GENETIC ENGINEERING IN FOREST TREES

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Abstract.--Gene transfer, using recombinant DNA technology, can be used to engineer new, improved trees in a fraction of the time required by traditional breeding methods. Genetic engineering requires isolation of genes, their multiplication in bacteria, their transfer to tree cells, and regeneration of the transformed cells into new trees. Success has already been achieved in cloning conifer genes and in developing a transfer system, and several genes of potential value to forestry have been isolated from bacteria. The inability to regenerate conifers from transformed cells is the major remaining barrier to application of genetic engineering in tree improvement.

Additional keywords: Agrobacterium tumefaciens, Pinus lambertiana, Pinus taeda, Cronartium ribicola, genetic transformation, isozymes, heterozygosity, microinjection, recombinant DNA, biotechnology.

INTRODUCTION

The long life and large size of trees have always been major barriers to progress in forestry, especially in forest genetics and tree breeding. To surmount these barriers, forest biologists tried to develop techniques to enable early evaluation of growth and disease resistance and to shorten the reproductive cycle (e.g., Kinloch and Comstock 1980, Ledig 1974). However, recent advances in molecular biology offer entirely new possibilities for tree improvement. Instead of devising techniques for early evaluation, it is now possible to direct genetic changes while bypassing the sexual cycle, at least in particular instances (Sederoff and Ledig 1985). Using new biotechnologies, improvements in forest trees can conceivably be made on the same time scale as those in agricultural crops, and the large size of trees, which presently restricts selection intensity, poses no difficulties for technologies that operate on the cellular or molecular level.

The new capability for biological manipulation using such tools as genetic transformation, parasexual hybridization by fusion of protoplasts, and multiplication of high value materials by cloning, have captured the public imagination like few other scientific developments. Our concepts of life are being changed as surely as they were by the public announcement of Darwin and Wallace's theory of evolution. If it is necessary to identify the beginning of the current revolution, then 1953 is a good candidate, when Watson and Crick published their classic paper on the structure of DNA. Since that time, knowledge of the genetic material and the ability to use that knowledge have been accelerating. The new genetic tools are much more powerful than the ones provided by Mendelism and its rediscoverers.

Application of the new technologies in forestry will require a major research effort. Our ignorance of the genetics, physiology, and biochemistry

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of forest trees, their pests and pathogens is as deep as our opportunities are broad. Until recently, fundamental research, such as studies of biosynthetic pathways, photosynthesis, and stress metabolism, had little application because they only explained how things worked, without providing ways to modify the genetic controls. Now, basic studies have greater utility because they provide information that can be used to modify processes to advantage. Many aspects of biology, physiology, pathology, and biochemistry, have been integrated by the new genetics. To narrow the subject, we concentrated on the possibilities of direct genetic manipulation of forest trees (i.e., genetic engineering) and the research needed to apply the technology.

Genetic engineering implies directed genetic change in individuals, and subsequently, in populations. Directed change is not new. During prehistory, early agriculturists brought about desirable changes in plants and animals despite little formal knowledge of genetics. With the discovery of the statistical laws of inheritance in the nineteenth and twentieth centuries, breeders accelerated the rate of change in agriculturally important plants and animals. However, genetic engineering now implies manipulations at the cellular or molecular level, and one of the most powerful tools of genetic engineering is transformation, the ability to insert new genes. Transformation provides both an applied tool and a method of studying the nature of the gene.

TRANSFORMATION OF FOREST TREES

The Process

The insertion of a gene into a new host, thereby genetically "transforming" the host, has four components: a DNA fragment consisting of a single gene or a small block of genes must be identified and isolated; the block must be inserted into a vector where it is multiplied; the foreign DNA must be transferred to the host cell where it is incorporated and expressed; and the transformed cells must be regenerated into a plant. The process of DNA-directed transformation was first discovered in bacteria (Avery, MacLeod, and McCarty 1944), but in recent years has been applied to cells of higher plants (Goldsbrough et al. 1983, Murai et Al. 1983), such as sunflower (Helianthus annuus L.) and tobacco (Nicotiana tabacum L.). Application to trees is not completely straightforward because of some unique aspects of their biology and because of the general lack of past effort in basic forest research.

The Genes

Single-gene traits in trees. Few simply inherited traits are known in forest trees, and most of these are of little or no economic interest. They can be divided into four major classes: isozymes, major visible aberrations, terpenes and other volatiles, and disease resistance factors. As many as 60 isozyme loci are known in some species (Conkle et al. 1982), but there seems to be no advantage in transferring them. Aberrations, such as albinism and dwarfism (Franklin 1970), are of negative value except perhaps for the narrow-crowned phenotype, considered to be inherited as a single gene in some European conifers (Karki 1983). Many volatiles are simply inherited and may have potential in conferring resistance to insects (Smith 1966), and in some cases, as valuable extractives. Disease resistance is the class of genes of obvious value for transfer among trees (Kinloch, Parks, and Fowler 1970).

Research at the Institute of Forest Genetics is directed toward transfer of genes that could improve yield or value of forest trees. One of these objectives is the eventual isolation and transfer of the major gene for white pine blister rust (Cronartium ribicola J.C. Fisch. ex Rabenh.) resistance from resistant sugar pine (Pines lambertiana Dougl.) to susceptible individuals and species.

Determining the mode of inheritance of genetic characteristics (single genic or polygenic) is especially difficult in forest trees because it is plagued with one of the traditional barriers confronting forest genetics; i.e., the long generation time. One or two generations of crosses must be made to demonstrate Mendelian segregation, and even then the simultaneous segregation of genes with pleiotrophic effects may make it difficult to draw definitive conclusions. Trees are among the most heterozygous of organisms (Hamrick 1979), so the genetic background in most species is highly heterogeneous, obscuring the effects of segregation at individual loci.

On the other hand, conifers have some advantages for genetics. Many genes code for enzymes that are active in the megagametophyte, the nutritive tissue or "endosperm" of the seed. The megagametophyte is a haploid tissue that is derived from one of the four cells produced by meiosis (e.g. Allen and Owens 1972). Segregation can be detected as variation among seed (megagametophytes) from the same cone or from different cones on the same tree. A sample of several megagametophytes will show a 1:1 ratio of allelic types in a heterozygous individual. In classic Mendelian genetics, a 1:1 ratio is usually demonstrated by a "test cross", but use of the conifer megagametophyte eliminates the need for test-crossing (Conkle 1974). Therefore, for allozyme loci, conifers provide the advantages of haplogenetics, pioneered in fungi such as the bread mold (Neurospora crassa; Barratt et al.. 1954).

Isolating genes. To isolate a gene, the DNA is cleaved with restriction enzymes and the fragments are spliced into the DNA of a self-replicating virus or plasmid, called a "vector", that infects bacterial cells. When the vector with its foreign DNA infects a bacteria, the fragment is multiplied, or "cloned", along with the vector's DNA. The colon bacteria (Escherichia coli) is a common organism used to clone DNA fragments, and a collection of bacterial colonies, each incorporating a different fragment, forms a "library" of the donor's DNA. There is often no way to tell which fragment carries the gene of interest unless a similar gene, previously isolated from another species, is available to "probe" for it with DNA-DNA hybridization techniques.

Isolation of genes in conifers would be difficult even if genes worthy of transfer were known. The conifer genome is very large, apparently 34.7 pg for 2C content in sugar pine (Dhillon 1980). By comparison, the genome of corn (Zea mays L.) is only about 11 pg (Bennett 1972), which itself is large compared to many animal species. The human genome is only 7.3 pg (Bachmannn 1972), and many insects have 2C contents that are another order of magnitude smaller, around 0.2 pg for fruit flies (Sparrow, Price, and Underbrink 1972).

Linkage mapping. Knowing where a gene is located is important if it is to be isolated. Linkage maps for conifers are very incomplete, and no genes have been associated with individual chromosomes. If genes could be identified to chromosome, it might be possible to rapidly sort out specific chromosomes with dual laser flow sorters (Dickson 1985). The task of constructing a fragment

library for a single chromosome would be less than one-tenth as difficult as constructing a library for the entire genome. Inserting entire chromosomes in plant cells, rather than fragments, is another possibility (Malmberg and Griesbach 1983), although aneuploids are unstable and usually aberrant in conifers (Mergen 1958, 1959). However, even isolating a chromosome would be difficult in forest trees, given present knowledge. For example, the 12 chromosomes of the haploid set that characterize most of the family Pinaceae are scarcely distinguishable with conventional stains (e.g., Saylor 1961). In most of the pines, spruces (Picea spp.), and firs (Abies spp.) only the smallest, heterobrachial chromosome can be identified with confidence. The others are all homobrachial and similar in size. Newer radiological and staining techniques employed in human cytogenetics may be fruitful. Recently, Hizume, Ohgiku, and Tanaka (1983) claimed to distinguish all of the chromosomes of Austrian pine (Pinus nigra Arnold) with fluorescent banding, but very little present research effort is focused on the conifer karyotype.

There is a chance of finding linkage between allozyme loci and genes controlling per characteristics, such as disease resistance. M.T. Conkle and B.B. Kinloch [1] (personal communication) have already demonstrated loose linkage (27 map units) between the major gene for blister rust resistance in sugar pine and a 6-phosphogluconate dehydrogenase locus. For isozyme loci to be really useful for isolating genes with unknown products, such as the gene for blister rust resistance, the two must be very tightly linked. Linkage maps are being constructed for several species in the Pinaceae (e.g., Conkle 1981). Because of the apparently high degree of conservatism in evolution of the conifer karyotype, linkage maps in one conifer are likely to approximate those in others. The same linkages are repeated in the pines, firs, and spruces investigated so far (e.g., Conkle 1981, King and Dancik 1983, Neale and Adams 1981).

Restriction site mapping using enzymes that cut the DNA at specific base sequences, combined with isozyme mapping, would provide an extensive map in a short time. The development of isozyme technology in conifers provided a rapid means for chromosome mapping, but its utility is limited; only about 60 isozyme marker loci are available. While 60 is a considerable number, especially compared to virtually none 10 years ago, restriction fragment mapping could expand the number of markers to hundreds. Recombinant DNA techniques do not depend on expression of a gene; fragments can be assayed at any time. By contrast, genes coding for enzymes, such as alcohol dehydrogenase, may be expressed only during a restricted period of development or in certain tissues (Conkle 1971). Furthermore, fragments need not include functional genes in order to be valuable markers. Any fragment can be used that can be recognized by its banding pattern in molecular hybridization analysis.

Genes in heterozygous combination. Of special interest is the relation between heterozygosity and growth. In trees, growth and fitness are closely related, and they are correlated with heterozygosity. The notion that vigor and heterozygosity are related is not new; explanations for hybrid vigor, or heterosis, go back at least to the work of East and Shull over three-quarters of a century ago (Shull 1952) and was the subject of Lerner's (1954) classic book, "Genetic Homeostasis". However, the development of enzyme

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electrophoresis revealed variation of such proportions that it was difficult to explain it all as a result of balancing selection; i.e., selection favoring heterozygotes (Lewontin 1974). Nevertheless, in a variety of organisms, including trees, growth and heterozygosity for isozyme loci are positively correlated in natural populations, a new finding (Ledig, Guries, and Bonefeld 1983). These results must be extended to additional species and to controlled environments, to determine their generality.

The newly found correlation between growth and heterozygosity in forest trees raises several questions regarding the conduct of tree improvement programs. In the initial stage of tree improvement programs, trees are selected one per stand and interplanted as grafted clones in seed orchards. Are realized gains from seed orchards the result of crossing among unrelated parents, which would favor heterozygosity? If so, will gains from a second generation of selection be much lower than expected? When trees are selected in natural populations based on growth, do heterozygotes have a greater probability of selection? Would a better scheme be to select and cross trees that differ at the maximum possible number of loci to produce highly heterozygous progeny?

Before the proper tree improvement strategy can be identified, research is needed to determine whether all the isozyme loci have an effect on growth or just specific genes. And, do all the loci involved have equal effects or are some more important than others? In fact, do the isozyme loci themselves control growth rate or are they simply linked to other, more important genes? Heterosis could actually be the result of inferiority of homozygotes at linked deleterious loci. Inbreeding depression is the converse of heterosis.

Transformation offers a way to investigate some of these questions. It would be relatively simply to isolate alternative alleles of isozyme loci and introduce them into a homozygous background to investigate the effect of heterozygosity at single loci. Torrey pines (Pinus torreyana Parry **ex** Carr.) are a prime target, because they seem to be completely homozygous (Ledig and Conkle 1983). Of course, regeneration of conifers from transformed cells is still a barrier to completing such a critical experiment.

Research needs. If genes are to be isolated from forest trees, forest biologists will need several types of knowledge: an understanding of the physiological and biochemical mechanisms of traits of interest, their mode of inheritance, and the gene products involved. Molecular geneticists will profit from better linkage maps of the conifer genome. Tight linkage with genes for which probes are available would facilitate isolation of the right fragment.

At first, genetic engineering in forestry will rely on genes from other organisms because of the paucity of economically important, single-gene traits identified in tree species. In fact, forestry will benefit from the much larger research effort in agriculture and medicine. Genes for insertion in conifers or hardwoods can come from any living system, bacterial, fungal, plant, or animal. Some candidates for transfer are herbicide resistance and salt tolerance (Chaleff and Ray 1984, Le Rudulier et al. 1984). Incorporation and expression must be investigated in tree species if forestry is to make use of genes from other organisms. At present we know little about the structure of the genome in tree species -- why do conifers have so much highly repeated DNA? Do conifer genes have introns? What are the promoters like? DNA content

may vary among populations and individuals: is DNA content itself adaptive, perhaps related to drought or cold hardiness? These questions are researchable and should be attacked early in any program of genetic engineering.

The Cloning-Vectors

Cloning conifer DNA. Few difficulties are anticipated in cloning conifer DNA. R.R. Sederoff and P.D. Hodgskiss have inserted two copies from a highly repeated fraction of the sugar pine genome into the bacterial virus M13 and multiplied them in the colon bacteria. They have sequenced segments of about 400 base pairs in length and will extend this in the near future. These DNA clones will be useful probes to determine where the sequence occurs in the sugar pine genome and its homology with the highly repeated fraction in other pines and more distantly related conifers.

The crown gall bacterium. Transformation is being approached from two directions: through the use of the crown gall bacterium (Agrobacterium tumeraciens) and by direct microinjection. Crown gall is the most widely-used system for transformation in higher plants (Barton and Chilton 1983). It carries a loop of DNA, the Ti plasmid. In an infected plant, part of the plasmid DNA takes up residence in a linear chromosome of the host. The plasmid genes are faithfully transcribed by the host, resulting in production of substances necessary for growth and reproduction of the bacterium. The plasmid DNA has been mapped, and can be modified to carry foreign genes, providing a means to transform selected host-plants. However, crown gall was not known to infect pines, although it had been reported on firs, incense cedar (Calocedrus decurrens [Torr.] Florin), and other conifers (de Cleene and de Lay 1976). Within the last year, R.R. Sederoff, A. Stomp, L. Moore, and W.S. Chilton have found a strain that will transfer and express genes from the crown gall bacterium in loblolly pine (Pinus taeda L.).

Microinjection. Microinjection is a direct way of introducing DNA into target cells, and has been used successfully in animal systems (Lo 1983). Very fine needles are guided into isolated, suspension-cultured cells, using micromanipulators. DNA is moved from the needle into the cell by altering the charge. Either vectors, such as the Ti plasmid, or "raw" DNA fragments can be "injected". There are still many technical difficulties in applying the procedure to conifer cells. Primary among these is the difficulty of penetrating the thick cell wall. It may be simpler to inject naked protoplasts (i.e., cells whose walls have been stripped by a cellulase enzyme). However, for most conifers it has not been possible to regenerate viable cell suspension cultures from protoplasts, although Teasdale and Rugini (1983) were successful with loblolly pine. And it is not at all certain that injected DNA will move into the nucleus, be incorporated in the conifer genome, or if incorporated, be expressed. D.E. Harry and M. Freeling of the University of California at Berkeley are working on these problems in cooperation with the Institute of Forest Genetics.

Selectin^{g} transformed cells. Transformation usually happens with low frequency, so transformed cells must be selected from among a larger population of untransformed cells. A common way to accomplish this is to engineer a vector that will permit easy identification of cells in which it has incorporated. An example is the use of the kanamycin-resistance gene (neomycin phosphotransferase, or NPT) from the colon bacterium, which has been spliced

into several plant vectors. When plant cells are plated onto agar with G418, an aminoglycoside antibiotic that can be inactivated by NPT, only those that have been transformed (i.e., those that have incorporated and expressed the gene) survive.

For some genes, like the major gene for resistance to white pine blister rust, direct selection may be possible. Fungal mycelia invade sugar pine cells in callus culture, and resistance is expressed on the cellular level by a hypersensitive reaction (Diner, Mott, and Amerson 1984). Following microinjection, cell lines could be multiplied, subdivided, and one replicate challenged by the fungus to identify transformed lines.

The Final Step: From Transformed Cells to Trees

The inability to regenerate whole plants from transformed cells is the greatest barrier to genetic engineering of conifers: there is no guarantee that research efforts will be rewarded in the near future. On the other hand, whole plantlets have been regenerated from cell and tissue culture in some hardwoods (Karnosky 1981). Several laboratories in the United States, Canada, and other countries are attempting to induce somatic embryogenesis in pines and Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco), so far without success. Perhaps, the problem should be sidestepped rather than met head-on.

For example, the megagametophyte of conifers has some features that might be used to circumvent the problem of regenerating plantlets from cells vitro. During an extended period of time, the megagametophyte is in a free nuclear state (i.e., the nuclei are not separated by cell walls). Mitotic divisions result in over a thousand nuclei before cell wall formation begins, and some of these nuclei differentiate into eggs (e.g. Allen and Owens 1972). If DNA fragments or vectors could be injected into the megagametophyte during the free-nuclear stage, they would be unimpeded by cell walls and, hopefully, incorporate in the conifer DNA at a high rate. The target is large, apparently up to 0.7 mm for an egg cell alone in sugar pine (Haupt 1941). Judging by the size of the free-nuclear megagametophyte in Douglas-fir (Allen and Owens 1972), the free-nuclear gametophyte in sugar pine may be several millimeters long. Injury from injection should not cause irreversible damage to the megagametophyte; e.g., seed bugs sometimes penetrate the megagametophyte without destroying it (Krugman and Koerber 1969). After differentiation of the egg and fertilization, the ovule could follow its normal course of development and mature an embryo. It may be better to use the system in this way rather than attempt to force conifer cells to do something they do not normally do (i.e., undergo somatic embryogenesis). However, research is needed to develop techniques for the direct injection of megagametophytes through the cone scales in such a way that the cone can continue its normal development.

Regeneration of plants from cell and callus cultures remains the most critical need in forest research. Other barriers to genetic engineering already show signs of cracking, but there have been no major breakthroughs in conifer regeneration. Without the capability of producing trees from cell culture, the full benefits of transformation will not be realized. The inability to regenerate trees from cells or callus *is* not only a block to the use of genetic engineering, it prevents forestry from making full use of the products of conventional selection and hybridization. There are several interspecific hybrids and some desirable intraspecific crosses that cannot be

economically multiplied, and mass cloning would be an especially valuable technique. One research approach would be the intensive study of embryogenesis to chart the path of normal development, providing a guide to the necessary steps in vitro.

CONCLUSION

Recombinant DNA technologies will make it possible to modify trees on a time scale comparable to that of annual crops. Furthermore, manipulations at the cellular level will result in greater gains than previously possible by eliminating the barrier posed by the large size of trees; as long *as* whole plants had to be evaluated in the field, selection intensity could never be as great for space-consuming trees as for relatively smaller agricultural plants.

Already there are indications that these technologies can be applied to conifers and hardwoods. Within the last year it has been possible to demonstrate the insertion of the Ti-plasmid from crown gall in pine and prove gene expression. DNA cloning and sequencing techniques have worked as well on conifers as on other plants. While there are still only a few valuable, single-gene traits known in forest trees, there are many markers, and linkage maps are being constructed for conifer genomes. The massive research effort in medical and agricultural sciences will provide valuable genes for the genetic engineering of trees just as it has provided the tools. However, forest biology cannot rely entirely on research in sister sciences.

Research should proceed on four parallel lines: 1) the genetic system of forest trees; 2) transfer systems; 3) the physiological and biochemical basis of valuable traits; and 4) the developmental path leading to regeneration from cell culture. Of these, work on the genetic system and transfer systems shows signs of progress.

With respect to research on physiological processes and gene products, it is time to stop treating tree growth, form, disease resistance, etc. as black boxes. While traditional breeding using the metrics of quantitative genetics has proved quite successful, more effort is needed to identify underlying mechanisms for important processes, their genetic control, and gene products if forest genetics is to realize its full potential.

Regeneration of trees from cell and tissue culture remains the major barrier to progress in forest genetics, and not because of lack of effort. Yet, there is no reason that this barrier too cannot be overcome. When it is, forestry will reap enormous benefits.

LITERATURE CITED

Allen, G.S. and J.N. Owens. 1972. The life history of Douglas-fir. Environ. Can., For. Serv., Ottawa. 139 pp.

Avery, 0.T., C.M. MacLeod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J. Exper. Med. 79: 137-158.

Bachmann, K. 1972. Genome size in mammals. Chromosoma 37: 85-93.

Barratt, R.W., D. Newmeyer, D.D. Perkins, and L. Garnjobst. 1954. Map construction in Neurospora crassa. Adv. Genet. 6: 1-93.

Barton, K.A. and M.D. Chilton. 1983. Agrobacterium Ti plasmids as vectors for plant genetic engineering. Pages 527-539 in: Methods in Enzymology, vol. 101. Academic Press, N.Y.
Bennett, M.D. 1972. Nuclear DNA content and minimum generation time in herbaceous plants. Proc. Roy. Soc. Lond., B 181: 109-135.
Chaleff, R.S. and T.B. Ray. 1984. Herbicide-resistant mutants from tobacco cell cultures. Science 223: 1148-1151.
Cleene, M. de and J. de Lay. 1976. The host range of crown gall. Bot. Rev. 42: 389-466.
Conkle, M.T. 1971. Isozyme specificity during germination and early growth of knobcone pine. For. Sci. 17: 494-498.
Conkle, M.T. 1981. Isozyme variation and linkage in six conifer species. Pages 11-18 in: M.T. Conkle (tech. coord.), Proc. Symp. Isozymes No. Am. For. Trees and For. Insects, Berkeley, CA July 1979. USDA For. Serv. Gen. Tech. Rep. PSW-48.
Conkle, M.T., P.D Hodgskiss, L.B. Nunally and S.C. Hunter. 1982. Starch gel electrophoresis of conifer seeds: a laboratory manual. USDA For. Serv. Gen. Tech. Rep. PSW-64. 18 pp.
Dhillon, S.S. 1980. Nuclear volume, chromosome size and DNA content in three species of Pinus. Cytologia 45: 555-560.
Dickson, S. 1985. National laboratories establishing human gene libraries. Genet. Eng. News 5 (1): 16, 35.
Diner, A.M., R.L. Mott, and H.V. Amerson. 1984. Cultured cells of white pine show genetic resistance to axenic blister rust hypae. Science 224: 407-408.
Franklin, E.C. 1970. Survey of mutant forms and inbreeding depression in species of the family Pinaceae. USDA For. Serv. Res. Pap. SE-61. 21 pp.
Goldsbrough, P.B., *S.J.* Karcher, S.B. Gelvin, and B.A. Larkins. 1983. Introduction of a zein gene into the Ti plasmid of Agrobacterium tumefaciens. Pages 35-43 in: Plant molecular biology. Alan R. Liss, Inc., N.Y.
Hamrick, J.L. 1979. Genetic variation and longevity. Pages 84-133 in: O.T. Solbrig, S. Jain, G.B. Johnson, and P.H. Raven (eds.), Topics in plant population biology. Columbia Univ. Press, N.Y.
Haupt, A.W. 1941. Oogenesis and fertilization in Pinus lambertiana and E. monophylla. Bot. Gaz. 102: 482-498.
Hizume, *M.,* A. Ohgiku, and A. Tanaka. 1983. Chromosome banding in the genus Pinus. I. Identification of chromosomes in P. nigra by fluorescent banding method. Bot. Mag. Tokyo 96: 273-276.
Karki, L. 1983. Crop tree idiotypes and harvest index should be the basis of selection in cultivated trees. Pages 26-27 Ix Metsanjalostussaatio, Ann. Rep. Found. For. Tree Breeding, Helsinki, Finl.
Karnosky, D.F. 1981. Potential for forest tree improvement via tissue culture. BioScience 31: 113-120.
King, J.N. and B.P. Dancik. 1983. Inheritance and linkage of isozymes in white spruce (Picea glauca). Can. J. Genet. Cytol. 25: 430-436.
Kinloch B.B. and M. Comstock. 1980. Cotyledon test for major gene resistance to white pine blister rust in sugar pine. Can. J. Bot. 58: 1912-1914.
Kinloch, B.B., G.K. Parks and C.W. Fowler. 1970. White pine blister rust: simply inherited resistance in sugar pine. Science 167: 193-195.
Krugman, S.L. and T.W. Koerber. 1969. Effect of cone feeding by Leptoglossus occidentalis on ponderosa pine seed development. For. Sci. 15: 104-111.

Ledig, F.T. (ed.). 1974. Toward the future forest: applying physiology and genetics to the domestication of trees. Bull. No. 85, Sch. For. Environ. Studies, Yale Univ., New Haven, Connecticut. 80 pp.

Ledig, F.T. and M.T. Conkle. 1983. Gene diversity anti genetic structure in a narrow endemic, Torrey pine (Pinus torreyana Parry ex Carr.). Evolution 37: 79-85.

Ledig, F.T., R.P. Guries, and B.A. Bonefeld. 1983. The relation of growth to heterozygosity in pitch pine. Evolution 37: 1227-1238.

Lerner, I.M. 1954. Genetic homeostasis. Oliver and Boyd, Edinburgh-Lond. 134 pp.

Le Rudulier, D., A.R. Strom, A.M. Dandekar, L.T. Smith, and R.C. Valentine. 1984. Molecular biology of osmoregulation. Science 224: 1064-1068.

Lewontin, R.C. 1974. The genetic basis of evolutionary change. Columbia Univ. Press, N.Y. - Lond. 346 pp.

Lo, C.W. 1983. Transformation by ionophoretic microinjection of DNA: multiple integrations without tandem insertions. Molec. Cell Biol. 3: 1803-1814.

Malmberg, R.L. and R.J. Griesbach. 1983. Chromosomes from protoplasts--isolation, fractionation, and uptake. Pages 195-201 in: T. Kosuge, C.P. Meredith, and A. Hollaender (eds.), Genetic engineering of plants: an agricultural perspective. Plenum Press, N.Y. - Lond.

Mergen, F. 1958. Natural polyploidy in slash pine. For. Sci. 4: 283-295.

Mergen, F. 1959. Colchicine-induced p[olyploidy in pines. J. For. 57: 180-190.

Mural, N., D.W. Sutton, M.G. Murray, J.L. Slightom, D.J. Merlo, N.A. Reichert, C. Sengupta-Gopalan, C.A. Stock, R.F. Barker, J.D. Kemp, and T.C. Hall. 1983. Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222: 476-482.

Neale, D.B. and W.T. Adams. 1981. Inheritance of isozyme variations in seed tissues of balsam fir (Abies balsamea). Can. J. Bot. 59: 1285-1291.

Saylor, L.C. 1961. A karyotypic analysis of selected species of Pinus. Silvae Genet. 10: 77-84.

Sederoff, R.R. and F.T. Ledig. 1985. Increasing forest productivity and value through biotechnology. In Weyerhaeuser For. Potentials Symp., Tacoma, Wash. (In press)

Shull, G.H. 1952. Beginnings of the heterosis concept. Pages 14-48 110 *J.W.* Gowen (ed.), Heterosis. Iowa State Coll. Press, Ames.

Smith, R.H. 1966. Resin quality as a factor in the resistance of pines to bark beetles. Pages 189-196 H.D. Gerhold (ed.), Breeding pest-resistant trees. Pergamon Press, N.Y.

Sparrow, A.H., H.J. Price, and A.G. Underbrink. 1972. A survey of DNA content per cell and per chromosome of prokaryotic and eukaryotic organisms: some evolutionaty considerations. Pages 451-495 H.H. Smith (ed.), Evolution of genetic systems. Gordon and Breach, N.Y.-Lond.-Paris.

Teasdale, R.D. and E. Rugini. 1983. Preparation of viable protoplasts from suspension-cultured loblolly pine (Pinus taeda) cells and subsequent regeneration to callus. Plant Cell Tissue Organ Culture 2: 253-261.

Watson, J.D. and F.H.C. Crick. 1953. Molecular structure of nucleic acids. Nature 171: 737-738.