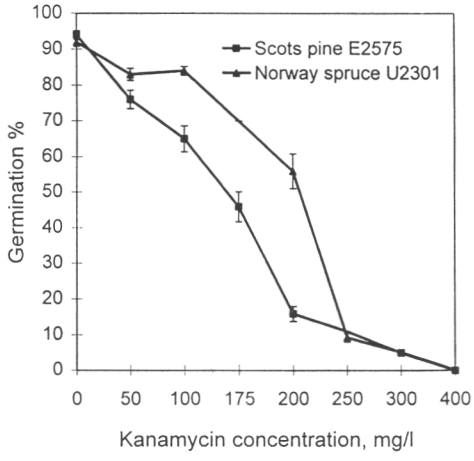

Fig. 6. The effect of different kanamycin concentrations on the germination ability of Scots pine and Norway spruce pollen.

Controlled pollinations

The characteristics of the bombarded Scots pine and Norway spruce pollen used for the controlled pollinations varied between the different pollination times. In Scots pine, the in vitro germination percentage of pollen was 91 % at the first pollination, 86% at the second one, and 82 % at the third one. In Norway spruce pollen the corresponding values were 87, 85, and 87 %. Also the GUS-expressing pollen grains as a percentage of germinated grains varied in both species. In Scots pine, it was 10.4 % at the first pollination, 22.3 % at the second one, and 4.4 % at the third one. In Norway spruce the corresponding transformation results were 1.7 %, 13.8 %, and 6.5 %.

Development of the cones after the controlled pollinations was followed at frequent intervals. Scoring of the cones was done four months after the pollinations, when 21 % of the Scots pine cones and 56% of the Norway spruce cones had developed normally (Fig. 7). The development of Scots pine cones takes two years, and therefore the Scots pine conelets were left to overwinter. The Norway spruce cones were collected after scoring, and stored at 2°C for three weeks. Seeds were extracted from the cones after the cold storage period. The total seed yield from nine Norway spruce cones was 6.8 g, i.e. approximately 1700 seeds. The seeds will be sown in greenhouse after four months of stratification at -5°C.

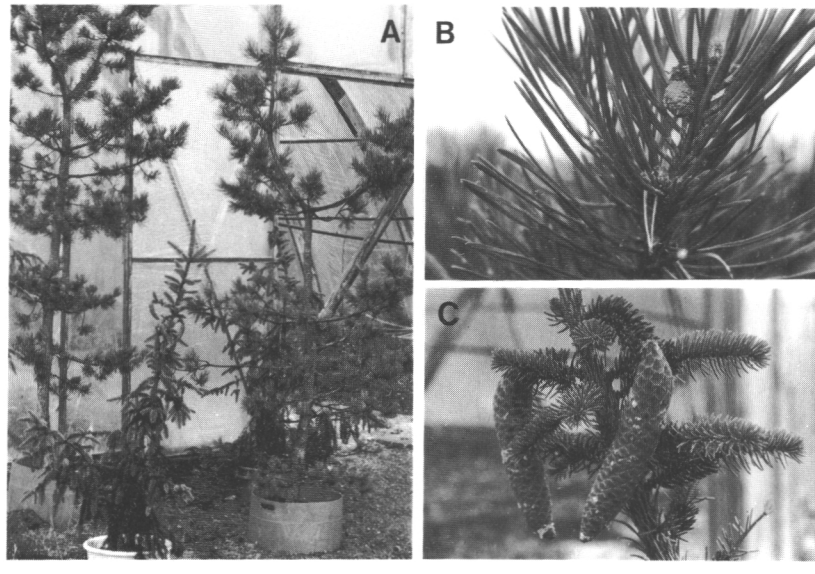

Fig. 7. Development of the cones in the potted grafts of Scots pine and Norway spruce after the controlled pollinations with the bombarded pollen. A) Grafts in the greenhouse. B) A Scots pine conelet after the first growing season. C) Maturing cones of Norway spruce at the end of the first growing season.

Discussion

Particle bombardment is a suitable method for transforming both Scots pine and Norway spruce pollen. Depending on the pollen source (tree genotype) and the gene construct used the transformation results in the present study were as high as 55 % in Scots pine and 44 % in Norway spruce. Hay and co-workers (1994) reported in their study with pollen of lodgepole pine, yellow cypress, western hemlock, jack pine, and black spruce the maximum transformation result in the best species, black spruce, to be 8.7%. Based on the data presented by Martinussen and co-workers (1994) the transformation percentage in Norway spruce pollen was less than one percent. Transformation parameters have also been optimized for white spruce pollen (Li et al. 1994), but transformation efficiency has not been reported. High level of transgene expression, 70 %, has also been reported in electroporated lily pollen protoplasts (Miyoshi et al. 1995).

In the present study, transformation results were calculated as GUS-expressing pollen grains as a percentage of the germinated ones. This was done to get a realistic view as to the possibility of producing transgenic progeny through pollen transformation, because the non-germinated pollen grains cannot participate in fertilization. For the same reason, the histochemical GUS assay was chosen instead of the fluorometric assay. By using the fluorometric assay the level of gene expression can be followed, as pointed out by Hay and co-workers (1994), but the histochemical assay more accurately indicates the frequency of transformed pollen grains. On the other hand, it should be taken into account that diffusion of a soluble intermediate of the dichloro-dibromoindigo from the test solution into germinating pollen grains causes a certain level of background staining.

Clonal differences in compatibility for transformation or in ability to express foreign genes found in the present study have been reported also in earlier conifer studies, e.g. with cotyledons of Douglas-fir (Goldfarb et al. 1991), several tissues of Norway spruce (Newton et al. 1992), and vegetative buds of Scots pine (Aronen et al. 1995). This means that for production of transgenic progenies the choice of the pollen source is important, and probably only part of the plus trees selected for the breeding program of the species will be competent for transformation.

Of the regulatory sequences tested in the present study the promoter of the sunflower polyubiquitin gene UbB1 and the abscisic acid (ABA) inducible promoter of the wheat EM gene proved to be the best for Scots pine and Norway spruce pollen. To our knowledge, the ubiquitin promoter has not been studied in coniferous pollen, but it has given high transient GUS expression results in the cotyledons of radiata pine (Rey et al. 1995). The ABA-inducible promoter has been introduced into pollen of several gymnosperms, in which it has promoted higher expression levels than the 35 S CaMV promoter or double 35 S promoter with AMV enhancer (Hay et al. 1994). This is in agreement with the present results.

Ubiquitin is one of the most abundant proteins in eukaryotic cells. It is a small, highly conserved polypeptide that covalently binds to proteins and marks them for destruction by a proteolytic complex. Enhanced proteolysis is required for repair of cellular damage caused by several stress-related factors, e.g. environmental insults. Ubiquitinylation of the histone proteins also appears to contribute to active transcription of DNA (Wilkinson 1995; Taiz and Zeiger 1991). In *Bryonia dioica*, mechanical pertubation induces a rapid increase in ubiquitin mRNA production (Galaud et al. 1995). High GUS expression levels in the Scots pine and Norway spruce pollen, bombarded with the pCGUΔ0 including the ubiquitin promoter, are thus probably connected to the conserved nature of ubiquitin proteins, as well as to stress and cell damage caused by penetrating gold particles.

Abscisic acid causes many physiological responses in higher plants, e.g. induces gene expression in different stress conditions (Bray 1991; Taiz and Zeiger 1991; Trewavas and Jones 1991). Therefore the bombardment stress may have contributed to the high expression results achieved with the ABA-inducible EM promoter of the pBM113 as well. ABA is also known to stimulate an increase in cytosolic free calcium ($[Ca^{2+}]_{cyt}$) in stomatal guard cells (McAinsh et al. 1991), and Ca^{2+} influx at the tip of the pollen tube and a Ca^{2+} gradient within the pollen tube are required for pollen tube growth (Malhó et al. 1995; Pierson et al. 1994). Thus it is probable that the germinating Scots pine and Norway spruce pollen contain ABA which explains the high GUS expression results after bombardment with the pBM113.

Pollen is an excellent target material for studying the effects of different regulatory sequences. Each pollen grain represents its own genotype, and thus a huge amount of different genotypes can be studied at the same time. As a target material pollen is easy to handle, since no complicated tissue culture methods, protoplast isolation etc. are needed. Pollen is also a natural vector for transporting genetic material, and moreover, the development of potentially transgenic progenies takes place through zygotic embryogenesis. To produce transgenic Scots pine and Norway spruce seedlings we used pollen bombarded with the pBI221 in controlled pollinations. The pBI221 was chosen as an introduced gene construct, because the results from the promoter experiments were not available at the time of the pollinations. High transient expression results, as achieved with the ubiquitin and ABA

inducible promoters in the present study, prove that large proportion of the pollen grains have received a functional foreign gene. Of course, this does not assure integration of the introduced gene into the genome of the zygotic embryo.

According to our results, high foreign gene expression levels reduced the germination capability of Scots pine pollen. In Scots pine, pollen chambers of ovules usually have room for at least two pollen grains (Sarvas 1962), and the better germination ability of non-transformed pollen grains can make them superior in the competition for fertilization. In Norway spruce, we did not observe any reduction in the germination ability of pollen due to foreign gene expression. Competition between normal and transgenic pollen grains, however, also takes place in Norway spruce, since the pollen chambers of the species can contain up to five pollen grains (Sarvas 1968). To limit pollen competition we tested the antibiotic kanamycin as a selective agent, and it proved to be effective in both species. However, enrichment of transformed pollen particles in the samples bombarded with pRT99, pBI410 or pBI426 failed. This was probably due to the promoters deriving neomycin phosphotransferase expression in these gene constructs, since the CaMV 35 S, 35 S with AMV, and double 35 S with AMV proved to be relatively weak regarding the GUS expression in our experiments.

The fact that coniferous pollen is multicellular consisting of a big tube cell and a smaller cell called either generative or antheridial cell (Moitra and Bhatnagar 1982) complicates the assessment of possibilities of producing transgenic seedlings through pollen transformation. The generative cell should be transformed in order to get transgenic progenies. In the present study, however, it was impossible to distinguish whether the blue stain in the histochemical GUS assay of the bombarded and germinated pollen grains originated in the generative cell or in the tube cell, owing to potential diffusion of the soluble intermediate of the dichloro-dibromo-indigo from one cell to another.

In Scots pine, the development of the cones reflects the success of pollination. Insufficient pollination causes deterioration of the female strobilus, and in most cases it drops during the first growing season or at the beginning of the second one. Normally, approximately one fifth of the conelets drops even if the pollen catch of female strobili is sufficient (Sarvas 1962). Thus, it can be assumed that 21 % of the pollinations performed with the bombarded pollen, were successful. Fertilization in Scots pine, however, takes place during the second growing season (Sarvas 1962), which means that in the present study the seeds will be available at the end of 1996, and the progenies can be studied in 1997.

In Norway spruce, both pollination and fertilization take place during the same growing season. Cone development does not reflect the success of pollination, because the ovules which fail to trap pollen develop into empty seed (Sarvas 1968). In the present study the degeneration and abscission of the developing cones in the potted grafts were probably caused by the environmental factors, such as temporary drought or deficiency of nutrients. The remaining cones produced a good seed yield, and the characteristics of the seeds and the progenies will be studied after a stratification period.

Our results show that high levels of transient foreign gene expression can be achieved in Scots pine and Norway spruce pollen by using regulatory sequences proper for the target material and its physiological stage. In Scots pine, this is the first report on the transformation of pollen. Moreover, it has been demonstrated that bombarded pollen can successfully be used for controlled pollinations in both species. Stable integration of the introduced genes and the characteristics of the progenies, however, remain to be investigated owing to the slow seed development of Scots pine and stratification requirements of both species.

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