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Clonal variation in Scots pine (*Pinus sylvestris*) and in transgenic silver birch (*Betula pendula*)

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Academic dissertation

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ABSTRACT

The aim of this study is to assess the clonal variation in Scots pine (*Pinus sylvestris* L.) clones and in transgenic lines of silver birch (*Betula pendula* Roth) and its causes, with special attention to the effects of cloning and transgenesis in tree breeding programmes. The parental effect on cloning success variation was studied in an experiment where Scots pine embryogenic lines were initiated from immature seeds of a full diallele cross. The evaluation was made after culture initiation, on maintenance medium and by mature embryo production. Growth and stem straightness of Scots pine clones were assessed in a 10-year field trial established with rooted cuttings. The effect of a single gene transfer, the sugar beet chitinase IV gene (*chiIV*), was assessed on plant growth, susceptibility to fungal diseases and development of root associated fungal communities and phenology, in a 3-year field trial established with micropropagated transgenic silver birch lines and wild-type clones. The results of the somatic embryogenesis experiment with Scots pine showed that the initiation success, as well as maturation, was more affected by the genotype of the mother than the one of the father, while during the proliferation period the mother's effect decreased and the father's increased. The field trial with Scots pine showed that the tree's genotype, more than the propagation method, has an effect on the plant behaviour in the field. In silver birch transgenic lines, the introduction of a single gene (*chiIV*) led to a reduction in growth and quality characteristics, although no significant changes occurred regarding fungal disease resistance, ectomycorrhizal colonization or fungal community structure, as compared to the natural variation occurring in wild type clones. The conclusion was that the variation in the success of Scots pine embryogenesis and in the growth of Scots pine rooted cuttings is strongly affected by genotype and, hence, the rooted cuttings are suitable for testing height growth in breeding programmes. A single *chiIV* gene transfer did not improve significantly fungal disease resistance in silver birch or interfere with root associated fungal community. However, the variation in adaptive traits among silver birch transgenic lines is at the same level with the variation detected in randomly selected wild-type silver birches and single transgenic lines may be selected in breeding programmes.

Keywords: clone, variation, gene transfer, fungal community, adaptive traits, tree breeding

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Helsinki, March 2013

Anna-Maija Niskanen

LIST OF ORIGINAL ARTICLES

This thesis is based on the following articles, which are referred to according to their Roman numerals I–IV. The articles are reprinted with the kind permission of the publishers.

I Niskanen, A-M., Lu, J., Seitz, S., Keinonen, K., von Weissenberg, K. and Pappinen, A. (2004) Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*). Tree physiology 24: 1259−1265.
doi:10.1093/treephys/24. 11.1259.

II Niskanen, A-M., Stenvall, N., Pakkanen, A. and Pulkkinen P. (2008). Comparison of growth and stem form characters of *Pinus sylvestris* clones and seedlings of the same origin in a 10-year field trial. Scandinavian Journal of Forest Research 23: 484−490.
doi:10.1080/02827580802512416.

III Pasonen, H-L., Lu, J., Niskanen, A-M., Seppänen, S-K., Rytkönen, A., Raunio, J., Pappinen, A., Kasanen, R. and Timonen, S. (2009). Effects of sugar beet chitinase IV on root-associated fungal community of transgenic silver birch in a field trial. Planta 230: 973−983.
doi: 10.1007/s00425-009-1005-4.

IV Niskanen, A-M., Kärkkäinen, K. and Pasonen H. (2011). Comparison of variation in adaptive traits between wild-type and transgenic silver birch (*Betula pendula*) in a field trial. Tree Genetics & Genomes 7: 955–967.
doi: 10.1007/s11295-011-0387-1.

Author contributions to the articles I−IV are given in table 1.

Table 1. Author contributions

Publication	I	II	III	IV
Original idea	KvW, KK	PP, AnP	HP	HP
Study design	AMN	AMN	HP, SS, ST, RK	HP
Data gathering	AMN, JL	*	JL, AMN, AR	HP,*
Analyses	SZ	AMN, NS	HP, AMN, JR	HP, AMN
Manuscript preparation	AMN	AMN, NS, PP	AMN, HP, ST	HP, AMN, KaK

KvW=Kim von Weissenberg, ArP=Ari Pappinen, AR=Anna Rytkönen, JL=Jinrong Lu, SZ=Sakari Seitz, KK=Kaija Keinonen, PP=Pertti Pulkkinen, AnP=Anne Pakkanen, NS=Niina Stenvall, HP=Hanna Pasonen, RK=Risto Kasanen, SS=Sanna-Kaisa Seppänen, ST=Sari Timonen, KaK=Katri Kärkkäinen, RJ=Juha Raunio, AMN=Anna-Maija Niskanen, *students/field technicians credited at the publication in question

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ABBREVIATIONS

AaXEG2	xyloglucan gene from *Aspergillus aculeatus*
ABA	abscisic acid
asl	above sea level
acp	gene encoding *Escherichia coli* acyl carrier protein
BA	benzyl adenine (6-bentzyl-aminopurine, BAP)
BAR	herbicide bialaphos resistance gene
barnase	gene coding cytotoxin barnase
barstar	gene coding barnase inhibitor
Bbchit1	chitinase gene from *Beauveria brassiana*
bgt	chimeric gene consisting of the insecticidal toxin gene from *Atrax robustus* and the C terminal of *cry*IA(b) gene from *Bacillus thuringiensis*
BwB	breeding without breeding -strategy
CaMVE35S	cauliflower mosaic virus promoter E35S
chiIV	chitinase IV gene from sugar beet
*chit*24	chitinase gene from *Trichoderma harzianum*
COMT	gene connected to lignin biosynthesis
*cry*IA(b)	gene from *Bacillus thuringensis*
*cry*IA(c)	gene from *Bacillus thuringensis*
Cu,Zn-SOD	chloroplastic superoxidase dismutase gene from *Solanum lycopersicum*
DCR	tissue culture medium (Gupta and Durzan 1986)
DEGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonuclear acid
*ech*42	chitinase gene from *Trichoderma harzianum*
ECM	ectomycorrhiza
GFP	green fluorescent reporter gene
GM	genetically modified
GUS	*β*-glucuronidase
IBA	indole-3-butyric acid
IndVal	indicator species analysis
ITS	internal transcribed spacer
leafy	gene regulating inflorescense in *Arabidopsis*
LM	tissue culture medium (Litvay et al. 1985)
LP	tissue culture medium (Quoirin and Lepoivre 1977)
mRNA	messenger RNA
MRPP	multi-response permutation procedure
MS	tissue culture medium (Murashige and Skoog 1962)
MSG	tissue culture medium (Brown and Lawrence 1968)
NAA	1-naphtalene acetic acid
NMS	non-metric multidimensional scaling
nos	nopaline synthase promoter
*npt*II	reporter gene coding neomysin phosphotransferase

OxOx	oxalate oxidase gene
pBKL4K4	binary plasmid used for chitinase transformation
PEG	polyethylene glycol
PR	pathogenesis related (proteins)
PsACS2	ACC (1-aminocyclopropane-1-carboxylate synthase) gene expressing during ethylene production in somatic embryo development of Scots pine
rDNA	ribosomal DNA
RGR	relative growth rate
RNA	ribonucleic acid
rol	genes (ABCD rooting oncogenic loci) from *Agrobacterium rhizogenes*
SE	somatic embryogenesis, somatic embryo
SH	tissue culture medium (Schenk and Hillebrandt 1972)
SSR	single sequence repeat
stsy	stilbene synthase gene from *Vitis vinifera*
ThChiI	chitinase gene from *Trichoderma harzianum*
ThEn42	*Trichoderma harzianum* endochitinase gene
Ugt	gene in the UDP glucuronosyltransferases (UGT) family
*uid*A	reporter gene encoding β-glucuronidase
WPM	tissue culture medium (Lloyd and McGown 1980)

1 INTRODUCTION

1.1 General framework

The basic question in tree breeding is how to define the objectives of tree improvement. The answer from forest owners is simply the best possible economic profit. The profit has been determined by buyers, i.e. by paper and plywood industry, which pay for wood by stem volume, and in much lesser amount by furniture and other timber utilising industries which value the quality and are − in theory − prepared to pay extra for high quality timber. Tree breeders for their part determine how to improve the volume and the quality of forest trees. The solution has been to select superior genotypes (plus trees) from natural forests and use them in traditional tree breeding programmes. The number of selected plus trees is crucial for the preservation of variation in the future generations to ensure the stability of the breeding population and the success of the breeding work. These four – productivity, quality, stability and effectiveness – have been the objectives of tree improvement in Finland since the 1940s. The latest Finnish long term tree breeding programme (Haapanen and Mikola, 2008) lists the main objectives of the Finnish tree breeding today: productivity (high genetic gain transferred to the next generation), predictivity (well adapted breeding material) and cost-efficiency (application of new breeding methods). Today about 50 % of the Scots pine (*Pinus sylvestris* L.) seedlings in southern Finland and nearly all the silver birch (*Betula pendula* Roth) seedlings originate from seed orchards.

The success of tree breeding depends on the variation found in the breeding population, which for its part mirrors the variation of natural forests, and on the heritability of the selected traits. Trees in boreal forests are in general long lived having long generation times, being highly outcrossing and non-domesticated with large, continuous and variable populations. The evolutionary forces − natural selection, mutations, random genetic drift − which promote, or those which limit − phenotypic plasticity and gene flow − the differentiation among populations, help the populations to adapt in varying environments (Falconer and Mackay 1996, Eriksson 1998). The Finnish long-term forest breeding programme recognises the future challenges connected with the climatic warming, which is predicted to have an effect both on the annual phenological rhythm of the trees and on the amount of pests and diseases in the future (Haapanen and Mikola 2008, Parviainen et al. 2010). It may be argued that if these changes are rapid, either natural adaptation or the traditional breeding methods have no realistic chance in producing adapted planting material in time. New breeding techniques, such as gene transfers, which create new variation beyond the species limit, molecular assisted selection, which helps to identify traits at an early age, the use of clonal material in testing, which speeds up the breeding process and finally the use of cloned material in planting may offer the only option in the changing environment.

1.2 Pine clones

The term ‘clone’ has been used to describe identical copies of molecule sequences (DNA, RNA), cells of specific tissues (e.g. stem cells) and whole single- or multi-cellular individuals which are genetically identical. In this research, the term clone is used to describe a group vegetatively propagated plants from a single genotype. The term ‘genetically modified line’ (GM-line) is used of vegetatively propagated group of plants induced from a single transformation event. Also, the term ‘embryogenic line’ is used of Scots pine embryogenic cultures which originate from a single immature seed from a controlled cross.

Pines, like conifers in general, are considered difficult to clone (Häggman et al. 1996) and the rooting percentage of Scots pine cuttings is lower than in some other pine species (Ragonezi et al. 2010). The cloning of Scots pine is based *in vivo* mostly on grafting and the seed-orchards in Finland base on grafted clones (Haapanen and Mikola 2008) and *in vitro* on either organogenesis (Häggman et al. 1996) or on somatic embryogenesis (Hohtola 1995, Keinonen-Mettälä et al. 1996, Sarjala et al. 1997, Häggman et al. 1999, Lelu et al. 1999, Filonova et al. 2002, Aronen et al. 2009).

1.2.1 Rooted pine cuttings

In the early 1970s research on the propagation of several pine species by rooted cuttings was reported in New Zealand (Sweet and Wells 1974, Cameron and Rook 1974, Shelbourne and Thulin 1974), Australia (Pawsey 1971), USA (Libby et al. 1972, Kiang et al. 1974, van Buijtenen et al. 1975) and Korea (Ok Hong 1974). The progress in the development of this method has been slow and radiate pine (*Pinus radiata* D. Don) is the only pine species today used in large quantities for reforestation as rooted cuttings (Ritchie 1991, Ragonezi et al. 2010).

Ragonezi et al. (2010) review adventitious rooting of conifers compare the rooting success of pine species *in vivo* and *in vitro*. The *Pinus* species with highest rooting percentages *in vivo* were *P. contorta* Douglas ex Louden (100%), *P. strobus* L. (97%), *P. caribaea* var *hondurensis* Morelet (95%), *P. radiata* (95%), *P. banksiana* Lamb (87−95%) and *P. taeda* L. (80−94%). The lowest rooting percentages *in vivo* were found in *P. virginiana* Mill. (47%), and in *P. sylvestris* (54%). Auxin − either indole-butyric-acid (IBA) or α-naphtalene-acidic-acid (NAA) − was used in varying concentrations to induce the adventive root development and the roots were grown on several substrates. Taking explants from mature trees, seedlings or micropropagated plantlets and rooting them as microcuttings *in vitro* is another method to induce adventive roots (Ragonezi 2010). The pine species with the highest rooting percentages *in vitro* were *P. roxburghii* Sarg. (97%), *P. pinaster* Aiton (86−92%), *P. brutia* Tenore *x P. halepensis* Mill. (84%), *P. canariensis* C. Sm. (83%), and with the lowest *P. sylvestris* (6−64%) and *P. virginiana* (18%).

Menzies et al. (2001) describe the radiate pine vegetative propagation method used in New Zealand, where the young seedlings (4–5 months old), used as stock plants, are pruned and the developing side shoots are used as cuttings. The stock plants may be planted outside in rows, which are pruned up to the age of eight years for cutting production. The cuttings are rooted without hormone treatments and used to bulk-up the limited progenies

from controlled crosses used in the breeding programmes and as planting material. The cuttings provided over 25% of the planting stock of radiata pine in New Zealand in the turn of the millennium (Menzies 2001). Ritchie (1991) reviewed the development of cutting production since 1970s and reported that the major single realization was the importance of the physiological status of the stock-plant, which led to the use of young donors, hedging and serial propagation in cutting production. Browne et al. (2001) studied the use of benzyl adenine (BA) in enhancing the number of shoots per stock plant by inducing the elongation of dwarf shoots. They sprayed pruned, young (6 months old) *P. banksiana* seedling stock plants with varying concentrations of BA and compared the number of cuttings to pruned controls. The number of cuttings increased from 14–17 in controls to 30–40. They found that higher concentrations of BA increased the number of cuttings, but lowered the rooting percentage. Frampton et al. (1999) studied the benefits of a commercial rooting compound containing IBA 10,300 ppm and NAA 6,600 ppm compared to boron and thiamine treatments in rooting loblolly pine (*P. taeda*) cuttings of full-sib families. They found out that the rooting percentage was the highest in water control but the morphology of the hormone treated roots was more symmetrical. The boron and thiamine treatments had no effect on the rooting, but family, rooting compound concentration and their interaction had significant effect on rooting.

In Finland experiments of the vegetative propagation of Scots pine by rooted cuttings started in 1962 (Mikola 2009). Boeijink and van Broekhuizen (1974) reported the use of mist chamber in improving the rooting of Scots pine cuttings. Kossuth (1978) studied the effect of cytokinin treatment on Scots pine pruned stems and found that the treatment with 225 ppm benzyladenine (BA) induced 100 % fascicular bud development. Improvements in the number of cuttings/stock-plant (Salonen 1994a) and in the rooting of the Scots pine cuttings have been reported (Niemi et al. 2000, Högberg 2005) in the last decades, but the large-scale cutting production of Scots pine still needs to be improved. Finland, Sweden and Latvia launced a joint research project on testing Scots pine cutting propagation methods (Högberg et al. 2011), where selected full-sib families with high breeding values were cloned. The tested methods included different pruning of the donor plants, IBA and gibberellin inhibitor treatment of the cuttings, rooting in high humidity, watering and rooting substrate tests. They reported in different locations considerable clonal variation within families in cutting production/donor plant (in average 7.8−21.5). The rooting of the Scots pine cuttings was low and erratical with total failures in some propagation occasions. However, in the best model and in the best location the rooting percentage was 53.2%. The variation in rooting success was suggested to be more dependent of technical solutions than variation within or between families. No effect of watering regime, rooting substrate type or gibberellin inhibitor with or without IBA treatment was detected on rooting success of Scots pine cuttings (Högberg et al. 2011).

1.2.2 Pine micropropagation/organogenesis

Young tissues (embryos/cotyledons) have been used for the initiation of tissue cultures for pine organogenesis. Aitken-Christie et al. (1985) used radiata pine seeds for induction of tissue cultures for micropropagated plantlet production. They obtained two types of shoots, wet and waxy, which varied in rooting ability and plantlet sturdiness. Aitken-Christie and

Davies (1988) developed a semi-automatized system for the production of micropropagated radiate pine plantlets. Saborio et al. (1997) produced Mexican white pine (*P. ayacahuite* var. *ayacahuite* Ehremb.) plantlets from mature seeds. Cuesta et al. (2008) used cotyledons of stone pine (*P. pinea* L) seeds for the initiation material of micropropagation cultures. They found variation in survival, microshoot production and rooting between selected families. RAPD analysis detected low polymorphism, uniquely detected among families and presenting the same pattern in clones, which was interpreted as a sign of clonal fidelity, but not necessarily absence of somaclonal variation.

Micropropagation has been successful in small-scale research purposes in Scots pine vegetative propagation. Chalupa (1985, 1989) used Scots pine axillary and adventitious buds from young, 1−2 year-old seedlings for initiation material on micropropagation experiments. He cultured stem cuttings on media supplemented with cytokinin and auxin to promote bud formation. The buds were elongated and multiplied on media containing lower concentrations of phytohormones and/or activated charcoal. When the shoots were 15−25 mm long they were rooted on media containing auxin and elongated on auxin-fee medium. Häggman et al. (1996) used cotyledons from germinated embryos as explants in producing early flowering Scots pine clones. They were successful in plantlet multiplication, but reported difficulties in rooting and most of the micropropagated plants were plagiotropic and highly branched in the greenhouse. It has been suggested that the micropropagation of Scots pine is a potential method to propagate adult trees to obtain clonal material (De Diego et al. 2010).

1.2.3 Pine somatic embryogenesis

Pine somatic embryogenesis (SE) has been induced using protoplasts of proembryonal cells (Gupta and Durzan 1987), immature (Becwar et al. 1988, Jain et al. 1989, Lainé and David 1990, Bercetche and Pâques 1995, Arya et al. 2000) and mature zygotic embryos (Gupta and Durzan 1986, Hohtola 1995, Bozhkov et al. 1997). Also, either dissected (Arya et al. 2000) or whole (Lainé and David 1990, Nagmani et al. 1993, Keinonen-Mettälä et al. 1996) female gametophytes (megagametophytes) have been used for tissue culture initiation. Becwar and Pullmann (1995) described how the immature embryos extruded from the megagametophyte through micropyle to the culture medium and formed proliferating embryogenic mass in loblolly pine. Becwar et al. (1991) found multiple genotypes in somatic embryogenic cultures of *P. taeda* initiated from megagametophytes in low frequencies (8 %).

Several culture media are used in Scots pine SE cultures at the initiation stage (Hohtola 1995), i.e. MS (Murashige and Skoog 1962), SH (Schenk and Hillebrandt 1972), WPM (Lloyd and McCown 1980), LP (Quoirin and Lepoivre 1977, Hakman and von Arnold 1985), LM (Litvay et al. 1985) and DCR (Gupta and Durzan 1986) with modifications in macro- and micronutrient compositions, vitamins and plant growth regulators, and addition of organic nitrogen and carbon source (Hohtola 1995, Keinonen-Mettälä et al. 1996, Sarjala et al. 1997, Häggman and Aronen 1998, Niemi et al. 1998, Häggman et al. 1999, Niemi and Häggman 2002, Park et al. 2006, Rodríguez et al. 2006, Lelu et al. 1999, Niemi et al. 2007, Burg et al. 2007, Lelu-Walther et al. 2008, Aronen et al. 2009, Abrahamsson et al. 2012).

The preferred initiation material for Scots pine embryogenic culture is zygotic embryo (Keinonen-Mettälä et al. 1996, Sarjala et al. 1997, Häggman and Aronen 1998, Niemi et al. 1998, Häggman et al. 1999, Niemi and Häggman 2002, Park et al. 2006, Rodríguez et al. 2006, Lelu et al. 1999, Niemi et al. 2007, Burg et al. 2007, Lelu-Walther et al. 2008, Aronen et al. 2009, Abrahamsson et al. 2012). The period of its competence to initiate somatic embryogenesis is narrow, 2−3 weeks (Keinonen-Mettälä et al. 1996). The optimal culture initiation time is one week after fertilization (Burg et al. 2007), when cleavage polyembryony is just starting (Filonova 2002). At that early stage single embryos cannot be isolated and the whole ovule/megagametophyte containing several embryos originating both single and cleavage polyembryony has been used for the culture initiation (Burg et al. 2007). Park et al. (2006) described, how Scots pine zygotic embryos extruded from the megagametophyte prior to the embryogenic mass initiation. Burg et al. (2007) analysed 325 new embryogenic cell lines from 15 mother trees using four nuclear SSR markers, but the results indicated no mixed cultures at the initiation stage. However, they observed mutations at all stages of the *in vitro* cultures, which resulted in mosaic cultures (Burg et al. 2007).

At the initiation stage the cultures have been incubated in the darkness (Sarjala et al. 1997, Niemi et al. 1998, Abrahamsson et al. 2012). The proliferation of the embryogenic tissue continues as long as the tissues are subcultured on fresh medium and the cultures may be cryopreserved as soon as they have been established (von Arnold et al. 2002). However, prolonged subculture period and/or cryopreservation increase the possibility of somaclonal variation (von Arnold et al. 2002, Burg et al. 2007). Lelu et al. (1999) tested both medium with and without growth regulators on initiation and proliferation of Scots pine somatic embryos and found no significant effect on the medium. A technique to obtain large quantities of somatic embryo mass involves a SE tissue suspension in liquid medium plated over filter paper, drained and cultivated on the surface of a culture medium (Klimaszewska et al. 2007, Aronen et al. 2009).

For maturation, the cultures may be treated with plant growth regulator-free medium (Rodríguez et al. 2006, Burg et al. 2007) or with medium containing activated charcoal (Häggman et al. 1999, Lelu-Walther et al. 2008). Lelu et al. (1999) reported that in some lines Scots pine somatic embryos, which had been initiated and maintained on growth regulator free medium, matured spontaneously. The critical factor for successful embryo maturation is reported to be the restriction of water availability on the maturation medium containing ABA (von Arnold et al. 2002, Klimaszewska et al. 2007), which can be achieved either by decreasing the water potential by high molecular weight polyethylene glycol (PEG) in the medium or with physical means, i.e. with high gelling agent concentration (Lelu et al. 1999, Klimaszewska et al. 2007). Embryo germination is achieved after either cold treatment or partial desiccation, which is not necessary when PEG is not used (Klimaszewska et al. 2007). To maximise the mature somatic embryo production, the embryonic mass can be suspended in plant growth regulator free liquid and cultured on a membrane support (Niemi and Häggman 2002, Klimaszewska et al. 2007, Aronen et al. 2009).

The mature somatic embryos have been germinated on half-strength growth medium without plant growth regulators in light (Häggman et al. 1999), or in darkness for the first week to elongate the hypocotyl before light conditions (Klimaszewska et al. 2007). It has

been reported, that it is beneficial for root development to place the embryos horizontally on the growth medium and place them vertically on the medium after the roots have developed (Aronen et al. 2009). Lu et al. (2011) studied the role of ethylene in Scots pine somatic embryo maturation and found that the expression of a potential genetic marker, *PsACS2,* corresponded during embryo development to ethylene production. Niemi and Häggman (2002) inoculated the Scots pine somatic embryos with mycorrhizal fungus *Pisolithus tinctorius* (Pers.) Cocker and Couch *in vitro* resulting in mycorrhiza formation, which potentially enhanced the germination and adaptation *ex vitro*. In later experiment (Niemi et al. 2007) it was reported that *P. tinctorius* together with spermidine induced somatic embryo maturation. Lelu et al. (1999) reported that some Scots pine embryos went through all stages of somatic embryogenesis without growth regulators, germinated and developed into plants. When rooted embryos, called emblings are adapted to greenhouse conditions, they can be planted on growth mixture and treated like seedlings (Häggman et al. 1999, Aronen et al. 2009).

Many factors in addition to the developmental stage of the zygotic embryo, growth medium composition and tissue culture procedures have been noted to have an effect on the success of Scots pine somatic embryogenesis, such as the storage time prior to the culture initiation and the genotype of the parent trees (Häggman et al. 1999, Lelu et al. 1999, Aronen et al. 2009).

1.3 Silver birch clones

Vegetative reproduction of silver birch may sometimes occur in the forest by dormant (epicormic) buds sprouting at the stump after the trunk is cut (Koski and Rousi 2005, Viherä-Aarnio 2008). The seeds for planting were up to the 1970s collected from plus trees in seed collection stands (Viherä-Aarnio and Ryynänen 1994, Viherä-Aarnio and Velling 2008). Today, silver birch seeds in Finland are produced in seed orchard grafts in polythene greenhouses, where the seed production is effective in controlled conditions (Viherä-Aarnio and Velling 2008). Silver birch has been propagated by rooted stem cuttings (Tervonen 1981, Salonen 1994b, Cameron and Sani 1994). The age of the stock plants has been reported to be a restriction in rooted cutting production and effective rooting has been achieved only when the cuttings were taken from trees under the age of 10 years (Tervonen 1981). Cameron and Sani (1994) rooted silver birch epicormic shoots to enhance the rooting capacity and to reduce the mature characteristics of the rooted cuttings. They reported that the growth of the cuttings from the epicormic shoots of 5-, 10-, and 30-years old trees had enhanced the rooting capacity, but it was still low compared to cuttings from young seedlings. Also, the cuttings retained a high degree of maturation. Salonen (1994b) reported that micropropagation of silver birch was clearly a more effective clonal propagation method than rooting of cuttings.

Micropropagation methods for silver birch were developed in 1970s and 1980s for *B. pendula* (Huhtinen and Yahyaoğlu 1974, Simola 1985) and for *Betula pendula* var. *carelica* (Merckl.) Hämet-Ahti (Ryynänen and Ryynänen 1986). The initiation material in micropropagation of silver birch, either bud meristem (Ryynänen and Ryynänen 1986), leaf disc (Simola 1985, Iliev et al. 2003) or stem cutting (Huhtinen and Yahyaoğlu 1974) is

placed on growth medium containing micro- and macronutrients, vitamins, and growth regulators, i.e. MS (Murashige and Skoog 1962) and WPM (Lloyd and McGown 1980). The surface sterilized explants go through three developmental steps, dedifferentiation, induction and differentiation. The auxin-cytokinin ratio on the medium may vary over a wide range on the first and third step, but the hormone composition is critical during induction.

Initiation material for silver birch somatic embryogenesis has been either young zygotic embryos (Kurtén et al. 1990) or mature leaf tissues (Kurtén et al. 1990, Hvoslef-Eide and Corke 1997). Both embryogenic and non-embryogenic cell lines have been observed in silver birch cell cultures (Kurtén et al. 1990, Nuutila and Kauppinen 1992). Puupponen-Pimiä et al. (1993) reported the characterization of the silver birch embryogenic *BP8* gene, which is a potential genetic indicator of embryogenic cells. The possibility to produce silver birch somatic embryos in large scale in liquid media using bioreactors has been discussed in connection of the rapid propagation of elite material (Hvoslef-Eide et al. 2005).

1.4 Gene transfer into silver birch

The development of successful micropropagation method has made silver birch a good candidate for genetic transformation. Both the particle bombardment (Valjakka et al. 2000, Tiimonen et al. 2005) and the *Agrobacterium* based (Aronen et al. 2002, Pappinen et al. 2002, Lemmetyinen et al. 2004, Lännenpää et al. 2005, Zeng et al. 2010, Zhang et al. 2012) gene transfer methods have been developed for silver birch. The transformed properties include modification of host-pathogen interaction (Pappinen et al. 2002, Pasonen et al. 2004), insect resistance (Zeng et al. 2010), flowering (Lemmetyinen et al. 2004, Lännenpää et al. 2005), lignin biosynthesis (Tiimonen et al. 2005, Sutela et al. 2009, Seppänen et al. 2007), nitrogen metabolism (Valjakka et al. 2000) and morphology, xylem structure and chemistry (Piispanen et al. 2003). Also, transient transformation system of birch is developed for the functional characterization of genes and protein production (Zhang et al. 2012).

In this research, the genetically modified (GM) silver birch lines were sugar beet chitinase IV (*chiIV*) transgenic and, hence, a broader introduction to plant chitinases, their role in plant-fungal interactions and the use of chitinase genes in genetic transformation is presented below.

1.4.1 Chitinases and their role in plant-fungal interactions

Chitinases are enzymes which catalyse the hydrolysis of chitin, a *N*-acetoglucosamine polymer, which is found f. ex. in the cell wall of true fungi and in the exoskeleton of arthropod insects and nematodes (Collinge et al. 1993). Several biotic and abiotic factors can induce the expression of chitinases and they are important factors in the induced defend mechanism of plants (Collinge et al. 1993, Sahai and Manocha 1993, Kasprazewska 2003, Grover 2012). Chitinases are expressed in the host plant during plant growth and development (Kasprazewska 2003), by pathogen stress (Shoresh and Harman 2008), during somatic embryogenesis (Dong and Dunstan 1997, Wiweger et al. 2003) and in mycorrhizal

symbiosis (Albrecht et al. 1994a, 1994b, Pozo et al. 1998, Slezack et al. 2001, Frettinger et al. 2006). Their physiological role in abiotic stress response is related to low osmotic potential and salt stress (Shoresh and Harman 2008), exposure to heavy metals (Békésiová et al. 2008) and cold (Pihakaski-Maunsbach et al. 2001, Atıcı et al. 2003, Jarząbek et al. 2009).

The mechanisms triggered by plant-fungal interactions involve different metabolic pathways and expression of several fungal/bacterial symbiosis and pathogenesis related (PR) proteins including chitinases (Hammond-Kossack and Jones 1996, Eyles et al. 2010). Albrecht et al. (1994a) compared the induction of chitinase activities in *Eucalyptus globulus* spp. *bicostata* (Maid. et al.) Kirkp. in response to pathogens (*Phytophthora cinnamomi* Rands, *Alternaria* sp., *Botrytis cinerea* (de Bary) Whezel and *Fusarium oxysporum* Schlecht. ex Snyder and Hansen) and mycorrhizal fungus *Pisolithus tinctorius*. They could not separate the two types of fungal contacts and both led to systemic chitinase activities. In a further study by Albrecht et al. 1994b, colonization by*Pisolithus tinctorius* induced a strong chitinase activity in eucalyptus roots in early stages of the establishment of symbiosis and remained constant for a week, suggesting rather support to the mycorrhizal colonization than defence mechanism. Slezack et al. (2001) purified a chitinase isoform induced by the colonization of *Glomus mossae* (Nicol. & Gerd.) Gerdeman & Trappe in *Pisum sativum* L. cv. Frisson roots and found a homology with class I pea chitinase. Frettinger et al. (2006) reported expression of a class III chitinases in *Quercus robur* L. lateral roots prior to interaction with ectomycorrhizal fungus *Piloderma croceum* J. Erikss. & Hjortstam *in vitro*. Vierheilig et al. (2001) treated arbuscular mycorrhizal fungus *Glomus mossae* hyphal tips grown *in vitro* by chitinase, which produced inhibition of the hyphal growth, lysis of the apex and changes in the growth pattern of the fungus. Heller et al. (2008) studied the molecular mechanism involved in *Pinus sylvestris – Laccaria bicolor* (Maire) P.D. Orton mycorrhizal symbiosis and reported down regulation of defence mechanism and cell wall modification related genes during ectomycorrhizal symbiosis development differentially expressed at different stages of the process.

The PR proteins, including chitinases, induce protein based defences in plants. Eyles et al. (2009) reviewed induced resistance to pests and pathogens in trees. Nagy et al. (2004) reported peroxidase and chitinase activity in Norway spruce (*Picea abies* [L.] Karst.) infected by the root fungus *Rhizoctonia* DC. Veluthakkal et al. (2012) reviewed the chitinases and their defence responses in trees. Chitinases are at low levels expressed constitutively in trees and their defence responses are induced by abiotic (salicylic acid, jasmonic acid, ethylene and ozone) and biotic (pathogens, pests, fungal cell wall components and oligosaccharides) factors (Veluthakkal et al. 2012).

1.4.2. Plants transformed with chitinase genes

Chitinase genes have been transferred to several herbaceous species in order to improve fungal disease resistance. Transferred chitinase genes originate either from plants (Lin et al. 1995, Kishimoto et al. 2004, Chye et al. 2005, Takashaki et al. 2005, Tohidfar et al. 2005, Vellicce et al. 2006, Xiao et al. 2007, He et al. 2008), fungi (Terakawa et al. 1997, Mora and Earle 2001, Kumar et al. 2009, Kern et al. 2010, Prasad et al. 2012) or viruses (Corrado et al. 2008).

Chitinase genes, either from plants or from antagonistic fungi, have been introduced to woody plants/trees and increased resistance has been reported to fungal diseases. Bolar et al. (2000) introduced endochitinase gene *ech42* from biocontrol fungus *Trichoderma harzianum* Rifai into apple tree (*Malus* x *domestica* Borkh.). Six of eight transgenic lines, grafts and own rooted plants, were more resistant to apple scab (*Venturia inequalis* [Cooke] G. Wint.) than the control. In later experiment *Trichoderma atroviride* P. Karst. (*Trichoderma harzianum*) genes encoding endo- and exochitinases were introduced into apple. The transgenic apple trees were tested in growth chamber where one line expressing both endo- and exochitinase was highly resistant to apple scap (Bolar et al. 2001). Noël et al. (2005) reported the introduction of endochitinase gene *ech42* from biocontrol fungus *Trichoderma harzianum* into black spruce (*Picea mariana* (Miller) Britton, Sterns & Poggenb.) and hybrid poplar (*Populus nigra* L. x *P. maximowiczii* A. Henry). Transgenic spruce seedlings demonstrated increased resistance against spruce rot (*Cylindrocladium floridanum* Sobers & C.P. Seym.) and leaves of transgenic hybrid poplar against leaf rust (*Melampsora medusa* Thüm.) *in vitro*. Maximova et al. (2006) reported that transgenic cacao plants (*Theobroma cacao* L.) overexpressing a cacao chitinase gene (*ThChiI*) were more resistant to *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. than controls. Distefano et al. (2008) reported that transgenic lemon plants with *chit*24 gene from *Trichoderma harzianum* showed less lesion development than control plants inoculated with *Botrytis cinerea*. Jia et al. (2010) transferred chitinase gene *Bbchit1* from *Beauveria brassiana* (Bals.-Criv.) Vuill. to Chinese white poplar (*Populus tomentosa* Carr.) and reported improved resistance to *Cytospora chrysosperma* (Pers.) Fr. *in vitro*.

The introduction of chitinase genes to silver birch and increased resistance to fungal diseases was reported by Pappinen et al. (2002). A sugar beet chitinase IV gene in transgenic silver birch increased resistance to *Pyrenopeziza betulicola* Fuckel in inoculation tests in controlled conditions. In later tests in the field the increased resistance to *P. betulicola* was no longer detectable, but the lines with high or intermediate sugar beet chitinase IV expression showed more resistance to birch rust (*Melampsoridium betulinum* (Pers.) Kleb.) than the lines with low expression (Pasonen et al. 2004). Pasonen et al. (2005) studied the effect of sugar beet class IV chitinase constitutive expression in transgenic silver birch in mycorrhizal symbiosis with *Paxillus involutus* (Batsch) Fr. They reported formation of a normal ectomycorrhizal symbiosis *in vitro*.

1.5 Potential risks of transgenic trees

The propagation of genetically modified (GM) crops and forest trees has raised questions of the possible risks involved in the use of transgenic plants (Devos et al. 2008, Strauss et al. 2009). Hoenicka and Fladung (2006) defined the biosafety term as ‘the prevention of large-scale loss of biological diversity and integrity due to human activities and reviewed the results of groups working in biosafety research and risk avoidance with forest trees. They included the use of exotics and bred trees in addition to the genetically transformed trees into the threads facing forests: biological invasions, horizontal and vertical gene transfer and effects on other organisms. From the tree-breeder’s point of view the stability of the expression of the transgene, its effect to growth related parameters and the possibility of

transgene escape (Ahuja 2009, 2011) are questions that have to be tested in field trials. Burdon (2003) discusses the operational use of GM trees and suggests critical examination of several economic aspects. One aspect is how fast and effective conventional breeding would be in reaching the same objectives. Also, could the same goals be achieved more cost-effectively by alternative approaches, e.g. by silvicultural practices or by better utilization and processing. It is also important to compare the costs of producing GM trees to the costs of trees produced by traditional tree improvement. Before the commercialization of transgenic trees, there is the question of the role of intellectual property (patented genes), which may increase the costs of the use of transgenic planting material (Doering 2004). There is a large number of legislation, which regulate the use of transgenic material (Bryson et al. 2004). Burdon (2003) also discusses the 'single-gene effects', which occur when single genes of large effects are used for transformation. Such genes have not been tested by evolution and may carry unexpected side effects on field fitness. Burdon (2003) uses as an example the corn blight epidemic that occurred in the USA in 1970s after a dominant use of Texas mail-sterility germplasm in hybrid maize production.

1.5.1 Transgene stability

The stability of the transgene expression in field conditions is necessary in commercial use of GM-trees. James et al. (1995) reported stable *nos* and *npt*II gene expression after 7 years and Mendelian segregation of the transgenes in the next generation in transgenic apple. Li et al. (2008) studied the stability of two reporter genes, *GFP* and *BAR*, over time in transgenic hybrid aspen clones (*Populus tremula* L. x *P. tremuloides* Michx. and *P. tremula* x *P. alba* L.). The expression of the transgenes was observed over 3 years in the greenhouse and field conditions. The transgenic cassette was eliminated during organogenesis in low frequency (2.5%), but no gene silencing (transgene without expression) was observed. The majority of the transgenic plants (85%) had single copies of the transgenes. All the plants with two or more copies showed direct repeats in 77 % of the plants. In plants with direct repeat loci the expression was higher than in other types of configuration. Zeng et al. (2010) screened 28 transgenic birch (*Betula platyphylla* Suk.) lines transformed by *Agrobacterium*–mediated transformation with a fused *bgt* gene consisting of an insecticidal toxin gene from a spider (*Atrax robustus* O. P.-Cambridge) and the C terminal of the *CryIA(b)* gene from *Bacillus thuringiensis* Berliner. They reported that the transgene copy number varied from one to four and indications of rearrangement or partial deletion was found in 68 % of the lines. Sequencing revealed deletions of 19−589 bp and one 45 bp filler sequence at the insertion sites and altogether 89% of the lines contained vector backbone DNA. Smolka et al. (2010) reported a stable expression of *rolB* gene in apple under field conditions for several years. They found no translocation of the transgene or *rolB* mRNA in the scion cultivars. Jarošová et al. (2011) monitored over four years in the field conditions the stability of post-transcriptional gene silencing in transgenic *Prunus domestica* L., which contained the plum pox virus (PPV) coat protein gene. They found that the transgenic line remained uninfected while wild-type trees had severe symptoms. Pons et al. (2012) monitored eight lines of two transformed citrus types (*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf. and *Citrus sinensis*). The integration and expression of two marker genes

*uid*A and *npt*II were confirmed after 7-years in the field and no rearrangements and/or silencing of the transgenes or its impact on relevant agronomic or phenotypic characteristics were observed.

Pathogenicity tests carried out in controlled environments have been reported to enhance fungal disease resistance in transgenic trees (Bolar et al. 2000, 2001, Noël et al. 2005, Maximova et al. 2006, Wei et al. 2007, Distefano et al. 2008, Jia et al. 2010). Results of *in vivo* and of field trials with transgenic trees have been controversial on transgene expression stability. Tzfira et al. (1999) engineered the growth and morphology of aspen by transferring the genes *rolA, rolB* and *rolC* which code for growth hormones in *A. rhizogenes* to aspen. In contrast to *rolC*-transgenic phenotype, which is producing dwarf individuals in hybrid aspen, the transgenic plants exhibited enhanced growth and higher stem production index than the controls. The transgenic plants had axillary bud growth from the main stem following the loss of apical dominance and their leaves exhibited in some degree *rol*-related wrinkling. Also, the transgenic plants exhibited dormancy break by better rooting and axillary bud break throughout the year compared to the controls. When transferred to the greenhouse the shoot morphology of the transgenic plants was similar to that of the controls, but their winter dormancy was delayed resulting taller stems. The plants were decapitated and when they emerged from dormancy no phenotypical differences were observed among them, suggesting the silencing of *rol* genes, which was confirmed by the absence of *rolB* and *rolC* transcripts in the 3-year old plant. Wei et al. (2007) reported that in a greenhouse experiment the relative growth rate (RGR) of attenuated *barstar:barnase* transgenic poplars had linear association with the ratio of cytotoxin barnase, used for engineering plant sterility, and barstar, a specific inhibitor of barnase and that the transgenic trees grew at the same rate with the controls. When the trees were tested in the field, the growth of the transgenic trees was reduced at a rate associated with the *barstar:barnase* expression ratio. Hu et al. (2001) reported that the insect-resistant transgenic poplars expressing a *Cry1Ac* gene were still resistant after 3 years in the field. Vishnevetsky et al. (2011) reported that endochitinase *ThEn-42* gene from *T. harzianum* expression together with the grape stibene synthase (*stsy*) gene and expression in transgenic banana, perennial monocotyledon, produced several lines with improved tolerance to Sigatoge leaf spot (*Mycosphaerella fijiensis* M. Morelet) in a 4-year field trial. The reason for the differences in transgene expression in the field compared to the expression in the controlled conditions was speculated by Meilan et al. (2004) and they concluded that the environmental factors affecting the transgene expression were far greater in the field than in the controlled conditions causing differeces within lines. However, they concluded that consistent differences between lines from year to year were of greater importance.

Sugar beet endochitinase IV transgenic silver birches were tested in pathogenicity tests in laboratory conditions for their resistance to leaf spot *Pyrenopeziza betulicola*. All the lines with high degree of sugar beet chitinase IV mRNA were resistant (Pappinen et al. 2002). When the same lines were tested in the field, the transgene expression was still high after three years, but the lines were not significantly resistant to the leaf spot (Pasonen et al. 2004).

1.5.2 Transgene escape

The possibility of transgene escape from a cultivar to wild relatives by pollen/seeds depends on the reproductive compatibility of the plants (Chapman and Burke 2006). Burke and Rieseberg (2003) studied transgene escape effects by back-crossing oxalate oxidase (*OxOx*) transgenic *Helianthus annuus* L. with wild sunflower. The transgene was introduced to increase resistance against *Sclerotinia sclerotiorum* (Lib.) de Bary. The back-crosses were planted in the field in three locations and half of the plants were inoculated with white mould. They found out that the transgene had effect on the likelihood of infection, but if the plants were infected the disease had a severe input on the seed production. They concluded that in their experiment, where they had given the wild sunflower some degree of resistance that it already naturally had, the transgene would have diffused neutrally after the escape. Kuparinen and Schurr (2007) used a modelling to study the possibility of transgene escape from GM-forest. They found out that the transgene escape is dependent of the modified trait transgene expression and the genotype used for transformation, and that the heterozygosity of the GM-tree reduces the transgene escape risk. In later study they suggested coupling the transgene with 'mitigation transgene', which is selectively disfavoured in natural populations in order to lower that escape risk (Kuparinen and Schurr 2008).

The risks of horizontal transgene escape from chitinase transformed silver birch to other species were studied by Lohtander et al. (2008). They compared the sequences from silver birch EST-library and studied their phylogenetic relationship to other chitinases. They found out that some bacterial chitinases grouped together with class IV chitinases in plants, indicating that some bacterial chitinases may have evolved from eukaryotic ones following horizontal gene transfer. They concluded by the results that this transfer has occurred only once and the likelihood of a similar event from chitinase transgenic silver birch is extremely low. The transgenic containment traits for mitigating gene flow from GM-trees to surrounding populations include induced prevention of flowering in transgenic silver birch (Lemmetyinen et al. 2001, Lännenpää et al. 2005).

1.5.3 Transgene effect on yield

The improved growth of trees has been one of the objectives in the genetic transformation of trees (Herschbach and Kopriva 2002). Shani et al. (2004) reported enhanced growth of transgenic *Populus tremula* overexpressing *Arabidopsis thaliana* (L.) Heynh. endoglucanase tested in a greenhouse. Also, the growth rate of *ugt* and *acb* transgenic *Populus tremula* has been reported to be higher than controls after 3 years in the field (Salyaev et al. 2006). They concluded that the enhanced growth was due to higher IAA production in the transgenic trees due to the transformation. Hjälten et al. (2012) reported that the increased resistance of Bt (*Bacillus thuringiensis*) toxin transgenic hybrid aspen to *Phratora vitellinae* L. lead to increased growth of one transformed line in an experiment under controlled conditions. They concluded that the result depended of better insect resistance and manifested only under herbivore pressure. Jing et al. (2004) reported that in a field trial transgenic hybrid poplars expressing a pine glutamine synthase transgene outgrew the control plants.

The transgene expression has been reported to have negative effect on yield, such as reduced growth vigor in apple (Bolar et al. 2000). Smolka et al. (2010) reported that the *rolB* transgenic rootstock reduced significantly scion growth, flowering and fruiting in transgenic apple. Taniguchi et al. (2012) reported that the constitutive overexpression of xyloglucan *AaXEG2* from *Aspergillus aculeatus* Iizuka in transgenic poplars (*Populus alba*) resulted in reduced aboveground biomass in a 4-year field trial. In addition to the growth reduction the transgenic poplars had reduced root sucker growth.

Pasonen et al. (2008) studied the growth of chitinase transgenic silver birches and reported that the transgenic trees were shorter and showed symptoms of physiological stress more than the control trees. They suggested that other factors than the chitinase transgene were responsible for the lower growth in transgenic trees.

1.5.4 The effect of the transgene to non-target species

Several GM-crops have been transformed to be resistant to herbicides or pesticides. Powles (2008) listed cases where transferred glyphosate resistance gene had been reported to cause herbicide resistance in local USA weed populations in areas where transgenic crops (soybean, cotton, maize and canola) are cultivated using glyphosate for weed control. It has been suggested that similar evolution of pesticide-resistance herbivores is possible in contact with GM-plants (Chapman and Burke 2006). Also, chitinase transgenes, aimed for improved fungal disease resistance in plants, have been reported to favour (to have positive effect on) herbivores. Saguez et al. (2005) reported a 1−1.5 days reduction in population's doubling time in peach-potato aphid, *Myzus persicae* Sulzer feeding on insect (*Phaedon cochleriaceae* F.) chitinase transgenic potato. Similarily, Post and Parry (2011) reported that one of three tested insect herbivores, gypsy moth *Lymantria dispar* L., feeding on oxalate oxidase transgenic American chestnut, *Castanea dentata* (Marshall) Borkhausen, was positively affected by the transgene and grew faster than on wild-type tree. However, Tiimonen et al. (2005) detected in controlled feeding experiments no direct association of lignin biosynthesis related *COMT* transgenic silver birch and feeding preference or growth performance of several insect herbivores (larvae of *Aethalura punctulata* D. & S., Llangattock., *Cleora cinctaria* Denis & Sciffermüller and *Trichopteryx carpinata* Borkhausen; adults of *Agelastica alni* L. and *Phyllobius* spp.). The impact of litter from chitinase transgenic silver birches to decomposing soil fauna was studied by Kotilainen et al. (2005) in controlled conditions. The results of their study was that the transgenic leaves lowered the number of nematodes, but the total decomposing rate was higher than in the control. The number of collembolans on transgenic litter was higher than in the control. They found that the transgene had no effect on the growth or survival of juvenile woodlouse (*Oniscidae* ssp.). Also, Burgess et al. (2011) detected no effects on the larvae of forest looper (*Pseudocoremia suavis* Butler) or its parasite *Meteorus pulchicornis* (Wesmael) feeding on *npt*II-*leafy* radiata pine. Vihervuori et al. (2008) evaluated the effect of sugar beet chitinase IV transgenic silver birches to the density and composition of insect communities in a field trial with control and wild-type trees (seven non-transgenic clones, see publications III and IV). They found out that the insect density was higher in transgenic trees than in the control and in the wild-type trees. The most common leaf insect was birch aphid, *Euceraphis betulae* Koch, which preferred the transgenic trees to the wild-type trees,

but no effect on the biodiversity of the insect communities was detected. Pasonen et al. (2005) studied in laboratory the effect of transgenic silver birch to the mycorrhizal symbiosis with *Paxillus involutus*. They found no effect of sugar beet chitinase IV gene expression in transgenic silver birch to the mycorrhiza formation compared to the control.

1.6 Variation

Several terms are used to describe biological variability, i.e. genetic diversity, biological diversity or biodiversity, variation and phenotypic plasticity. Genetic diversity is used for genetic variation within species and biological diversity implies usually the species richness in an ecosystem. Variation describes either heritable (genotypic, additive, dominance and interaction components) or inheritable (environmental) differences on individual or population level (Falconer and Mackay 1996). The heritable variation in a population decreases when close relatives mate (inbreeding depression). The following reduced vigour in the progeny is explained by the increased homozygous condition of the alleles in a given locus and, hence, the expression of the deleterious recessive genes (Falconer and Mackay 1996, Wright 1976). Clonal reproduction lowers the genetic variation in a population even faster than inbreeding. The opposite of inbreeding depression is heterosis (hybrid vigour) which occurs when non-related individuals mate and the heterozygous state of alleles is higher in the progeny than in either of the parental genotypes. Phenotypic plasticity was defined by Bradshaw (1965) as 'shown by genotype when its expression is able to be altered by environmental influences', and later by de Kroon et al. (2005) as a plant's response at a sub-individual level to the environment.

1.6.1 Somaclonal variation

The term 'somaclonal variation' was given by Larkin and Scowcroft (1981) for the variation observed in plants regenerated by plant cell cultures. Somaclonal variation is reviewed in several publications i.e. by Larkin and Scowcroft (1981), Kaeppler et al. (2000), Rani and Raina (2000), Bairu et al. (2011). Gymnosperm cell cultures are considered to be more stable than angiosperm cell cultures, but somaclonal variation has still been observed in several conifer species (rewieved by Ahuja 1998). Ahuja (1998) lists the types of somaclonal variation observed: variation in phenotypic traits, chromosome number and structure, and in biochemical traits in plant tissues/regenerates derived from protoplasts, cells, anthers or other tissue types. Rani and Raina (2000) reviewed the reported somaclonal variation in meristem-derived micropropagated plants and found variation in plant morphology, in chromosome number and structure, in genome size, in proteins and isoenzymes and in nuclear and organelle genomes.

The genetic changes observed in connection with somaclonal variation include autopolyploidy, aneuploidy, deletions and point mutations (Ahuja 1998). Aderkas et al. (2003) reported varying chromosome numbers in larch (*Larix decidua* Mill.) derived from megagametophytes and maintained up to 17 years as embryonic cultures. Both chromosome number and DNA content had stabilized around 24 chromosomes in most lines, but a few lines still had both lower and higher chromosome numbers, varying from 12

to 48. Burg et al. (2007) reported continuous mutations in four nuclear SSR loci during Scots pine somatic embryogenesis. The karyotype changes caused by tissue culture are reviewed by Larkin and Scowcroft (1981) with speculation of selection against gross karyotype changes during plant propagation. Rani and Raina (2000) summarize in their review the factors causing somaclonal variation as culture method and environment, explant source, genotype and ploidy level and the time of *in vitro* culture. They suggest that cultures initiated from callus, cells and protoplasts are genetically less stable than single-node cultures, regeneration of adventitious buds/shoots and somatic embryogenesis, as organized meristems are considered to be genetically stable and immune to changes that may arise *in vitro* cultures during cell division or differentiation. They conclude from literature that the tissue culture conditions act as a stress environment where for ex. the presence of hormones and the release of cytotoxic products induce a loss of cellular control.

The term epigenetic is used for changes in gene expression or in plant habitus, which are transferred to the next cell generation but not caused by changes in the DNA sequences. An example of epigenetic changes is tissue differentiation during plant morphogenesis, when − depending of the developmental stage of the plant − some genes are activated and others are inhibited. This regulation of gene functions is suggested to be a combination of DNA methylation, histone modifications and functions of small RNAs (Turner 2007). It is suggested to be responsible of a type of adaptive phenotypic plasticity, which is triggered by external stress (e.g. temperature, day length) and is preserved as an epigenetic memory affecting DNA replication, recombination, repair and gene expression (Yakolev et al. 2010, 2011, Fisher and Franklin 2011). Rani and Raina (2000) reviewed genetic fidelity in micropropagated plants, and DNA methylation has been suggested to be one of the causes that explain the changes occurring *in vitro* conditions, such as gene silencing and inactivation of transposons.

1.6.2 Variation in transgenic lines

The variation found in transgenic plant lines can be listed in four categories (Ahuja 2011): 1) transformation methodologies induced, 2) tissue culture related, 3) integration patterns dependent, and 4) inactivation/silencing of the transgene.

Genetic transformation may be achieved either by physical or biological means. Selected tissues are bombarded with tungsten or gold particles covered with the transgene. This method is suitable for all tree species (reviewed by Poupin and Arce-Johnson 2005). The method is relatively simple, but it has some undesirable side-effects, such as complex DNA integration and production of large number of transgene copies in the transformed plant, which generally results in different stages of tree development in transgene silencing. In the biological transformation, trees are transformed using bacteria, such as *Agrobacterium tumefaciens*, which is currently the favoured method for genetically engineering the trees, as it normally produces stable integration patterns with one or only a few transgene copies. Zeng et al. (2010) reported that 89.3% of the *Betula platufylla* Suk. from *Agrobacterium*-transformed lines had vector backbone DNA in the transformants. Also, the transgene expression may vary depending on the promoter used in the transgenic plant. The CaMV 35S promoter, used in this research to drive the expression of sugar beet

chitinase IV (*chiIV*) gene in transgenic silver birch, seems to work well in forest trees (Ahuja 2009).

Tissue culture related variation in transgenic lines is associated with somaclonal variation. The literature, reviewed by Rani and Raina (2000), reveals that several factors during tissue culture, such as the presence of phytohormones, act as stress factors inducing loss of cellular control and hence, variation in transgenic lines.

The third type of variation in transgenic lines is caused by integration patterns of the transgene. Zeng et al. (2010) studied the integration and transgene *locus* structure in *Betula platufylla* Suk. from *Agrobacterium*-transformed lines with a resistance gene against insects (*bgt*). They found that the copy number varied from one to four, and the T-DNA integration had caused partial deletions and rearrangements. Also, they found both deletions and filler sequences between the T-DNA repeats.

Post-transcriptional gene silencing in plants involves short RNAs that target homologous mRNA in the cytoplasm (Aufsatz et al. 2002). Most transposons are silenced post-transcriptionally. Hamilton and Baulcombe (1999) found in transgenic tomato post-transcriptional gene silencing of an endogenous mRNA, which had a close homology to the transgene. They could also purify a small antisense RNA, which was complementary to the endogenous mRNA. Distefano et al. (2008) found out that the transcription of *chit42* gene from *Trichoderma harzianum* in lemon tree induced down-regulation of the tree's own chitinase and glucanase genes.

1.7 Silvicultural and tree breeding aspects

1.7.1 History and current status of clonal forestry

The cloning of trees has been practised for centuries in China and in Japan. In the 1990s, it was estimated that of the Chinese fir, *Cunninghamia lancelota* (Lamb.) Hook., plantations in China about 5.4% of the total planting area of 400 000 ha, was planted by about 56 million stecklings (Ritchie 1991). In 2009 the number of rooted cuttings of Chinese fir produced in China was 65 million. (Aronen 2011). In the 1980s the production of sugi, *Cryptomeria japonica* D. Don, stecklings in Japan per year was close to 30 million, roughly 25% of the total sugi production, which amounted in 1985 to 41.4 million stecklings (Ritchie 1991, Minghe and Ritchie 1999a, 1999b,). The production of *C. japonica* in 2009 was 17 million stecklings (Aronen 2011). In Finland the Foundation for Forest Tree Breeding started experiments with rooted cuttings in 1962 with several conifer and broad-leaf tree species, but the interest shifted in the 1970s to Norway spruce (Mikola 2009). Clonal forestry was discussed in 1973 in the Vegetative Propagation Meeting in Rotorua, New Zealand and the New Zealand Journal of Forestry Sciences published a special issue on vegetative propagation in 1974 in which the existing clonal projects were covered world-wide. Only in three countries the production was over 100 000 rooted cuttings annually, sugi in Japan 120 million (Toda 1974), Norway spruce in Germany 1 million (Kleinschmit 1974), and Norway spruce in Finland 150 000 (Lepistö 1974).

The commercial use of conifer cuttings was reviewed by Ritchie (1991). Relatively large clonal forestry programmes were implemented in New-Zealand and Australia, Africa,

South-America and Malaysia. In New Zealand approximately 25% of the radiate pine nursery plants used in plantations was produced by cuttings and tissue culture. In 1998, the total radiata pine planting area in New Zealand was 81 100 ha (Menzies et al. 2001). The annual production of radiata pine rooted cuttings was 600 000 in New Zealand and 9.5 million in Australia (Ritchie 1991). The production of rooted cuttings of radiata pine in New Zealand for forestry had increased in 2009 up to 20 mills. a year and 2 million stecklings were produced by somatic embryogenesis (Aronen 2011). In Australia the production of pines by stecklings in 2009 was 6 mills. (Aronen 2011).

In North America (USA and Canada) programs of the rooted cuttings included in 1991 several conifer species, *Picea abies* with annual production of 100 000 cuttings, *Picea mariana* (3.1 million), *Chamaecyparis nootkaensis* Spach (650 000), *Pinus taeda* (100 000) and *Pseudozuga menziesii* (Mirbel) Franco (1 million), which were produced for several objectives. The spruce cuttings were produced mainly for clonal orchards, for bulking up seed sources and for plantations. The Alaskan yellow cedar was cloned by rooted cuttings for restoration purposes, loblolly pine from improved families in breeding programmes to be applied in combinations with recurrent selection aiming for fusiform rust resistance and production traits and Douglas fir from tested full-sib families to bulk up the best performing orchard seeds (Ritchie 1991). The total production of spruce cuttings (*Picea mariana, P. glauca, P. abies*) and larch hybrids for forestry in Canada was in 2009 4−10 mills. a year and the number of spruce and pine emblings varied from 1.2 to 2 million annually. In the USA the number of *P. taeda* planting material produced by somatic embryogenesis was 10−30 mills. (Aronen 2011).

In Scandinavia and Finland, the production of rooted conifer cuttings, all Norway spruce (*Picea abies*), was in 1991 approximately 8 million. In the United Kingdom and Ireland approximately 5.1 million Sitka spruce, *Picea sitchensis* (Bong.) Carr. and 1 million hybrid larch were produced from seed orchard seedling stock plants, with the age up to 6 years. In 2009 the number of *Picea sitchensis* stecklings produced in the United Kingdom was 7 million, and 3 million stecklings were produced in Ireland by somatic embryogenesis (Aronen 2011). In Germany, France, and Belgium the estimated annual production of Norway spruce, maritime pine *(Pinus pinaster* Ait.) and hybrid larch rooted cuttings was 2.1 million. In Eastern Europe and Russia the production of rooted Norway spruce cuttings was estimated to be annually 200 000 (Ritchie 1991).

Leakey (2004) reviewed the clonal use of hardwoods in the tropics. The production of fast growing hardwood *Eucalyptus* spp. had been increasing and in Australia the first clonal *Eucalyptus* plantations were established in 1994. Today, the Australian Government sector produces about 13 million *Eucalyptus* cuttings in mist houses and the total clonal plantation area in 2012 was 40 400 ha (http://apfdc.apts.gov.in/APFDC-Eucalyptus-Plantations.htm). Clonal production of *Eucalyptus* spp. has been implemented outside Australia, i.e. in China, India, South Africa and Chile (Leakey 2004). In Africa, in the People's Republic of Congo, where the total planting area of *Eucalyptus* in 2003 was 42 000 ha and the production of planting material was 600 000 cuttings/year (Leakey 2004). In South-Africa the production of pine stecklings in 2009 was 5 mills. (Aronen 2011). In South America, in Brazil the rooting of Eucalyptus cuttings was started by a company, which owned in 1999 plantations of 132 147 ha for bleached wood pulp (Leakey 2004). In 2009 the number of *Pinus taeda* and *Pinus radiata* stecklings produced by somatic embryogenesis in Brazil and Chile was

70 100 mills. (Aronen 2011). The vegetative propagation of *Acacia senegal* (L.) Willd. was studied by Badji et al. (1991) and Danthu et al. (1992) after the recurrent droughts in Sahel in the 1970s. In 1981, a breeding project of *Gmelina arborea* Roxb. combined with clonal plantations was started in Malaysia and in the same year 500 000 cuttings were planted from 3000 selected clones (Leakey 2004). The clonal production of trees in large scale is carried out by companies, i.e. Eucalyptus production in Brazil, that does not publish the number of cuttings used annually for planting. However, the intensive wood production and the large planting areas for ex. in China and South America suggest that the production of rooted cuttings is millions if not billions every year.

Mikola (2009) reviewed the situation of forest tree cutting production in Finland. The research, which had started in the 1960s continued in the 1990s in the Foundation of Forest Tree Breeding. Salonen (1994b) reported cytokinin spraying of young Scots pine, contorta pine and Norway spruce for the elongation of dwarf shoots for cuttings rooted with or without auxin treatment. Also, micropropagation experiments of Scots pine, Norway spruce and hybrid larch were carried out and regardless of the reported lack of success, about 100 micropropagated Norway spruce plantlets were planted in the progeny test. The best results of conifer micropropagation were achieved by hybrid larch (*Larix leptolepis* (Siebold & Zucc.) Gordon x *L. sibirica* Ledeb., *L. decidua* Mill. x *L. leptolepis, L. leptolepis x L. decidua*) and about 200 plantlets were rooted. Also, Salonen (1994b) reported the micropropagation experiments of silver birch special forms and the number of rooted plantlets in 1991−1993 was about 58 000. At the same time, lime tree (*Tilia cordata* Mill. and *T. vulgaris* cv. Pallida), hybrid aspen (*Populus x wettsteinii* Janch.), hybrid alder (*Alnus incana* (L.) Moench x *A. glutinosa* L.) and a special form of alder (*A. incana* var. *gibberosa*) were cloned by micropropagation and about 1000 rooted lime trees, 1500 alders and 800 hybrid aspen were produced (Salonen 1994b). The micropropagated hybrid aspen and alders were ordered and forwarded to a Norwegian company. Also another cooperation project with commercial sector was carried out in Haapastensyrjä tree breeding station during 1995−1996 involving clonal production of hybrid aspen for Mesäliitto-yhtymä (Lepistö et al. 1996a, 1996b, 1996c). By the spring of 1996, the production of hybrid aspen in the project was about 5000 and after that no production numbers were reported. Also other companies were interested in clonal material in Finland in the 1990s. Hagqvist (1991) reports the production of 500 000 micropropagated birches annually by Enso-Gutzeit Oy and the start of production in another company, Metsätyllilä Oy. Jokinen (1991) reports the optimization of micropropagation methods for silver birch elite trees and somatic embryogenesis of Norway spruce in Kemira Oy.

Today, the marketing of forest reproductive material, including clones, is regulated in the European Union by Coucil Directive 1999/105/EC (2000). The tree species with registered clones in Finland are *Populus tremula* (3 clones), *P. tremula* x *P. tremuloides* (34 clones) and *Betula pendula* var. *carelica* (31 clones). Hybrid aspen is planted for the material production for paper industry and about 50 new clones were in field tests for registration (Koivuranta et al. 2008). There are no registered clones of Scots pine in Finland, and the registered silver birch clones are curly birch, suitable for timber production for special purposes.

In the future, the goals in breeding programmes are expected to remain mainly economical, but the predicted climatic warming has already directed long term breeding

efforts towards wider climatic adaptation to ensure production in future conditions. Wider adaptation includes expectations of better disease and insect resistance of trees and, in short, more evolutionary flexibility, i.e. adaptability to a wide range of environmental conditions (Haapanen and Mikola 2008). How do the tree breeding objectives and cloning of trees fit together?

1.7.2 Lowered genetic diversity in clonal forests

Clonal plantation forests are planted with identical genotypes – how many, depends on the number of clones on a plantation site. The suitable number of clones has been debated and basing on earlier analyses and models by Roberds and Bishir (1997) suggested that 30 to 40 unrelated clones provide similar protection against catastrophic events than a large number of clones. Muona and Harju (1989) studied the genetic variation of Scots pine comparing natural stands and clonal seed orchards and they detected no difference in variation at allozyme loci or in inbreeding level in spite of decreased effective population size in clonal seed orchards. Inbreeding depression occurs in the nature either by self-pollination or when related individuals with copies of a gene from a common ancestry cross and produce offspring with genes which are identical homozygotes, i.e. carry identical alleles (Falconer and Mackay 1996). This increased homozygosity can be harmful if the alleles are recessive, detrimental mutations or if the alleles are at loci with overdominance, i.e. in which heterozygosity is advantageous (Charlesworth and Willis 2009). Similarly, the consequences of the mating of clonal trees may be observed in the following generations, where the effects of inbreeding may be lowered fertility, survival, and growth rate (Chrleswoth and Willis 2009).

1.7.3 Potential of inbreeding in Scots pine and silver birch populations

Inbreeding depression in the natural populations of Finnish Scots pine is generally low due to efficient mechanisms, which eliminate selfed seeds (Kärkkäinen and Savolainen 1993). The location of male and female flowers in trees and the timing of flowering (spatial and temporal isolation) limit the amount of self-pollination. However, the self-pollination level in Scots pine in Finland has been estimated to be 10 – 25 % (Sarvas 1962). The number of lethal recessive genes in Scots pine was estimated by Kärkkäinen et al. (1996) vary from 6.3 to 8.0 in general and from 3.0 to 20.0 per tree with less lethals in the northern than in the southern populations, which may indicate more frequent self-pollination in the north (Kärkkäinen et al. 1996). The lethal recessive genes act after pollination and cause embryo abortion and empty seeds in Scots pine (Wright 1976). Inbred embryos are usually eliminated by genetic causes at an early stage of embryo development (Kärkkäinen and Savolainen 1993). Polyembryony in Scots pine diminishes the abortion rate at the later stage of embryo development as competition between embryos, either selfed without lethal recessive genes or out-crossed, allows natural selection without lowering the seed set. Scots pine (Kärkkäinen and Savolainen 1993, Koelewijn et al. 1999) had differences in the amount of inbreeding depression among families and individuals.

Silver birch, unlike Scots pine, has an effective incombatipility mechanism, which prevents the growth of pollen tube in the same genotype pistil's stigma. Inbreeding is not a

problem in the natural stands, but the incompatibility mechanism is not complete and allows selfing for ex. in greenhouse. Wang et al. (1999) studied the inbreeding depression in three selfed generations of silver birch in a field trial of 13-year-old trees. They compared the survival and growth related traits of selfed progenies to the out-crossed controls and found out that the inbreeding depression in growth related characteristics increased in every inbred generation, but the low survival rate was achieved in the first selfed progeny and maintained thereafter in about the same level (Wang et al. 1999). Like Scots pine also silver birch (Wang et al. 1999) had differences in the amount of inbreeding depression among families and individuals.

1.7.4 Inbreeding and tree breeding programmes

The use of inbreeding in tree breeding programmes by crossing inbred lines is expected to create improved vigour in growth, heterosis. However, when well-adapted populations cross they fail sometimes to show positive heterosis, which is explained by epistatic interaction (Falconer and Mackay 1996). Wang et al. (1996) crossed selfed lines of silver birch and compared in a field trial the growth related parameters of the progeny to the parental inbred lines. They found that the degree of inbreeding in the parental generation correlated with the heterosis in the progeny, indicating that the inbred material could be used to improve the yield in silver birch. Park and Gerhold (1986) estimated heterosis in Scots pine population hybrids. They found that, even if the progenies were less inbred than the parent populations, the heterosis effects found were positive and negative, mostly non-significant and suggested that the explanation could be that individual Scots pine populations maintain a number of favourable linkages, which are lost by hybridization.

1.7.5 The use of clones in tree breeding programmes

The objective in tree breeding programmes is to increase the mean breeding value of important traits, such as stem volume, wood properties and disease resistance in the breeding populations. Breeding value is a measure of a single genotype as a parent determined by the mean value of its offspring (Falconer and Mackay 1996). The increased productivity, which follows changes in gene frequencies, is called genetic gain (Wright 1976). It usually follows genetic improvement and can be calculated from the progeny as yield increment. The ability of a parent to transmit its excellence to the offspring in crosses with other parents in general is called general combining ability, which is half of the breeding value. Tree breeding programmes base on selection of genotypes by their general combining ability, which means that the selected trees have been tested in half-sib progeny tests and the parents which have transferred their good qualities to the progeny are selected. Sometimes trees are tested in full-sib progeny tests, which reveal the specific combining ability, i.e. the average of the full-sib family is better than the breeding value of both of the parents (Wright 1976). The candidate trees for breeding population, plus trees, are selected phenotypically, tested in progeny (or clonal) tests for their breeding value and the best parent genotypes are selected to production populations, seed orchards, for seed production. The trees in breeding population are crossed in controlled crosses and the offspring of the

best tested parents form a new population from which the candidates for the next breeding cycle are selected (Haapanen and Mikola 2008).

The use of clones in breeding programmes for genetic testing is expected to be more efficient than the use of seedlings. The properties of an elite tree can be transferred directly to clonal material, and the testing cycle is shortened as the time needed for the flowering of the seedling candidates and the propagation of the progeny can be saved in clonal tests (Haapanen and Mikola 2008, Haapanen 2009). Further advantages of the use of clones are the possibility to test the genotypes in different environments and use even destructive treatments in tests (Haapanen 2009).

Isik et al. (2005) estimated genetic gain from clonal selection of four full-sib families of loblolly pine grown in two locations. The genetic gain was 27% and 31% in the two locations respectively when the best eight clones were selected regardless of their relationship to each other. When the best clones of each four families were selected the genetic gain estimation was only slightly lower. They observed that the genetic gain for volume decreased when the number of selected clones was increased using either of the selection type. In an earlier experiment Isik et al. (2004) compared genetic gain estimates for loblolly pine using seedling and clonal testing in polycross breeding and testing for general combining ability followed by controlled crosses for within family selection. They found out that the clonal testing of full-sib families increased the expected genetic gain for volume faster than seedling testing.

Baltunis et al. (2007) studied the genetic gain of two positively correlated traits of loblolly pine, rooting ability and early height growth, when four selection intensities were used. Both traits showed large clonal variation and, as they were positively correlated, selection of either of them was expected to bring gain in the other. They selected both for rooting ability and early growth the best half-sib and full-sib family, best clone from the 10 best full-sib families and the best overall clone. They found out that the best option in selection for a single trait was the single best clone, but the best overall clone selected for rooting ability resulted negative genetic gain in early height growth. Also, they discovered that the selection of best clones of half- and full-sib families tended to bias to the same families and produce related clones. However, they recognized the problems of undesirable lowering of genetic diversity arising from the use of related clones or single clones in plantation and selected the best clone from each 10 best families for the two traits. By using this strategy they doubled the genetic gain which they had achieved by using the single best full-sib family.

Weng et al. (2009) simulated three testing and deployment strategies for white spruce [*Picea glauca* (Moench) Voss] and black spruce (*Picea mariana*): seedling tests with clonal seed orchards deployed as seedlings and two clonal tests, one with clonal seed orchards deployed as seedlings and the other deployed as clone mix. The breeding populations were formed using balanced within-family selection and the production populations had no common parents. They reported faster accumulation of additive effects combined with loss of additive variance in the breeding population when clonal tests were used, resulting higher gain in clonally tested production populations. The most effective strategy was the use of clone mix and the gain increased with generations. They concluded that the use of clonal tests for selection and clonal deployment in spruce breeding will maximize genetic gain (Weng et al. 2009).

Lindgren (2009) suggests the use of a novel breeding strategy called 'breeding without breeding (BwB)', which relies on wind pollination in clone collections followed by molecular marker based paternity tests instead of controlled crosses of the best clones followed by progeny tests and selection in Norway spruce breeding programmes. Shimono et al. (2011) tested the pedigree construction of seeds collected from wind pollinated Norway spruce clone collection using eight microsatellite (SSR) loci as markers, as suggested in the BwB breeding strategy. They reported that 42 % of the fathers of the progeny could be assigned with high confidence within 30-m distance from the mother tree and that the close neighbour (within 6 m) mating was in average 13 % of the seeds. Also, they reported that 57.5% of the pollen was outside the sampling block and the self-pollination rate was 0.5%. It is suggested (Lindgren 2009, Shimono et al. 2011) that BwB strategy could be used in tree breeding programmes for easily clonable species. The benefits would be both economical and time saving, as the breeder saves the time between selection and recombination, there is no need for controlled crosses and the irregularity of flowering is not a problem in clonal testing. The starting point for BwB breeding programme would be to establish a clonal test and wait for it to flower. The clones are measured and the best clones selected for seed collection to establish the next generation (Lindgren 2009).

In traditional breeding programmes the breeding populations and production populations are kept separate, but Lindgren (2009) suggests that another option for the use of BwB strategy is to place ramets from breeding population to seed orchard in the hope of combining the whole population. The seeds are collected from the extra ramets, fathers identified and genotypes selected and cloned for the next recruitment population. Shimono et al. (2011) calculate that Norway spruce BwB strategy can be executed with 25% of the annual costs of traditional breeding, as there is no need for grafted clones, clone archives or controlled crosses. Also, the breeding cycle will be 25% shorter than in the traditional breeding programme, which would compensate the lowered genetic gain from the male side.

The use of clones is already included in the Finnish Norway spruce breeding strategy, where according to the breeding plan, the 2nd generation candidates, selected either from full-sib families or progeny trials, undergo clone testing (Haapanen 2009). Also, the clonal field tests are included in the hybrid aspen breeding programme. Scots pine and silver birch have both long breeding cycles, 40 and 30 years respectively. The clone testing would be a good option to shorten that in both cases (Haapanen 2009). Cloning methods for Scots pine, basing on rooted cuttings, are being optimized for further use (Högberg 2005, Högberg et al. 2011). Silver birch has properties that make it easier to breed than pines, such as good flowering and seed production, fast seedling growth and early greenhouse flowering (Viherä-Aarnio and Velling 2008) and the breeding of silver birch is further advanced than the breeding of Scots pine (Haapanen and Mikola 2008). Also, the functioning micropropagation methods for birch make it a good candidate for clonal propagation and gene transfers. In the late 1980s and early 1990s, the silver birch micropropagation was expected to be a viable propagation method, but the high costs soon ended the commercial production (Mikola 2009).

1.8 Paradigm shift

The forests represent in the minds of the Finns a common natural resource, which exists in a stable world and is accessible for all. The trees have adapted in their natural environment to local temperature, photoperiod, soil conditions, pests and diseases. During the last decades, we have encountered a new situation, as the climate change caused by air pollution and increase in atmospheric carbon dioxide has become a reality. How will that affect forests, how soon will the effects become visible and how can we counteract future forest damages? Can breeding programmes produce adapted trees for the future forests?

If the scenarios of predicted 1.0–5.5 ºC temperature rise and 40% of rainfall increase by the end of the 21st century in North-Europe, become a reality, our presently existing forests will meet not only difficulties with phenology, but with new pests and diseases and harsher climate conditions. The Finnish annual temperature rise predictions are 2–6 ºC by the end of 2100 which includes the temperature increase of 3–9 ºC in the winter and 1–5 ºC in the summer (Parviainen et al. 2010). The expected impacts to the forests are 1) extended growing season with species dependent growth increase up to 20–50%, 2) increased risk of some wind damages – Finland's geographical location is sheltered from south-western storms, 3) higher risk of pathogens and insects, 4) species invasion from the South and 4) the timberline shift to the North in the long run (Parviainen et al. 2010).

The adaptation of natural forests to the predicted climatic warming is not fast enough to become materialized in decades and the traditional breeding methods, basing on crossing and selection, are too slow to produce adapted seedlings locally. The future forests in Finland during and after the climatic warming may not be very different from the present, only the trees may originate from areas which are 200−300 km south of Finland. However, the new breeding methods − gene transfers, molecular assisted selection, clonal trials and the use of cloned planting material – may become necessary in securing the productivity of the future Finnish forests. In that case the forests themselves may look different from the present as cloned trees are used for plantation forestry and the surviving natural forests serve as gene banks where genetic variation is maintained.

1.9 Aims of the study

The use of clones is restricted by the absence of good cloning methods suitable for Scots pine and more research is required to develop a functional propagation method. Also, the public opposition against clonal forestry whether it uses genetically modified trees or not, is strong. In case of transgenic planting material, there is an overall fear that transferred genes will escape from the transformed trees and create environmental risks and that genetically uniform stands are liable to suffer from pests and diseases. The predicted climatic warming will put tree breeders in a new situation where the traditional breeding methods may not be fast enough to produce suitable planting material. Therefore, new breeding methods – including cloning and gene transfers – may offer a functional tree breeding option in the future.

In this study, the aim was first to examine the variation of clones and the factors responsible for that variation and to assess the effect of a single gene transfer in the field performance of clones. The hypothesis was that the variation in clonal material is affected by parental genotypes and stress and that the single gene, *chiIV*, used in this experiment in silver birch has very little or no unexpected side-effect on the genotype's behaviour. Two model species were used: Scots pine (gymnosperm, conifer) and silver birch (angiosperm, broad-leaf deciduous tree), both economically important forest trees and the hypotheses were tested by:

1. Study of the factors affecting the variation in the success of Scots pine somatic embryogenesis and silver birch field trial.
2. The field performance of Scots pine clones compared to seedlings of the same origin in a field trial.
3. The effect of transferred gene to Silver birch mycorrhizal community structure and clonal variation of the adaptive traits.

2 MATERIAL AND METHODS

2.1 Outline of the experiments and data collection

The detailed description of material and methods is found in each separate original publication (I–IV). In this section only an outline of the genetic background of the clones and GM-lines, experimental design and methods are provided for each experiment indicated by roman numerals. Scots pine and silver birch (*B. pendula*) clones were propagated either *ex vitro* (II) or *in vitro* (I, III, IV). The pine clones were produced both through rooting of cuttings (II) and through somatic embryogenesis (I). The silver birch GM-lines/clones were produced through organogenesis (III, IV), initiated either from leaf discs (GM-lines and positive control) or from bud meristems (wild type clones and negative control) and propagated by axillary shoot multiplication method. The mature pine somatic embryos (I) were used for data collection and sent to tree nursery for further cultivation. The rooted pine cuttings (II), micropropagated birch clones and GM-lines (III, IV) were planted in field trials for testing the field behaviour of the cloned material.

2.1.1 Scots pine somatic embryogenesis

Seven Scots pine plus trees were pollinated in controlled crosses in a full diallele mating design. Immature cones (5–8) were collected from each family. Altogether 5264 megagametophytes from immature seeds were used for culture initiation on three different culture media: modified MSG, modified W-pat., and modified DCR20 (see publication I). The successfully initiated cultivations and the success percentages were calculated after two weeks and the cultures were transferred to maintenance culture medium, where they were

subcultured every 2 weeks and evaluated after 2 months. When the cultures were incubated in average for 6 months on maintenance medium they were transferred to maturation medium and the mature somatic embryos were counted every 2 weeks by subculture. The mature embryos were rooted on rooting medium and sent to tree nursery.

2.1.2 Scots pine rooted cuttings

Young Scots pine stock plants were sprayed with varying concentrations of cytokinin 6-benzyladenine (BA) solution to induce the elongation of the brachyblasts. After the shoot elongation all 15 mm or longer shoots were cut and treated with 0.5 mM Indole-3-butyric acid (IBA) solution for 20 hours to enhance the rooting. The rooted cuttings were planted in a field trial with seedlings of the same origin as controls. The experimental design was a completely randomized test of single ramet/seedling plots (1.8 m x 1.8 m) with 10−30 replications and 100 seedlings from each origin as controls. The height and diameter of both cuttings and seedlings was measured after 1, 2, 9, and 10 years and the stem straightness was assessed on a scale of 1−3. Relative growth rate and stem volume were calculated from the measurements (II).

2.1.3 Silver birch GM-lines and clones

The silver birch transgenic lines were planted in a three year field trial with one (III) or two (IV) controls and seven wild-type clones (III, IV). The experimental design was a complete block of randomised single ramet plots (1.8 m x 1.8 m) with 15 replications. The tree height and stem basal diameter were measured (III, IV) and relative growth rate calculated (III), and observations of fungal disease resistance (III, IV) and phenology (III) were made in the field trial. Broad sense heritabilities of growth and phenology related parameters were calculated (III). Root samples were collected at the end of the experiment. The colonization intensity of ectomycorrhizas was calculated from the root tips and the total DNA was extracted, fungal rDNA ITS region sequences amplified using GC-clamped ITS1F primer (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA-3') together with ITS2 reverse primer (5'-GCT GCG TTC TTC ATC GAT GC-3'). Fungal sequences were separated by denaturing gradient gel electrophoresis (DGGE) and identified by comparing them to public databases EMBL, DDBJ, GeneBank and UNITE to assess the root-associated fungal community structure and clonal variation between the transgenic and wild-type trees (IV).

2.2 Genetic background of the clonal material

2.2.1 Scots pine

Seven Scots pines were used in diallele crosses for production of full sib-families for the pine somatic embryo production in the spring of 1998 (I). All plus trees (P451, K738, K770, K801, K828, K1005, K1011) originated from the geographic range 66º 21'−62º 52'N, 25º 40'−30º 57' E, 120–200 m above sea level (Table 2).

Table 2. Origins of the Scots pine plus trees used in diallele crosses for pine somatic embryo production. The letter before the plus tree number indicates the latitude: K = between 62–64ºN, P = north from the 64ºN, asl = above sea level.

Plustree	Origin	Description	Selection year
K770	Ilomantsi, 62º 50' N, 30º 57' E, 150 m asl	Plustree in natural stand, very narrow crown	1962
K801	Joroinen, 62º 25' N, 27º 30' E, 120 m asl	Plustree in natural stand, narrow crown	1962
K828	Jäppilä, 62º 17' N, 27º 37' E, 135 m asl	Plustree in natural stand, conical crown	1963
P451	Kuusamo, 66º 21' N, 29º 26' E, 200 m asl	Plustree in natural stand after forest fire, narrow crown	1953
K738	Ilomantsi, 62º 52' N, 30º 48' E, 150 m asl	Plustree in natural stand, wide conical crown	1959
K1005	Äänekoski, 62º 43' N, 25º 40' E, 145 m asl	Plustree in natural stand, very narrow crown	1964
K1011	Äänekoski, 62º 42' N, 25º 42' E, 145 m asl	Plustree in natural stand, very narrow crown	1964

The seeds were collected from grafted trees in Forest Research Institute collections no. 21, 24, and 26 in Punkaharju Research Unit (60º48'E, 29º17'E). Immature seeds for the initiation of embryogenic cultures were produced by full diallele mating pattern of seven plus trees. Four of the trees were tested earlier as mothers for their capacity to produce somatic embryos (Keinonen-Mettälä et al. 1996) and for their resistance against *Gremmeniella abietina* (Terho et al. 2000).

The rooted pine cuttings in a 10-year field trial (II) were cut from young (2 or 5 years old) seedling stock-plants, which originated either from 10 genotype seed orchard seedlings (half-sibs) from Imatra (2 stock plants) and Varkaus (5 stock plants) or from a controlled cross (full-sib) of two plus trees E104 x E1101 (1 stock plant). Control seedlings were of the same corresponding origin, 100 seedlings from each.

2.2.2 Silver birch

Transgenic silver birches were engineered by transferring the chitinase IV (*ChiIV*) gene from sugar beet to 15.76 Kb binary t-plasmid pBKL4 K4 (Figure 1), which was provided

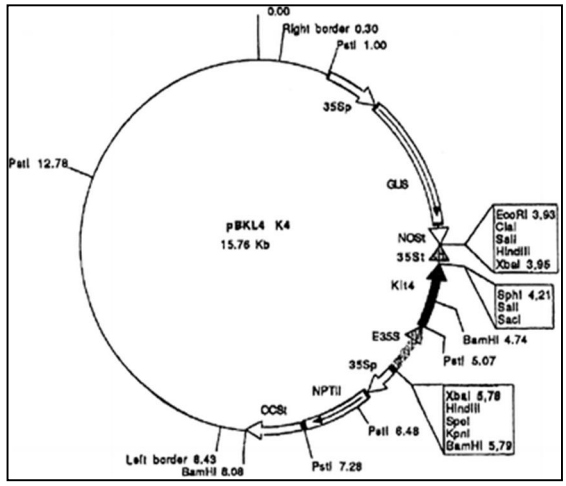

Figure 1. Binary t-plasmid pBKL4 K4 with an introduced (*Kit4=ChiIV*) gene under viral CaMV E35S (4x) promoter, an *npt*II gene for kanamycin selection and a *uid*A reporter gene coding for β-glucuronidase (GUS) (Pappinen et al. 2002).

by Dr. J. D. Mikkelsen, Danisco A/S, Denmark. The t-plasmid was introduced to silver birch clone JR1/4 by *Agrobacterium tumefaciens* (strain LBA4404) mediated gene transfer method (Pappinen et al. 2002) and 15 sugar beet chitinase transgenic silver birch lines were initiated from the leaf discs.

The plantlets were propagated by axillary shoot multiplication method. The number of inserts was confirmed by southern analysis to vary from 2 to 4 (Pappinen et al. 2002). The sugar beet chitinase IV gene expression (transcript accumulation) was assessed by northern blot analysis from leaves collected in the greenhouse (Pappinen et al. 2002) and after three years in the field from leaf samples (Pasonen et al. 2004) and from the roots (Figure 2). The total endochitinase activity was measured by fluorimetric assay for endochitinase from the roots from selected GM-lines and control after 3 years of field trial. The transformed lines were tested for resistance against *Pyrenopeziza betulicola*, but no correlation was found between the resistance and the number of insets or mRNA accumulation (Pappinen et al. 2002).

Figure 2. Northern blot analysis of the sugar beet chitinase IV gene expression level on chitinase-transgenic birch line root samples (Kit1–Kit15) and on the control clone (JR ¼) collected after three years in the field trial (III, Fig. 1).

The lines were grouped according to the expression level (Figure 2) to lines with high expression (Kit8, Kit10, Kit11), intermediate expression (Kit4, Kit12, Kit14), low or very low expression (Kit1, Kit2, Kit3, Kit5, Kit15) and no expression (Kit6, Kit7, Kit9, Kit13). Total endochitinase activity was low and only slightly higher in the seven tested transgenic lines than in the corresponding wild-type control. Only line Kit15 showed significantly ($p < 0.001$) higher total endochitinase activity than the control.

Only one genotype was used as a recipient clone in the gene transfers, a tree with code JR-1. The tree grows in Imatra (61º08'N, 28º49'E, 55 m above sea level) and it is a cross of two plus trees E1970 (selected from planted stand of Suonenjoki seeds in Kangasala, 61º26'N, 24º08'E) x E1980 (Pusula, 60º36'N, 23º57'E), both selected by Dr. Jyrki Raulo (hence the JR code) in the early 1960s. The progeny of these two parent trees was produced during 1979–1998 for the cultivation in Southern Finland for its good specific combining ability. The code JR1/4 was given to the clone initiated from the 4th bud meristem taken from the tree JR-1 by axillary shoot multiplication method.

The non-transformed controls of clone JR1/4 were initiated either from leaf discs (IV) or from bud meristems (III, IV) and micropropagated by axillary shoot multiplication method. The wild-type silver birch clones were initiated from bud meristems (III, IV) and multiplicated in similar conditions and with similar methods than the GM-lines and control clones. All the parent trees were South and Central/Eastern Finnish plus trees from the geographical range 60º30'−62º47'N, 24º25' – 30º05'E, 55 –120 m asl (Table 3).

Table 3. Origins of the silver birch plus trees used as wild-type control clones for chitinase GM-lines in the field trial.

Plus tree	Origin	Description	Selection year
E4212	Ristiina, 61º24'N, 27º24'E, 90 m asl	Plus tree in natural stand	1971
V5952	Imatra, 61º08'N, 28º49'E, 55 m asl	Open pollinated progeny of plus tree E2818 from Valkeakoski (61º12'N, 24º00'E)	1991
K2674	Eno, 62º47'N, 30º05'E, 120 m asl	Plus tree in natural stand	1988
V5834	Nurmijärvi, 60º30'N, 24º42'E, 100 m asl	Open pollinated progeny of plus tree V596 (seed orchard 288 in Loppi)	1988
V5818	Loppi, 60º39'N, 24º25'E, 120 m asl	Controlled cross of V590 (Loppi) x V554 (Rautalampi)	1985
K1316	Rautalampi, 62º30'N, 26º45'E, 120 m asl	Plus tree in natural stand	1971
V5920	Punkaharju, 61º48'N, 29º17'E, 84 m asl	Open pollinated progeny of plus tree K1257 (Varkaus)	1988

2.3 Statistical methods

2.3.1 Scots pine somatic embryogenesis

The differences in initiation percentages between the different culture media were calculated using analysis of variance basing on additive model and including the significant (0.05) first-order effects. The binomial data was transformed to normally distributed by Arcsine transformation. The factors in the analysis were mother's genotype, father's genotype and cold storage time. As the numbers of explants were different for different factor combinations, the frequencies were used as weights. The effects of the mother's and father's genotype were separated in the analysis to exclude the non-genetic maternal effects (e.g. physiological) biassing the results when the same genotypes were used as fathers. The arrangement made it possible to compare the ranking of genotypes as mothers and fathers. Selfed lines were excluded from the analysis of induction percentages. The survival data of the cultures was analysed using similar analysis of the variance as the initiation success, but the mature embryo production data was analysed using ranks instead of frequencies per line because of the large variation in mature somatic embryos in different lines.

2.3.2 Scots pine rooted cuttings

The variation in height between the Scots pine cuttings and seedlings at planting in 1992 and the effect of the stock plant age on height in 2003 was analysed by independent sample *t* test. Variation in height in the field was analysed by analysis of variance where the factors were hormone treatment, stock plant genotype within origin and cutting/seedling origin. The planting height was used as covariate in the analysis. The effects of plant type (seedling or cutting) and origin and their interactions in height, diameter at breast height, stem volume and stem taper was analysed by analysis of variance of random factors where the planting height was used as a covariate. The variation in bole straightness was analysed by Pearson's chi-square test.

2.3.3 Fungal communities in transgenic silver birch

The variation in ectomycorrhizal colonization intensity between the silver birch transgenic lines and non-transgenic control and wild-type clones was analysed by analysis of variance. Each transgenic line was compared to the control clone using Mann-Whitney *U* test. Variation in total endochitinase activity between the transgenic lines and the control was analysed by Dunnett's test. The differences in fungal community structures between the transgenic lines, control and the wild-type clones was studied by multi-response permutation procedures (MRPP), where in the first comparison the transgenic lines were compared to the wild-type clones and the dissimilarity was measured by Sørensen (Bray-Curtis) distance measure. In the second comparison the fungal community in each transgenic line was compared to the control clone using Jaccard's distance measure. The differences between plant groups were identified by fungal DNA sequences using indicator species analysis (IndVal) and the significance of each indicator value was tested with a Monte Carlo randomized test with 1000 permutations and hierarchical clustering was used

to classify the transgenic lines and the wild-type clones according to the fungal sequences. The data was divided into two groups using again Sørensen (Bray-Curtis) distance measure and flexible data linking method in order to detect deviations from the *a priori* plant classification. The differences in fungal communities between different plant genotypes were visualized using non-metric multidimensional scaling (NMS).

2.3.4 Variation in transgenic silver birch adaptive traits

The variation in adaptive traits between the transgenic lines, two controls and the wild-type clones was analysed by analysis of variance where the effect of the block was included in the model and tree height was used as covariate. In the cases where the data was not normally distributed Kruscall-Wallis tests were used. Pairwise comparisons between the lines/clones were made using Mann-Whitney tests. The broad sense heritabilities, their standard errors and coefficient of variation were derived from the sum of squares of analysis of variance. The variance components were divided into variation between lines/clones and to variation between ramets within line/clone, which were used to calculate the broad sense heritabilities.

3 RESULTS AND DISCUSSION

The detailed results and broader discussions are found in each separate original publication (I−IV). In this section only the major results are briefly outlined and discussed.

3.1 Parental effects on Scots pine somatic embryogenesis

Whole immature megagametophytes were used as the initiation material in the Scots pine somatic embryogenic cultures and the initiation procedure was similar to that described by Becwar and Pullmann (1995) in *P. taeda*. The immature cones were collected at a time which coincides with Scots pine simple polyembryony (Sarvas 1962) and the possibility of more than one genotype in the developing embryo culture was conceivable as was reported in low frequency in loblolly pine (Becwar et al. 1991). In our experiment the genotypes of the cultures were not determined, but the controlled pollinations ensured that in all the cultures the pollen came from a single known paternal genotype.

The initiation percentages varied between families (1−42%) and were in average higher in the cross-pollinated families (14%) than in the self-pollinated families (4%). The average initiation success was 13%, which is slightly higher than the typically low initiation percentages reported in Scots pine somatic embryogenic cultures (Keinonen-Mettälä et al. 1996, Häggman et al. 1999, Lelu et al. 1999, Aronen et al. 2009). Lelu et al. (1999) reported significant mother tree effect in the initiation phase of somatic embryogenic cultures of Scots pine and loblolly pine. In our experiment, the variation in initiation success was higher among the mothers than among the fathers and the ranking of the

mothers by initiation percentage was not the same than the ranking of the fathers. The maternal and paternal effects during the initiation period of the Scots pine somatic embryogenesis were both detectable, but the maternal effect was more pronounced than the paternal. Park et al. (1993) reported that more than 20% of the variation on the initiation of the white spruce somatic embryogenesis was caused by general combining ability and no specific combining ability was detectable. We found no specific mother x father combining ability with high initiation percentages, indicating that the father's effect is difficult to evaluate. Other factors, like cold storage time of the immature cones prior to the initiation had no effect on the initiation success alone, but the mother's genotype x storage time interaction was significant indicating that the zygotic embryos in cones continued to develop during the cold storage period. Häggman et al. (1999) found out that the cold storage temperature of +5 ºC was high enough for the immature zygotic embryo to develop. Our results indicate that the variation in initiation stage of Scots pine somatic embryogenesis can be explained by the mother tree's genotype and the megagametophyte's developmental and /or physiological stage (possibly including the variation between cones) and by the maternal alleles of the zygotic embryo.

During the maintenance culture and proliferation stage both mother's and father's effect was significant on survival. There was a large variation in the number of surviving lines (1□27) between families. The father with the highest surviving percentage had better survival with all the mothers than the father with the lowest survival percentage, indicating that even if both father and mother had significant effect on the survival, the earlier more pronounced mother's effect seems to diminish revealing the paternal effect at this point. Reasonable survival of lines with variation between lines was reported in *P. strobus* (Finer et al. 1989, Garin et al. 1998) and in Scots pine (Häggman et al. 1999) during proliferation. In our experiment the elimination of the lines during the proliferation stage was lower than at the initiation, indicating that any well-established line at the initiation stage may survive. Also, the survival of the self-pollinated lines did not differ from the cross-pollinated.

About 2/3 (69%) of the surviving lines in our experiment produced mature embryos. Similar proportions were reported in Scots pine by Häggman et al. (1999) and in *P. strobus* by Garin et al. (1998). We found out that the maternal effect was significant at this stage again. However, there was a large variation between lines within families and the variation in the frequencies of mature somatic embryos between families was only about 10% higher than within families, indicating that the highest ranking lines were scattered among different families. In general, the maternal effects were highest at the initiation stage, diminished by proliferation stage revealing the paternal effects, which were again covered by maternal effects at the embryo maturation stage. The lack of specific combining ability at any stage of the process indicated that any successful mother pollinated by any fathers may produce mature somatic embryos.

3.2 Field performance of Scots pine clones

The stock plants in our experiment were 2- and 5-years old seedlings. We found no detectable effect of age, stock plant hormone treatment or planting height on the field performance of rooted cuttings after 10 years in the field. The survival of both cuttings and

seedlings was very high in the field, after 2 years 99.6% and after 10 years still 98.0%. Sweet and Welles (1974) compared the growth of radiata pine cuttings and seedlings. They reported that the growth rate of initially smaller seedlings was higher than the relative growth rate (RGR) of the cuttings after 5 years in the field. Foster et al. (1987) reported equal RGRs for loblolly pine seedlings and cuttings from 4 years old stock-plants after 4 years in the field. Stelzer et al. (1998) reported similar RGR for loblolly pine seedlings and cuttings from 1,5-years-old stock-plants. In our experiment, the Scots pine cuttings were shorter with higher RGR than the seedlings throughout the trial period, but the difference started to even out already after the first growing season in the field. By planting the seedlings were twice as tall as the rooted cuttings, the height of the cuttings was 57.7% of the height of the seedlings, after 9 years 84.6% and after 10 years 85.9%. The variation in height between clones of the same origin (Varkaus) was statistically significant in the first two years in the field, but not detectable in later measurements. The relative growth rate of the rooted cuttings stabilized in the field between 2 and 9 years. No differences in stem taper or pole straightness between the rooted cuttings and the seedlings could be detected in the field.

Struve et al. (1984) reported that the rooted cuttings of eastern white pine (*P. strobus*) grew adequately compared to seedlings in one site but the seedlings were taller on the other site. Struve and McKeand (1990) reported that the shorter seedlings by planting outgrew the rooted cuttings of eastern white pine in 4 years and maintained their advantage for the next 5 years to the end of the trial. In our experiment, the height difference of Scots pine taller seedlings and shorter cuttings was statistically significant after 10 years in the field. The origin of the seedlings and the cuttings had more significant effect on the height than the plant type x origin interaction or plant type alone. We concluded from the similar growth pattern of Scots pine seedlings and cuttings, that the cloned material is suitable in breeding programmes and in experimental plantations.

3.3 Root-associated fungal communities in GM silver birch

The heterologous expression of sugar beet chitinase IV had no effect on the intensity of the colonization of ectomycorrhizal fungi in transgenic silver birch lines in the field. The colonization in our experiment was slightly lower in 7 of 15 transgenic lines, but the decrease was not related to transgene activity or total endochitinase activity. The indicator species analysis detected differences between the fungal communities in transgenic lines and wild-type clones, but there were no differences among the wild-type clones in root-associated fungal communities. Five sequences belonging to three different fungal genera (*Hebeloma*, *Inocybe, Laccaria*) were indicative of wild-type genotypes, and one sequence (*Lactarius*) indicated one transgenic line. The species *Hebeloma*, *Inocybe* and *Laccaria* are ‘early-stage’ fungi colonizing the seedlings by basidiospores and *Lactarius* a ‘late-stage fungus colonizing by hyphal contact (Read 1991), which may indicate the transgenic silver birches inability to form symbiosis with the ‘early-stage’ fungi. However, in cluster analysis, non-transgenic control grouped together with the transgenic lines indicating that the tree's genotype was a more important factor determining the structure of fungal communities than the single transgene in the plants. With the tested transgenic birch lines,

no clear evidence of the effect of sugar beet chitinase IV gene on ECM colonization or the structure of fungal communities was found.

The ectomycorrhizal (ECM) fungi colonize all or nearly all the root tips of plants (Harley and Smith 1983) and the number of the species colonizing one tree varies from 3 to 30, from which 1−4 species are dominant (Kauppinen 2001). The EMC fungi colonization in transgenic aspen in field conditions has been studied by Kaldorf et al. (2002). The transgene in their experiment was *rolC* gene from *Agrobacterium rhizogenes,* and they found it had no effect on the rate of mycorrhizas in the trees. Also, they reported intense colonization of the transgenic trees and that few species were dominant in the fungal community. Pasonen et al. (2005) studied *in vitro* the colonization of the roots of chitinase transgenic silver birch by *Paxillus involutus.* All tested lines formed ectomycorrhizal symbiosis and two lines with high transgene expression had slightly lower EMC root-tip percentage than the controls and other transgenic lines. In our experiment, the percentage of ectomycorrhizal root tips was high (98.3−100.0 %) in all types of the silver birches in the field trial. The ECM colonization was slightly lower in seven out of the fifteen transgenic lines than in the control clones but the slight decrease in ECM colonization could not be related to the transgene expression or total endochitinase activity. The CaMV E35S promoter was expected to give constitutive expression of the *chiIV* gene in different parts of the transgenic silver birches (Lemmetyinen et al. 1998), but the level of expression in leaves and roots was not similar. This discrepancy may have been caused by the promoter, which may not have functioned in birch as well as other promoters have been reported to function, i.e. *ubiquitin* promoter from sunflower or *rolC* promoter from *Agrobacterium rhizogenes* (Keinonen-Mettälä et al. 1998). Another explanation could be the transgene insertion site effects.

3.4 Adaptive traits in transgenic silver birch

In our experiment, the variation detected in transgenic silver birch lines was equal or larger than variation of both the target and non-target traits in randomly selected wild-type silver birch clones. The characteristics of the recipient clone (JR1/4) could be seen in the transgenic lines. The lowered growth and quality characteristics of the transgenic lines compared to the wild-type clones was partly likely to depend on the lowered fitness characteristics due to the random locations of the transgene in the birch genome.

Pappinen et al. (2002) reported that in pathogenicity test in a greenhouse the chitinase transgenic silver birches inoculated by *Pyrenopeziza betulicola* showed better resistance in the lines that had high transgene mRNA level than control clones. In the field conditions it was found out (Pasonen et al. 2004) that the silver birch lines with high transgene expression were more resistant than the lines with low expression against leaf spot. In our experiment we found out that the variation in transgenic lines was equal or higher than in the wild-type clones in fungal disease resistance. We concluded that the introduction of a single transgene can create highly variable lines in their resistance characteristics.

In silver birch the length of the growing season, a quantitative adaptive trait, is controlled by time and day length (Viherä-Aarnio et al. 2005). In our experiment, the transgenic silver birch lines started their growth later than the ones of the control clones and the wild-type clones. Also, the growth cessation in the autumn was delayed in the

transgenic lines compared to the wild-type clones, indicating poor adaptation and/or physiological difficulties during the winter hardening process. Billington and Pelham (1991) found large variation in the heritability of the length of the growing season in Scottish birch populations. Rousi and Pusenius (2005) reported variation in phenological characteristics in silver birch populations in Finland. In our experiment the heritabilities for bud burst and development of autumn colours of silver birch varied from very low to moderate both in the transgenic lines and in the wild-type clones. The importance of ample variation in the phenology related traits has been discussed in relation to climatic warming (Scotti-Saintage et al. 2004, Derory et al. 2010). In our experiment the heritabilities for stress related traits indicate an inner disturbance in addition to environmental stress, possibly caused by the functioning of the transgene.

Ahuja (1998) indicates that epigenetic changes may cause somaclonal variation during tissue culture. According to Kaeppler et al. (2000), cultures initiated from bud meristems may be more stable than from more differentiated tissue. We compared two types of initiation explants in silver birch axillary micropropagation, bud meristems and leaf discs. The clones initiated from leaf discs resembled in their adaptive traits more the transgenic lines than the control from bud meristems, and the clones initiated from bud meristems were closer to the wild-type clones.

4 CONCLUSIONS

Cloning is a powerful tool for tree breeders, which in combination with gene transfer opens new possibilities by widening the available variation beyond the species limits. The use of clones instead of seedlings in testing will allow one genotype to be tested in varying environments and in destructive tests. Furthermore, if a candidate for selection can be tested without waiting for the plant to flower and produce seeds it will shorten the breeding cycle. The prospect of clonal forests planted by genetically transformed genotypes is an outlook which raises many questions on the reliability and economic profitability of the methods used today, safety of the genetically transformed and/or cloned planting material and general scepticism towards gene technology. Experimental studies, i.e. field tests, provide the means to assess the risks and benefits of clonal forestry.

My thesis presents some answers to the questions raised by the clonal forestry option. The two articles on Scots pine clones (I, II) address the two cloning methods available for Scots pine today based on somatic embryogenesis and rooted cuttings. We demonstrated that the cloning of Scots pine by somatic embryogenesis (I) is affected by maternal effects more than by paternal and that there is no detectable mother x father interaction affecting the success of the method used. Also, there was a wide variation in the success of somatic embryogenesis within and between the lines and elimination of lines occurred in every step of the procedure. Our conclusion was that the success of Scots pine somatic embryogenesis is too much affected by genetic effects and too many lines are eliminated especially at the initiation stage and, hence, the method needs further development in order to be a suitable

large scale propagation method. The other tested Scots pine cloning method based on organogenesis, the use of rooted cuttings (II), was time and labour consuming, but produced suitable material for a field trial. The propagation method we used – spraying young seedlings with cytokinin to elongate the brachyblasts and rooting the cuttings with auxin treatment – needs to be optimised for tree nursery use. The chitinase transformed silver birches were cloned using axillary micropropagation method using leaf discs for initiation. Two control clones of the same genotype were initiated either from leaf discs or bud meristems. The propagation method is working well and there were no difficulties in producing enough material for experiments. However, the control clones initiated from bud meristems resembled the wild-type clones more than the clones initiated from leaf-discs suggesting the possibility of some degree of somaclonal variation in the clones initiated from leaf-discs.

The introduction of sugar beet chitinase IV gene (*chiIV*) with an enhanced (4x) viral CaMV E35S promoter into silver birch genome is an interesting option, where we added into the tree something very similar of its own chitinase genes. Another option would have been a chitinase gene from a biocontrol fungus *Trichoderma* or from mycoparasitic *Clonystachus*. For three years we collected data from plant-fungal interaction with pathogenic and mycorrhizal fungi in a field trial. We found out that the transgene had only marginal effect in the field conditions on the resistance against birch rust (*Melampsorium betulinum*) and no detectable effect against birch leaf spot (*Pyrenopeziza betulicola*) and it did not interfere with mycorrhizal colonization or with the root associated fungal community structure (III).

We compared the growth characteristics of cloned Scots pines (II) to seedlings and transgenic silver birches (IV) to control clones and wild-type clones in field conditions. The Scots pine clones in our experiments were shorter than seedlings and the transgenic silver birch lines were shorter than the control clones and wild-type clones. However, the growth pattern of Scots pine clones resembled that of the seedlings of the same origin and the ranking of the genotypes was the same in both groups after 10 years in the field, indicating that the genotype more than propagation method affected the field performance of the trees and that the height growth of Scots pine can be tested by rooted cuttings reliably. In silver birch, the transgene, sugar beet chitinase IV (*chiIV*) gene, was transferred only to one genotype (JR1/4), which was shorter than the wild-type clones. We concluded that part of the poor growth of the GM-lines was due to the original genotype and part of it was caused by the position effects of the transgene, as the indication of stress in transgenic lines could be due to disturbances in metabolism caused by the transgene. Also, the tissue culture initiation explant type, bud meristem or leaf disc, affected in some extent the growth of silver birch control clones.

The variation in height between Scots pine clones of the same origin (Varkaus) was statistically significant after two years in the field, but not any more in later measurements (II). We concluded from the vigorous growth of the rooted cuttings in the first years after planting in the field, that the height growth of Scots pine can be reliably evaluated from rooted cuttings only when the growth rate has stabilised, which in our experiment was between 2 and 9 years in the field. We attempted to evaluate the wood quality of rooted Scots pine cuttings by comparing it to seedlings, but the trees after 10-year field were still too young for quality evaluations. The pole straightness and the stem taper of the rooted

cuttings were similar to those of the seedlings. The variation in disease resistance between the chitinase transgenic silver birch lines was higher or equal to that of the wild-type clones, and we concluded that the introduction of a single gene into a silver birch genotype can create enough variation for the selection of resistant lines (IV). The effect of the sugar beet chitinase IV (*chiIV*) gene on the colonization intensity of ectomycorrhizas and on the root associated fungal community structure was studied from samples collected from the field at the end of the field trial (III). We detected three fungal species indicative of wild-type silver birch clones (*Hebeloma, Inocybe, Laccaria*) and one genotype indicative for the chitinase transgenic lines (*Lactarius*). Although 7 of the 15 transgenic lines had less intense ectomycorrhizal colonization than the control clones, we concluded from the results of the cluster analysis which grouped the control together with the transgenic lines that the genotype − not the transgenic status of the trees − was the most important factor in determining the EMC colonization intensity and the fungal community structure in the field trial of chitinase transgenic silver birch.

The broad-sense heritabilities of growth and leaf phenology related traits were slightly higher in transgenic silver birch lines than in the wild-type clones in average. We concluded that the introduction of a single chitinase gene had fitness consequences (lower growth and quality characteristics) but could not create variation above the variation found in wild-type clones of the same characteristics (IV).

The introduction of new breeding methods, the use of clones, molecular selection, and transgenes opens new possibilities for tree breeders. The benefits of clonal testing seem to be clear: higher genetic gain and shorter breeding cycle followed by lowered costs. The costs would be even lower if a novel tree breeding strategy without controlled crosses combined with molecular selection could be used for Scots pine and silver birch. The use of transgenes in tree breeding is a still developing strategy, which could be a powerful method if 1) the effect of the transgene exceeds the effect found naturally in the species, 2) the integration of the transgene, stable expression and copy numbers were precise in the tree genome, and 3) the risks of the transgene escape and effect on other than target species could be controlled by mitigating genes or other means. The cloning of trees, with or without gene transfers, is not always successful. In Scots pine, propagation of clones by somatic embryogenesis is possible, but not all the genotypes can be cloned reliably by the present methods, which are labour-intensive, costly and need more development to be suitable for tree nurseries. The same can be said of the rooting of Scots pine cuttings: the rooting methods used are not reliable enough to ensure that all selected genotypes can be rooted and, hence, more research is needed in this area. The cloning of silver birch by axillary shoot multiplication method is today reliable propagation method. However, the micropropagated birches are produced in sterile laboratory conditions, which make the production much more expensive than seedling propagation. Nevertheless, I can see the integration of the new breeding methods into the future Finnish tree breeding programme in phase with advancements in molecular biology and cutting edge breeding research.

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