Micropropagation and Biotechnology in Forestry: Preliminary Results From the Danish Christmas Tree Improvement Programme©

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INTRODUCTION

The principal methods of micropropagation (in vitro propagation or tissue culture) are axillary budding, induction of adventitious buds, and somatic embryogenesis (where individual cultured cells or small groups of cells undergo development resembling that of the zygotic embryo. These methods are very expensive compared to conventional vegetative propagation due to the costly equipment and the technical staff that is needed to perform the techniques. An additional disadvantage of micropropagation is that it may lead to unwanted genetic alterations, e.g., somaclonal variation (George, 1993; Barbara, 1984). However in vitro propagation technology of forest trees has several potential benefits:

- Allow mass propagation of trees that are difficult to propagate by conventional means.
- Provides suitable material, e.g., somatic embryos for cryostorage in liquid nitrogen, permitting year-long field evaluation of stored clones.
- Micropropagation, e.g., somatic embryogenesis, is required for the development of genetic engineering techniques that allow rapid introduction of traits that are neither available in the breeding population nor in the original genetic resource.

Consequently, the importance of micropropagation is increasing in large-scale multiplication of superior trees for clonal forestry. Commercial micropropagation of forest trees for wood and pulp production is currently being developed in Canada, New Zealand, and U.S.A. for species like loblolly pine (*Pinus taeda*) and radiate pine (*Pinus radiata*), for eucalyptus (*Eucalyptus* sp.) in Brazil (de Freitas, 1996), and poplar (*Populus* sp.) in, e.g., China.

In Denmark traditional timber production is declining, while the production of Christmas trees and greenery is becoming increasingly important. Although Caucasian fir or Nordmann fir (*Abies nordmanniana*) covers less than 6% of the total forest area, the sale of Christmas trees and greenery constitutes more than one-third of the total income from Danish forestry (Danish Forest and Nature Agency, 2004).

The following preliminary results constitute the outcome of a long-term programme on micropropagation and genetic engineering of Caucasian fir.

MICROPROPAGATION AND GENETIC ENGENEERING OF CAUCASIAN FIR: FROM LABORATORY TO NURSERY

Caucasian fir is the preferred Christmas tree in Denmark and much of Europe. It grows naturally in mountains around the eastern end of the Black Sea at 900–2100 m. Denmark produces about 12 million of the 70 million Christmas trees annually

marketed in Europe. Ten million trees are exported every year making Denmark the primary country in Europe with Christmas tree exports.

Traditional vegetative propagation of Caucasian fir is difficult and the production of Christmas trees for plantations is primarily derived from seeds collected in the forests in Caucasus. The production of Christmas trees is cost- and labour-intensive, and each single plant represents a high value. The seed supply is unstable and many plants are of low quality, being either too broad, too open between the whorls of branches, or have a poor colouring.

Some of the problems in Caucasian fir production may be alleviated using cloning by somatic embryogenesis. Due to the large returns on the sale of Christmas trees, growers will be willing to pay a notably higher price for cloned plants.

Table 1. Increase in value per planted tree (DKr.) with the use of clonal propagation compared to the use of traditional seed propagation. Estimated by the Danish Christmas Tree Growers Association, 2003.

	Percentage distribution			Estimated price/ 100 trees	
Category of tree quality	Present	Clonal propagation	Price/tree	Present	Cloning
Prime	15	60	90	1350	5400
Standard	40	20	55	2200	1100
3rd class	20	5	30	600	150
Discarded due to shape	20	10	0	0	0
Discarded due to damage	5	5	0	0	0
Total	100	100		4150	6650

Average increase in price per planted tree with cloning is 25DKr.

Somatic Embryogenesis. Establishment of embryogenic cell cultures of Caucasian fir was reported for the first time in 1991 (Nørgaard and Krogstrup, 1991). Since then the protocols have been further developed (Nørgaard and Krogstrup, 1995, Nørgaard, 1997) including development of standard methods for cryopreservation (Nørgaard et al., 1993) and genetic transformation (Find et al., 2004, in press). A significant improvement in the quantity and quality of somatic embryo maturation has been achieved by including an anti-auxin, p-chlorophenoxyisobutyric acid (PCIB), during maturation step to prevent proliferation (Find et al., 2002). A multi-year project was initiated in 2001 in order to evaluate the possible implementation of somatic embryogenesis of Caucasian fir in the Danish tree-breeding program. The project includes field trials of clones on multiple sites to identify, e.g., disease and frost resistance and the response capacity of different clones towards available resources like water and nutrients.

Genetic Transformation. Growing monocultures with a rotation period of 8–12 year increases the risk of damage caused by insect predation. This is a considerable problem in the Christmas tree production as both form and appearance is of

major importance for the quality and value of the product. At present, the problem is mainly countered by application of pesticides. However, both public and political acceptance of this solution is declining, due to the impact on nontarget species and the associated risk of environmental contamination.

An alternative strategy may be to selectively target certain insect pests through introduction of specific genes via genetic engineering. The present study focuses on the development of protocols for stable genetic transformation of Caucasian fir and provides evidence that the transgenes are stably integrated into embryogenic cultures and that transgenic plants can be regenerated.

RESULTS AND DISCUSSION

Somatic Embryogenesis. A total of 146 cell lines were established in 2002 from mature 7-month-old, stored seeds (Table 2). In 2003, 157 cell lines were established from freshly harvested, mature seeds (Table 3). Fifty-nine of 60 cell lines selected in 2003 for maturation produced mature embryos (7341 embryos in all); 4846 plantlets were transferred to the nursery. A total of 27 cell lines have been selected for maturation in 2004 so far. All lines produced mature embryos (5391 in all) and about 5000 plantlets have been transferred to peat-plugs. In addition 305 cell lines were cryopreserved (not shown) of which 270 survived (89%).

The standard micropropagation method resulted in high initiation and high maturation frequencies of somatic embryos (Fig. 1). However, preliminary results indicate that plantlets derived from somatic embryos are very small compared to plants from seeds and less than 25% survived transfer to a commercial nursery (not shown). Accordingly future micropropagation work on Caucasian fir must aim at improving protocols for transfer to peat plugs and investigate factors affecting growth and development of somatic plantlets in nursery culture.

Genetic Transformation. Six transclones were recovered from a total of 215 of bombardments. *Uid*A expression was confirmed by histochemical analysis and the expression of the *npt*II gene was quantified by ELISA. All transclones expressed both genes in proliferating tissue, in mature embryos, and in regenerated plants. As expected, the expression of the transgenes varied between the transclones as well as during different developmental stages of the same transclone throughout a period of 5 years. The presence and integration of the *npt*II gene was confirmed by Southern blotting in embryogenic tissue after 5 years of culture. More than 2500 transgenic plants from the 6 independent transformation events were regenerated and of these approx. 100 were transferred to soil in the greenhouse. The results demonstrate that it is possible to genetically engineer embryogenic cultures of Caucasian fir by Biolistic® transformation. Evidence is established that the cultures and plants regenerated from transgenic cultures, express the transgenes over a period of at least 5 years (Find et al. 2004, in press.).

CONCLUSION

There are several benefits associated with micropropagation of forest trees, e.g., mass propagation of elite trees that are difficult to propagate by conventional means, production of transgenic trees with, e.g., increased pest resistance, and cryopreservation of clones during field testing. Taking these factors and the preliminary results from the present article into consideration we believe that micro-

Table 2. In 2002/2003, 146 cell lines were established in 2002 from 7-month-old, heavily contaminated seeds. From a total of 146 established cell lines, 60 cell lines were selected for test of maturation capacity. Maturation was induced in 59 of the 60 selected cell lines. 7341embryos were produced in total, 4846 plantlets were transferred to peat plugs, and about 1000 plant were transferred to the field in 2003.

Families from 2002/2003	Excised embryos	Excised sterile embryos	Initiated cell-lines	Established cell-lines	Selected cell-lines	Mature embryos (no.)	Transplanted to peat plugs
J87.2	325	170	85	30	12	1233	957
J87.3	261	47	17	12	10	683	535
J87.4	311	138	111	66	12	1340	1050
J87.5	209	46	11	8	8	911	718
J87.6	315	214	40	23	11	2156	1026
J87.7	252	58	8	7	7	1018	560
Total	1673	673	272	146	60	7341	4846

Table 3. In 2003/2004, 157 new cell lines were established in 2003. 27 cell lines have been selected for maturation in 2004 so far. All lines produced mature embryos, 5191 in total, and about 5000 plantlets have been transferred to peat plugs in a commercial nursery. Surviving plantlets (less than 25%) will be transferred to the field in 2005.

Families from 2002/2003	Excised embryos	Excised sterile embryos	Initiated cell-lines	Established cell-lines	Selected cell-lines	Mature embryos (no.)	Transplanted to peat plugs
J90.1	285	225	41	36	5	1035	N/A
J90.2	234	182	19	17	6	1617	
J90.3	220	210	24	23	6	1127	
J90.4	212	187	42	39	0	0	
J90.5	200	199	5	5	4	698	
J90.6	215	211	39	37	6	914	
Total	1366	1214	170	157	27	5391	

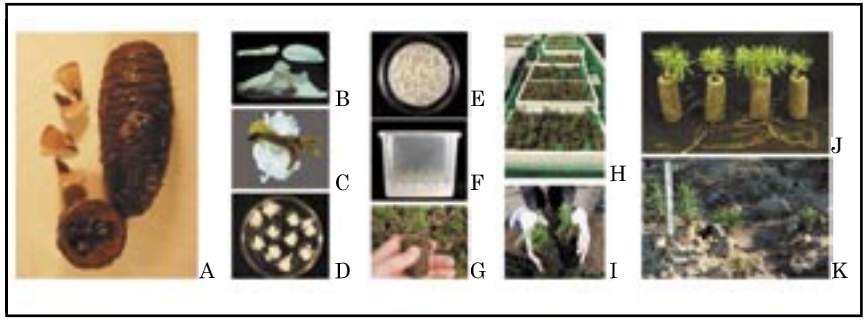

Figure 1. Stages of in vitro propagation of Caucasian fir.

A. The process begins with the selection of improved conifer seeds from a conventional tree breeding program. The starting material is, e.g., 2000 seeds from six families in order to generate a large number of superior cell lines (genotypes).

B. Sterilized seed (bottom), excised, approximately 8-mm-long zygotic embryo (upper left) and the white, oily megagametophyte (reserve food) on the upper right.

C. The zygotic embryo is placed on a culture medium (BLG) to induce somatic embryogenesis. The white translucent embryogenic masses are initiated from the swollen, brownish zygotic embryo after 6 to 8 weeks.

D. The resulting embryogenic culture, now a distinct cell line, will continue to proliferate as immature somatic embryos. Each petri dish, 8.5 cm in diameter, holds several thousand embryos. These cultures are transferred to fresh medium every 2 weeks or put into cryogenic storage in liquid nitrogen (-196 °C).

E. In order to induce maturation somatic embryos are transferred to a medium containing abscisic (ABA). Caucasian fir requires an anti-auxin, PCIB, in order to activate the switch from proliferation to maturation. The mature embryos are identical genetic copies of the zygotic embryo in the original seed embryo.

F. The mature somatic embryos are germinated for 8 weeks on a medium without plant growth regulators.

G. The plantlets are transferred to peat plugs (Jiffy plugs). The photo was taken 8 months after transfer.

H. Plantlets 20 months after transfer to peat plugs.

I. Plantlets 24 months after transfer to peat plugs.

J. Plantlets in the field. Elite genotypes may be identified following 56 years of field trials and the corresponding frozen cell lines can be restored from liquid nitrogen.

propagation and genetic transformation of Caucasian fir could have a supportive role within the framework of existing traditional breeding programmes.

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