Application of biotechnology to forest tree breeding

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Impact of biotechnology on forest tree breeding

Biotechnology has created a virtual revolution in different fields of biology. The research advances in basic molecular, biochemical and physiological research give tremendous promises for the application of biotechnological methods in the genetic improvement of plants. Many of these new techniques are already in use of the breeders for agricultural and horticultural plants. When comparing the economically important crops of agriculture and forestry the application of the new techniques to forest tree breeding has been much slower. This is the sum of different factors; less amount of basic research, which means less amount of understanding of different biological processes, the special characters of trees as crop plants, newness of the technology, lack of skilled human resources, public acceptance etc.

Forest tree breeding involves manipulation of the genetic composition of populations and individuals. The primary breeding unit is the population, which is a repository of desirable alleles, reconstituted in each generation. Biotechnology focuses on specific genetic modification of selected individual genotypes (Fig. 1) and these selected elite genotypes form a link between traditional breeding work and biotechnology (Riemenshneider et al. 1988, Cheliak and Rogers 1990). The consequences of modern tree breeding techniques on breeding programs of the main tree species in Finland is discussed in review article of Mikola (1990).

In addition, breeding progress can be accelerated when the basic knowledge of the structure, regulation and function of genes of biological processes like hardening and resistance increases.

What are these biotech methods in forest tree breeding? Depending on the field of biology the term biotechnology can get slightly different definitions. When talking about forest trees biotechnology involves the cooperation between different independent disciplines; molecular genetics, vegetative propagation, and genetic transformation. In this article the biotechnological methods which have or may have some impact on forest tree breeding are presented as well as the state of this kind of research in Finland.

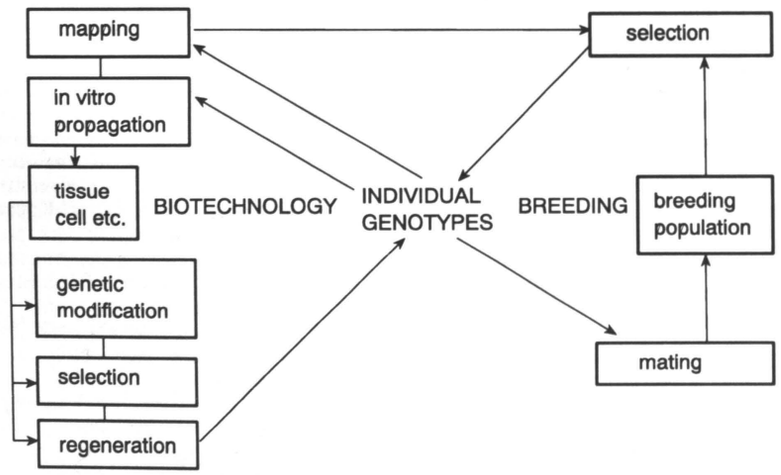

Fig. 1. Relations between forest tree breeding and biotechnology.

Molecular genetics

Historically speaking, it was the discovery of specific restriction endonucleases in late sixties (Arber and Linn 1969, Meselson and Yuan 1968) that actually gave birth to DNA (deoxyribonucleic acid) manipulations and made the recombinant DNA technology possible. The fundamental aspect of gene technology is the creation of new combinations of genetic material from DNA molecules of different origin. Such recombinant molecules are introduced into appropriate hosts, where they can be multiplied and selected. These techniques have contributed much to the elucidation of basic mechanisms in plants at the molecular level and furthermore enabled the identification and eventual manipulation of genes controlling important plant functions.

The huge growth of plant molecular genetics over the past decade has created opportunities for their use in tree breeding. The potential impact lies on two fronts ; the use of molecular markers and the cloning and characterization of genes and their promoters controlling development and function of biological processes.

Molecular markers

Genetic markers most often used in studies of forest trees have been morphological markers, monoterpene variants and isozymes. Determining the mode of inheritance of both monoterpenes and morphological markers requires controlled crosses and the analysis of seedling progeny. Isozymes were first utilized in genetic studies of forest trees in the middle of seventies (Feret and Bergmann 1976) and since then they have been widely used for certifying the identity of parent trees, clones and seed lots, determining the validity of controlled crosses, studying the genetic efficiency of seed orchards etc. In Finland isozyme technique is used in genetic studies of forest trees in the University of Oulu, Department of Genetics; in the University of Helsinki, Department of Plant Breeding; in The Foundation for Forest Tree Breeding and in The Finnish Forest Research Institute.

However, basically due to the small number of isozymes available, much interest has been focused on using DNA markers in forest tree breeding. When comparing isozyme polymorphism with that of DNA, DNA sequence does not necessarily have to express a protein in order to be identified by polymorphous cleavage sites. DNA polymorphism can occur in any DNA sequence.

From the middle of eighties restriction fragment length polymorphism (RFLP) probing techniques have been used for dicot and later on also for forest tree genome mapping (Gorzo and Neale 1989, Bradshaw and Stettler 1991). RFLPs are promising because a large number of these markers may be isolated and mapped covering the whole genome and on the other hand they also can be used as DNA markers to identify genotypes. A simple overview of this technique and its relation to forest tree breeding is given in Fig. 2.

In the case of conifers the high DNA content presents some difficulties in detecting single copy sequences and also the number of RFLPs required to cover the genome, at any given spacing, can be expected to be very large. Thus also in the RFLP mapping conifers are more difficult than the hardwoods with small contents of DNA and shorter generation times.

In Finland RFLP technique has been used to study the genetic structure and variation of Scots pine (*Pinus sylvestris*) and to find markers assisting in the genetic improvement of this species. The first achievements have been made in mapping the genes of rDNA (ribosomal DNA) and the work with single copy sequences is beginning (Outi Savolainen, Päivi Karvonen & Matti Karjalainen, University of Oulu, Department of Genetics, pers. comm.).

The detection of RFLPs by Southern blot hybridizations are laborious. This is maybe one of the reasons that during the recent years other techniques have been used to study DNA polymorphism. Most of these are based on polymerase chain reaction (PCR). Minisatellite technique is based on the use of the probes which hybridize to multiple tandem-repetitive or hypervariable minisatellites of DNA (Jeffreys et al. 1985). This technique has been used by Kvarnheden and Engström (1991) to study DNA polymorphism of Norway spruce (*Picea abies*).

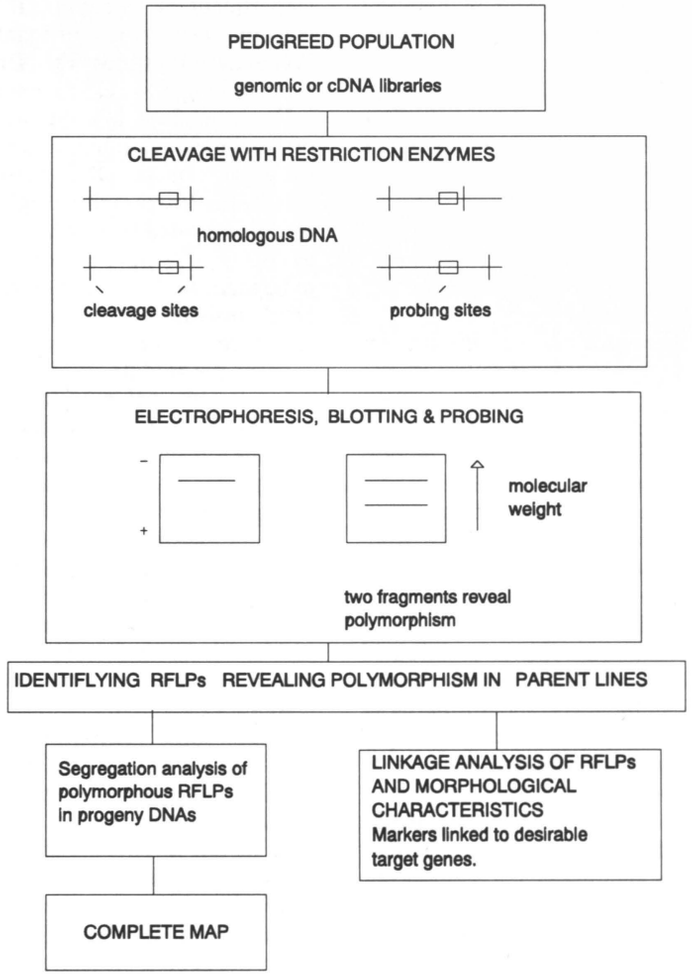

Fig. 2. Principle of restriction fragment length polymorphism (RFLP) and its use in forest tree breeding.

Just lately a new DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence has been developed (Williams et al. 1990, Caetano-Anolles et al. 1991). It remains to be seen if this new technique can be adopted in the research of DNA polymorphism of forest trees. At this moment it seems to be a very promising one.

Cloned plant genes and quantitative trait loci

The list of cloned plant genes and also functionally useful genes in plants isolated from other organisms is increasing really fast. This list might include e.g. the following areas of plant metabolism (as presented by Von Arnold et al. 1991) : 1) growth regulation, 2) disease resistance, 3) herbicide resistance, and 4) organ specific/regulating genes. As an example of growth regulatig genes the auxin and cytokinin synthesis from *Agrobacterium tumefaciens* can be mentioned (Klee and Estelle 1991). There are also many disease resistance genes like several genes of phenylpropanoid metabolism, chitinase genes (Dunsmuir and Suslow 1989), proteinase inhibitor genes (Thornburg et al. 1987) and toxin producing genes from *Bacillus thuringiensis* (Barton et al. 1987). In addition the genes like PAL (phenylalanine ammonia-lyase) and CAD (cinnamyl alcohol dehydrogenase) of phenylpropanoid metabolism have an important function in the lignification during the development of vascular plants (O'Malley et al. 1991). Of the herbicide resistant genes the best known is the EPSP (5-enolpyruvylshikimate) synthase gene conferring resistance to glyfosate (Fillatti et al. 1987). The pollen specific genes could be the example of organ specific genes (Mascarenhas 1989).

One approach to quantitative genetic research in plants with particular promise for trees is the use of detailed genetic maps for counting and identifying quantitative trait loci (QTLs) by the existence of linkage between an RFLP and a quantitative character. Several QTLs have been mapped for tomato (Paterson et al. 1988) and the work is going on with *Populus* (Bradshaw and Stettler 1991).

Vegetative propagation

Vegetative propagation can contribute to the improvement of forest trees through exploitation of existing genotypes and production of new commercially valuable genotypes (Haissig et al. 1987). For forest trees the main vegetative propagation methods are cuttings and *in vitro* techniques; organogenesis and somatic embryogenesis. The methods used vary between different species and within species, depending on objectives etc. Because of the possibility of the genetic instability of the *in vitro* regenerated plants the field tests are of major importance.

Cuttings

Cuttings are used in considerable amounts for instance in propagation of Norway spruce, and microcuttings (i.e. fascicular shoots produced with cytokinin spraying treatments) to some extent in propagation of Scots pine. The situation in Finland is presented in Table 1. The main obstacles in the use of cuttings are the limitation in the number of shoots produced from each plant, ageing and the possibly inadequate rooting.

Organogenesis

Organogenesis is still today the main way to *in vitro* regenerate forest trees. This can be seen for instance in Table 1 which presents the ways and places of *in vitro* propagation of forest trees in Finland. The now classic experiments of Skoog and Miller (1957) demonstrated that organogenesis was controlled by the phytohormones, cytokinin and auxin, in the medium.

The hardwoods are most often propagated, in some cases even mass propagated, using existing axillary meristems as explant material. The technique is relatively simple and the risks for aberrations are small. In the case of silver birch (B*etula pendula*) these kind of plantlets have been used in several research experiments in Finland (e.g. Poteri 1991).

It is also possible to propagate the hardwoods through callus cultures, which could mean the problems of genetic instability. For instance Cameron (1990) found some autotetraploids in a population of plants derived from callus cultures of silver birch. The autotetraploids on the other hand have potential value as breeding material for fast growing triploid birches. Another technique of producing polyploid birches is the treatment of tissues or seeds with colchicine. The seed treatment produces good growing polyploid birches (Terho Valanne, University of Turku, Department of Botany, pers. comm.) which in their turn can be vegetatively propagated (Särkilahti 1990).

Conifers are most often propagated by inducing adventitious buds on young explant material. Generally speaking the limitations of this technique can be caused by difficulties in elongation, rooting, acclimatization, and early maturation. This technique may also cause somaclonal variation (Larkin and Scowcroft 1981, De Klerk 1990). But this does not have to be the case, which is indicated by mass propagation of radiata pine (*Pinus radiata*) in New Zealand (Aitken-Christie and Jones 1987). Loblolly pine (*Pinus taeda*), a model system for tissue culture of conifers, is propagated by adventitious buds using cotyledons as explant material (Amerson et al. 1988, Mott and Amerson 1981). Modified loblolly pine system seems to work for Scots pine (Häggman and Stomp 1990a), too. Conifers can, however, also be propagated through callus cultures as shown by Gladfelter and Phillips (1987) in the case of Afghan pine (*Pinus eldarica*).

Somatic embryogenesis

Somatic embryogenesis is based on the fact that somatic cells can be stimulated to develop into

Table 1.Vegetatively propagated forest trees in Finland. M = mature, Y = young (embryo or seedling), C = cuttings, MC = microcuttings (fascicular shoots produced with cytokinin spraying treatments), O = organogenesis and SE = somatic embryogenesis.

Hardwood species	Tree age	Propagation type
Betula pendula	M	O
The Finn. For. Res. Institute, Punkaharju Res. Stat. (Ryynänen, pers. comm.) The Foundation for Forest Tree Breeding (Salonen, pers. comm.) Kemira, Hortus & Enso-Gutzeit OY (Jokinen et al. 1991) Univ. of Joensuu, Dept. of For. (Keinonen-Mettälä, pers. comm.) Univ. of Kuopio (Paasisalo, pers. comm.) Univ. of Turku, Dept. of Biology (Särkilahti 1990)		
	Y	SE
Technical Research Centre of Finland, Biotechnological Laboratory (Kurten et al. 1990)		
Betula pendula f. *purpurea*	M	O
Univ. of Helsinki, Dept. of Botany (Simola 1985)		
Betula pendula var. *carelica*	M	O
The Finn. For. Res. Institute, Punkaharju Res. Stat. (Ryynänen and Ryynänen 1986)		
Betula pubescens	M	O
The Finn. For. Res. Institute, Punkaharju Res. Stat. (Ryynänen, pers. comm.) Univ. of Turku, Dept. of Biology (Särkilahti 1990)		
Alnus sp.	M	O
The Finn. For. Res. Institute, Punkaharju Res. Stat. (Ryynänen, pers. comm.) Kemira (Jokinen, pers. comm.) Univ. of Helsinki, Dept. of Botany (Simola and Tomell, pers. comm.)		
Tilia sp.	M	O
Univ. of Helsinki, Dep.t of Botany (Simola, pers. comm.)		
Conifers	**Tree age**	**Propagation type**
Picea abies	M & Y	C
The Foundation for Forest Tree Breeding (Napola 1992)		
	M	O
Kemira (Jokinen, pers. comm.)		
	Y	SE
Kemira (Jokinen, pers. comm.) Univ. of Helsinki, Dept. of Botany (Simola and Santanen 1990) Univ. of Joensuu, Dept. of For.(Keinonen-Mettälä, pers. comm.)		

Table 1 continued.

Conifers	Tree age	Propagation type
Pinus sylvestris	M	O
Kemira (Jokinen, pers. comm.) Univ. of Oulu, Dept. of Botany (Hohtola 1988)		
	Y	O
The Finn. For. Res. Institute, Punkaharju Res. Stat. (Häggman and Stomp 1990a) The Foundation for Forest Tree Breeding (Salonen, pers. comm.) Kemira (Jokinen, pers. comm.)		
	Y	SE
Univ. of Joensuu, Dept. of For. (Keinonen-Mettälä, pers. comm.) Univ. of Oulu, Dept. of Botany (Hohtola, pers.comm.)		
	Y	MC
The Foundation for Forest Tree Breeding (Salonen 1990)		
Pinus contorta	Y	MC
The Foundation for Forest Tree Breeding (Salonen, pers. comm.)		
Larix sp.	Y	O
The Finn.For.Res.Institute, Punkaharju Res.Stat. (Niskanen, pers.comm) Kemira (Jokinen, pers. comm.)		

somatic embryos and further on into plants. The number of forest tree species from which somatic embryos can be obtained is rapidly increasing. The somatic embryogenesis of Norway spruce has been studied during the last few years most intensively (Hakman and von Arnold 1985, Gupta and Durzan 1986, Simola and Santanen 1990). The case in Finland is seen in Table 1. However, to my knowledge, this technique has not been used for practical forestry. The main problem seems to be that despite high yields of somatic embryos, only comparably few go through complete germination. It is also suspected that somaclonal variation can be one of the limitations of this technique (De Klerk 1990) but on the other hand in the case of interior spruce (*Picea glauca engelmannii* complex) the embryogenic cultures showed a high degree of genetic stability (Eastman et al. 1991). Somatic embryogenesis gives great promises for the future for several reasons: somatic embryos can be produced in large quantities; they can be encapsulated to form "artificial seeds"; they can be cryopreservated; rejuvenation can be obtained if somatic embryos are regenerated from mature trees.

Maturation and micropropagation

Maturation or the transition from the juvenile to the adult phase can be defined as the developmental process inducing changes in morphological and physiological characteristics leading to the reproductive state (Pierik 1990). At the moment maturation is the major problem preventing a wider application of tissue culture technology among woody species. Cloning of adult trees is adversely affected by characteristics accompanying maturation as reduced growth rate, reduced rooting ability and plagiotropy. However, rejuvenation in *in vitro* propagation of adult trees has been achieved in some cases (*Pinus radiata* and *Tectona grandis*) by using special starting material and special pre-treatments. This means that rejuvenation is difficult but not im-

possible. In the future the somatic embryogenesis may be the method to choose for obtaining rejuvenation.

Genetic transformation

The conventional gene transfer method involving hybridization is effective but takes a number of years and is limited to those tree species which are sexually compatible. The new gene transfer techniques make it possible to avoid these problems.

Somatic hybridization

Protoplast (i.e. single plant cells lacking a cell wall) fusion offers prospects for gene transfer in sexually incompatible species and, in addition, random distribution of organelles in cytoplasm allows new and rearranged combinations of traits coded by the organelles. Using this technique it is possible to transfer for instance polygenic traits or traits whose genetic background is unknown. At the beginning of seventies the first tobaccos were regenerated from the protoplasts. Since then the amount of especially herbaceous species which can be regenerated by protoplasts as well as the amount of somatic hybrides produced by protoplast fusion techniques has been increasing rapidly.

In most forest trees it is still difficult to regenerate viable plants from the protoplasts. Especially protoplasts of conifers are regarded as recalcitrant to culture. However, there are some exceptions such as plantlet regeneration from embryogenic protoplasts of white spruce (Attree et al. 1989) and Norway spruce (Gupta et al. 1990). Up to my knowledge there is no report of somatic hybrids of forest trees right now but in the future this technique may be used in genetic transformation of forest trees.

Gene transfer methods

There are several ways to transfer genes to somatic plant cells. These are discussed for instance in the review articles of Klee et al. (1987), Joersbo and Brunsted (1991) and Potrykus (1991). The methods can be divided into the vector mediated and direct methods.

Potential vectors for gene transfer are for instance A*grobacterium tumefaciens* and *rhizogenes*, different viruses, transposable elements. From these the most common one is *Agrobacterium tumefaciens* mediated gene transfer. When comparing dicots, monocots and gymnosperms the host range of *Agrobacterium* is the most extensive in dicots. However, it has been proved that *A. tumefaciens* can infect several gymnosperms (Sederoff et al. 1986, Clapham et al. 1990, Häggman and Stomp 1990b, Loopstra et al. 1990, Stomp et al. 1990).

Agrobacterium mediated gene transfer has been used for transforming several hardwoods. The best known is herbicide glyfosate tolerant *Populus* (Fillatti et al. 1987). In Finland this technique is used in silver birch transformations (Jokinen et al. 1991, Kim von Weissenberg. University of Joensuu, Department of Forestry. pers. comm.).

The direct gene transfer methods are microinjection, electroporation, chemical methods and biolistic methods. Electroporation is in most cases used for transformation of protoplasts (e.g. Shillito et al. 1985) but the technique may have wider applications (Dekeyser et al. 1990), too. The biolistic methods are shown to be very promising in the transformation of trees (e.g. McCown et al. 1989, Stomp et al. 1990, Ellis et al. 1991). The results of our studies (Häggman and Stomp 1990b) have shown that when using the biolistic method the foreign genes are expressed in Scots pine cotyledons. So far a transformed conifer has not been introduced but it will be interesting to see what laboratory will have that honour.

Gene transfers, prospects and limitations

First attempts to transform plants with foreign DNA were made during the 1970s. A major advance occured when chimeric genes were constructed in which the coding regions of foreign genes were inserted between the signals controlling gene expression in plants — upstream promoters and downstream polyadenylation sites (Herrera-Estrella et al. 1983). At first the natural gene transfer ability of A*grobacterium tumefaciens* was exploited to insert into plant cells the bacterial Tn5 neomycin phosphotransferase coding sequence under the control of nopaline synthase expression signals from the T-DNA of *Agrobacterium*. This gene confers resistance to antibiotic kanamycin. The results established the integration and expression of chimeric gene in plant cells and further on the sexual transmission of the foreign DNA to progeny of transgenic plants in segregation ratios typical of simply inherited Mendelian genes.

Thus from the very beginning the gene tranfer approach has included three parts 1) isolation and characterization of spesific genes , 2) gene transfer into plant, and 3) testing of transformed plants and their progeny. At the moment the basic importance of the gene transfer technique is based on the increasing knowledge of gene regulation of specific traits. Especially when talking about trees with long rotation times gene transfer techniques promise revolutionary possibilities for acceleration of breeding work. One of the limitations of the use of this technique is the fact that until know it has not been possible to insert DNA at specific locations within plant genome. The different locations mean both variable levels of expression in individually transformed plants and that some other genes can be switched on or off. The testing of transgenic plants and their progeny is vitally important. The significance of the technique to practical forest tree breeding will be seen in the future. Guidelines for in field testing of transgenic plants in Finland are under development and they will resemble the ones of EEC.

Conclusion

Forest tree breeding involves manipulation of genetic composition of populations and individuals, and biotechnology focuses on selected individuals. The new techniques cannot replace the conventional breeding techniques but both need effective cooperation of each other. Thus the distinction between conventional breeding and biotechnology is artificial. Of course all of the biotechnological methods are new and fast developing and the future with field and progeny testing will show which techniques will be permanently adopted into tree breeding. For instance, the great hopes of the use of somaclonal variation as a new source of variability and a powerful tool for the breeder about ten years ago seem today be quite the opposite. Somaclonal variation constituting a major problem in present-day micropropagation is due to the unpredictable variation. Based on the knowledge of today especially micropropagation via somatic embryos, transgenic trees and the identification of major genes seem to be good candidates to be permanently adopted into tree breeding. It will be interesting to see how these candidates have done after ten years.

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