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Pines as Model Gymnosperms to Study Evolution, Wood Formation, and Perennial Growth

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Abstract

Pines provide a model system for the gymnosperms, an old and successful group of vascular plants that last shared a common ancestor with the angiosperms about 285 million years ago. Gymnosperms are distinct from angiosperms in their reproduction, development, metabolism, adaptations, and evolution. Pines cover vast areas of the globe, are one of the most important genera of forest trees, dominate the ecology of many temperate and subtropical forest ecosystems, and provide a major fraction of the world's wood. Here, we summarize many features of pine that make it a useful model for gymnosperms and woody plants. We also describe the influence of its reproductive system on methods for genetic analysis and the prospects for genomic studies and genetic engineering. Pines are limited as model systems by their long generation times, large size, large genomes, and the long time from fertilization to seed set.

Key words: Forest biotechnology; Genomics; Gymnosperms; *Pinus*; Plant longevity; Plant reproduction; Wood formation; Wood structure; Xylogenesis

Introduction

What is needed to become a model plant? *Arabidopsis thaliana* is a model plant for genetic studies because of its short generation time, small genome, and the ease of genetic manipulation (Meyerowitz 1989). The small size permits large populations to be easily grown and analyzed. Nevertheless, it took several decades for *Arabidopsis* to become the leading model plant (Rédei 1992). "Know your organism" should be the first principle of a biological study. A thorough understanding of the biology of any organism should be the basis for choosing research questions. A model system should have specialized features that provide general information on fundamental biology.

Pines are valuable as model organisms because they are the best characterized gymnosperms, one of the two major groups of seed plants. Their development, reproduction, ecology, and genetics are well documented. Unique features for genetic analysis are provided by the haploid megagametophyte and the ability to carry out intensive genetic analysis on individual trees in natural populations or in breeding programs. Pines also provide a biochemical model for the biosynthesis of plant cell walls because of the large amount of differentiating xylem that may be obtained and the highly specialized process of wood formation (xylogenesis).

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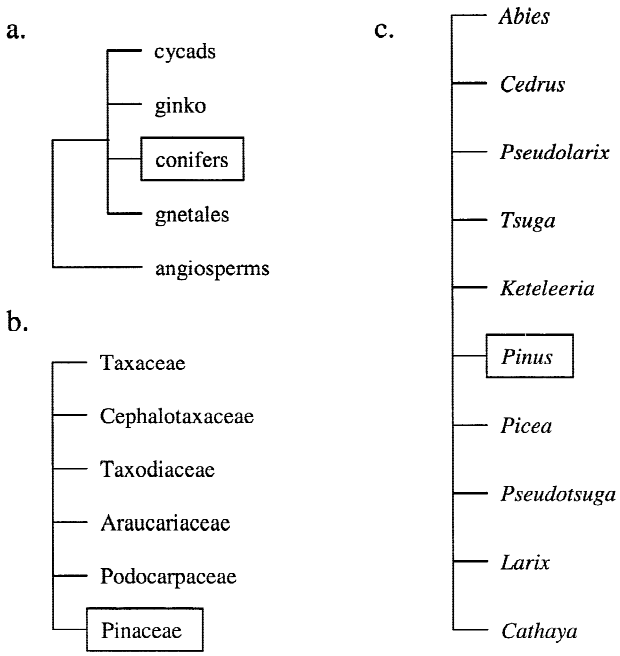

Figure 1. Relationships of pines. (a) Relationships of gymnosperms and angiosperms; (b) families of conifers; (c) genera of the Pinaceae. Compiled from Shaw (1914), Mirov (1967), Gifford and Foster (1989) and Price and others (1998).

Gymnosperms are a taxon with about 600 perennial species, which represent an ancient and extant link between the seedless ferns and the flowering angiosperms. The genus *Pinus* comprises at least 111 tree species and belongs to the Pinaceae, a family with about 200 tree species in 10 to 11 genera (Figure 1). Pines are dominant or important members of the vegetation in vast areas of Europe, Asia, and North and Central America (Richardson and Rundel 1998). All pines are evergreen, whether they grow north of the polar circle in northern Norway, with winter temperatures approximating -50°C, or in subtropical regions with summer temperatures over 40°C. Their leaves are long needles, carried in fascicles of one to five. Pines grow naturally or are planted in considerable areas of the world and are of great economic importance, probably second only to cereals.

Five pine species, in our view, are the most significant, on the basis of their biology, geography, and economic importance. Three of these species lead in economic importance: *P. radiata* (monterey pine), *P. taeda* (loblolly pine), and *P. sylvestris* (scots pine). We have also included the two pines with the greatest longevity, the bristlecone pines, *P. aristata* and *P. longaeva*. The economically dominant pines (*P. radiata, P. taeda,* and *P. sylvestris*) differ greatly in their natural distribution (Critchfield and Little 1966; Richardson and Rundel 1998). The natural range of *P. radiata* is restricted to several small areas with limited ecological variability, in California, Mexico, and Guadalupe island. *Pinus taeda* has a wide distribution in the southeastern United States. *Pinus sylvestris* has the largest distribution of all pines and covers vast areas of considerable ecological diversity in Asia and Europe. *Pinus sylvestris* has been planted widely, but the extent of its natural populations far exceeds the planted areas. *Pinus taeda* is probably the most widely planted pine species in the world, covering approximately 134,000 km^2, mostly plantations, cutover forest, and abandoned farmland (Schultz 1999). *Pinus radiata* is now widely planted outside of its natural range and is the most commonly planted pine in the southern hemisphere. These pine species of economic importance have been the best studied and have been under genetic selection for several generations.

The most important commercial product of pines is wood. Wood is one of the world's leading industrial raw materials and is probably the most common renewable natural resource. Understanding the structure and the formation of wood is essential for improving wood to increase its economic value. Anatomically, wood is secondary xylem, the major product of cambial activity, and the basic component that characterizes trees. Most pine wood is composed of tracheids and a minor component of ray cells and resin ducts. Wood is the largest carbon sink in trees and is a significant component of the terrestrial carbon cycle.

The life history of pines, as with other trees, represents an evolutionary compromise between the survival and fecundity of individuals, which is affected by biotic and abiotic factors. Many pine species perform well in various types of disturbed environments, affected, for example, by fire, flooding, or wind. Pines are tolerant of stressful environments, probably because of their xylem structure, which is less sensitive to cavitation. They exploit poor soils, have winged seeds (Lanner 1998), and are able to invade disturbed habitats. Certain pines have a thick bark, serotinous cones, and early reproduction, which enable them to occupy habitats where fire is common. The life history of pines was recently and extensively reviewed (Keeley and Zedler 1998).

Bristlecone pines attain exceptional age among nonclonal organisms. *Pinus aristata* and *P. longaeva* from the southwestern United States may become 5,000 years old (Currey 1965; Ferguson 1968). Although individuals of certain pine species such as *P. lambertiana* (sugar pine) may be 80 m high (Fowells

1965), the oldest bristlecone pines are usually only 5 to 15 m tall. Pines normally show a gradual increase in tracheid length and width with age, with distance from the pith, and with distance from the young leaves. In *P. longaeva,* tracheid length continued to increase even at the age of 2,200 years (Baas and others 1986).

Strengths of the Model System

1. Pines as gymnosperms represent an important link in the evolution of higher plants.

2. The developmental and reproductive biology of pines has been well characterized.

3. Pines have well-developed structural and chemical defense systems.

4. Pines provide a model for wood structure and biosynthesis.

5. Differentiating xylem is an abundant and metabolically active tissue, specialized in the formation of secondary cell walls and in programmed cell death.

6. Pines are tractable for culture, propagation, and transformation.

7. Pine provides a model for genomics of gymnosperms.

8. The haploid pine megagametophyte has advantages for genetic analysis for tree breeding and for studies of genetics of natural populations.

9. Pines provide a renewable resource of wood and enrich the biosphere and the human environment.

The special evolutionary position of pines as gymnosperms requires study of their developmental and reproductive biology to understand the basis for their success and the early evolution of angiosperms. Pine tree development and reproduction are described in considerable detail by Mirov (1967) and Gifford and Foster (1989) (Figure 2). Cotyledons, which are leaves formed during embryogenesis, emerge above the soil surface by a germination hook (epigeous germination). The cotyledons exploit the nutrients stored in the endosperm, then the seed coat is shed. Pine embryos have many cotyledons. In the young stem above the cotyledons, juvenile needles (always single) develop. Later, mature needles appear in the axils of the primary needles and then gradually, only secondary needles develop. The number of mature needles per fascicle varies and is species specific. At the base of the needles, there is an intercalary meristem, that is, their apical growth is limited, and they elongate from their base (Mirov 1967). In some pine species, needles fall at the end of the second growing season. In other species they persist for 3 to 5 years, and in *P. aristata* for more than 30 years (Ewers 1982; Mirov 1967).

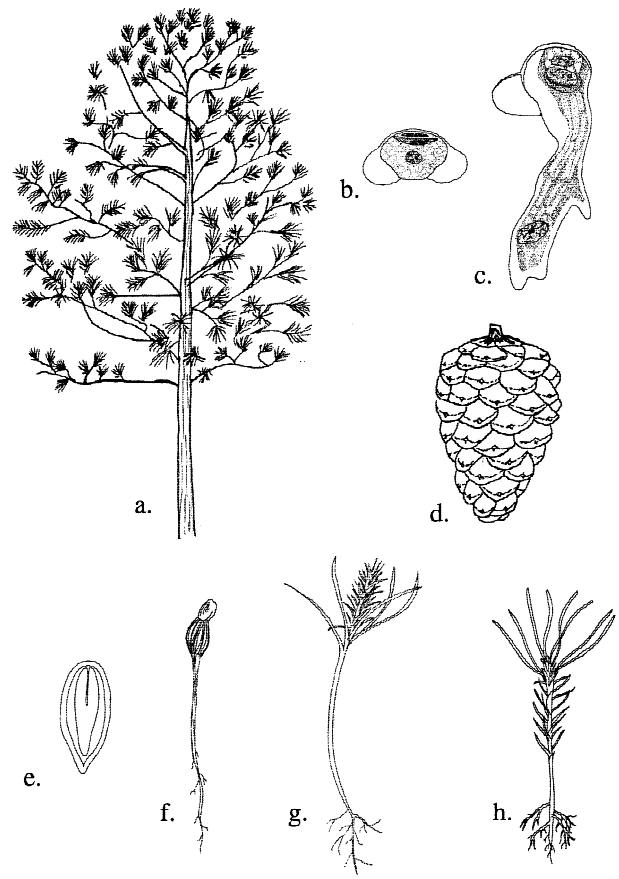

Figure 2. Some generalized life history stages of pine. (a) Mature tree; (b) pollen grain; (c) germinating pollen grain; (d) female cone (closed); (e) mature seed; (f) germinating seed; (g) seedling showing cotyledons and primary needles; (h) seedling showing secondary needles. a and e are original drawings; b and c are redrawn from Coulter and Chamberlain 1917; f, g and h are redrawn from Chamberlain 1935 (by R. Schaffer).

Only about 10% of the whole wood mass of the pine tree comprises the root system (Mirov 1967). Compared with stems, roots lack pith; their growth rings are less defined; and the cells are wider, longer, and have thinner walls. The woody tissue of roots is less lignified and less dense than stem wood (Fayle 1968). Primary growth is found at and near the tip, and secondary growth is distal to the tip (Mirov 1967). The root apex is not terminal as in the shoot, but protected by the root cap, which is formed by special initials of the root. The root cap consists of living parenchymal cells that often contain starch and may be involved in the gravitropic response of the growing root. Root cap cells secrete a polysaccharide slime that helps in penetrating the soil (Fahn 1990). The vascular cylinder (xylem and phloem)

occupies the center of the root. When roots mature, cork is formed in the outer surface, which isolates the root from the external environment. A root cambium forms secondary wood and growth rings. Many pine roots are associated with symbiotic mycorrhizal fungi.

The hormonal status that regulates differentiation in trees may vary as a result of internal and external factors, including photoperiod, light quality, water availability, temperature, and wounding (Fink 1999; Kozlowski and others 1991; Larson 1994). However, trees may also be influenced by wind action, snow load, late frost, and flooding, which affect tree growth and differentiation of wood and other tissues (Kozlowski and others 1991; Larson 1994; Timell 1986).

Structural Defenses Include Cork, Resin Ducts, and Sclereids

Pines have three major structural/chemical defense mechanisms: (1) The periderm (cork) (in roots, trunks and branches); (2) resin ducts (in all plant organs); and (3) sclerenchyma layers (within the periderm and in the female cones). The periderm is a secondary, complex, protective tissue, which in pine is composed of a meristem (phellogen, also known as cork cambium), live parenchyma cells (phelloderm), cork cells and sclereids. The periderm is part of the outer bark, and its physical and chemical properties protect the inner, living tissues from damage by abiotic agents, such as fire (Ducrey and others 1996; Vines 1968) or pathogens. Resin ducts also serve as a defense mechanism in pines (Hodges and others 1977). The constitutive production of both axial and radial resin ducts is a basic aspect of wood formation in all pines.

Wood Biosynthesis

The secondary xylem (wood) and phloem in both shoot and root are formed by the vascular cambium. The cambium differentiates to form fusiform initials that give rise to the tracheids and other axial components of the vascular system and ray initials that give rise to the radial component. In conifers, fusiform initials occupy about 90% of the cambium (Kozlowski 1971). Cambial activity and regulation of differentiation of cambial derivatives determine the morphology and composition of the cells of xylem and consequently, wood. The cambium is active in cell division. Its differentiating derivatives have high biosynthetic activity, which is limited in duration. Division is followed by cell expansion, which is followed by the ordered deposition of cell wall components. Many of the cells in the cambium have higher than usual levels of ploidy because of DNA endoreduplication (Larson 1994; Roberts and others 1988). Phytohormones, especially auxins, giberellins, cytokinins, and ethylene, regulate differentiation in the secondary xylem (Lev-Yadun and Aloni 1995; Roberts and others 1988; Savidge 1988; Savidge and Wareing 1981; Tuominen and others 1997; Uggla and others 1996, 1998; Wareing and Phillips 1981).

The ray system is the radial component of the secondary xylem and phloem (Lev-Yadun and Aloni 1995). In pines, the rays are radial multicellular sheets of parenchyma cells with some radial ray tracheids. Rays in pines are usually only one cell wide but may be from 1 to 20 or more cells in height. In the wider rays, radial resin ducts are formed (Fahn 1990; Larson 1994).

Differentiation of conducting xylem elements involves cell death. Cell death also occurs during the differentiation of many of the fibers, sclereids, and all phellem (cork) cells (Fahn 1990). Apoptosis occurs in xylem differentiation (Fukuda and others 1998; Groover and others 1997; Mittler and Lam 1995; O'Brien and others 1998), but has not been studied in fibers, sclereids, and cork cells. Arrested apoptosis occurs in the conducting cells of phloem (sieve cells), which lose their nuclei but maintain a functioning cytoplasm (Fahn 1990). In some plants, such as palms, anucleate sieve elements may function for a century.

Wood formation varies greatly during the growing season. Earlywood and latewood tracheids differ greatly in diameter and cell wall thickness. The latewood, having narrower lumens in the tracheids, is much less vulnerable to water-stress–induced xylem embolism, and so increases the safety of water conductance. The desired properties of wood for different applications require differences in fiber length, wall thickness, lumen diameter, and chemical composition (Biermann 1993; Megraw 1985). Wood is also modified by damage from pathogens and by wounding (Fink 1999).

Reaction wood is unusual in structure and composition and occurs in response to mechanical stimulation, which changes the hormonal balance. In conifers, reaction wood is typically formed in the lower side of branches or leaning stems and is termed compression wood (Timell 1986). In dicotyledons, reaction wood is usually formed in the upper side of branches and leaning stems, is characterized by gelatinous fibers, and is termed tension wood (Fahn 1990). The formation of reaction wood is regulated mainly by auxin (Kozlowski 1971; Timell 1986). High levels induce compression wood in co-

nifers and low levels induce tension wood in dicotyledons.

Two major transitions are typically found in the wood during the maturation of trees. These are the transitions from juvenile to mature wood and from sapwood to heartwood. The wood formed in young trees and near the crown of older trees is known as juvenile wood (Zobel and Sprague 1998). When old trees are cut, the inner part of the wood is often dark, whereas the outer parts are lighter. The dark-colored region is called heartwood and the light region, sapwood (Hillis 1987). Although sapwood of pines contains living ray cells, 90% of its mass is comprised of terminally differentiated nonliving tracheids. There are no living cells in heartwood. Sapwood acts as a water transport and storage system, a mineral and carbohydrate storage compartment, and a wound-healing tissue. Ethylene is the major signal for the synthesis of polyphenols that give heartwood its color and make it resistant to decay.

Biochemistry of Wood

The chemical and physical properties of pine wood are well characterized (Lewin and Goldstein 1991; Sjöström 1993). Cellulose is the major chemical component of wood (45%) and is the major strength-bearing component. The water-insoluble cellulose microfibrils are associated with mixtures of soluble noncellulosic polysaccharides, the hemicelluloses, which account for about 25% of the dry weight of wood. The third major component of wood (27%) is lignin, the phenolic polymer that embeds the polysaccharide matrix as a stiffening agent within the fibers. It is highly insoluble and provides mature xylem with the hydrophobic surface needed for the transport of water. The remaining minor components of wood are extractable volatile oils, terpenes, fatty acids, resin, and pectin, and some nonextractable protein (Bao and others 1992).

Secondary Cell Walls of Pines are Amenable to Biochemical and Molecular Studies

Pines have been extensively studied as a biochemical system to investigate enzymes and structural proteins involved in the formation of the secondary cell walls (Higuchi 1997). Wood formation in pines is a specialized case of differentiation of immature xylem. The tissue mass is primarily composed of axial tracheids with primary cell walls, which proceed through secondary cell wall biosynthesis, followed by programmed cell death. Differentiating xylem is rich in cell wall biosynthetic enzymes. Immature xylem may be readily collected in kilogram quantities from fast-growing trees and used for biochemical and molecular studies (Sederoff and others 1994). Many enzymes and proteins have been identified and characterized, including enzymes involved in lignin precursor biosynthesis (Whetten and others 1998), cell wall–associated proteins (Bao and others 1992; Zhang and others 2000), and cell wall–associated oxidases (Bao and others 1993; O'Malley and others 1993; Ugaramandeniya and Savidge 1994). The tissue from differentiating xylem has also been used as a source of libraries for the identification of genes involved in cell wall biosynthesis (Allona and others 1998; Loopstra and Sederoff 1995).

Pine is a Tractable Model for Studying Gymnosperm Reproduction and Evolution

The reproductive structures of pines (like most conifers) are unisexual cones. There are male (staminate, or microsporangiate, or pollen) cones and female (ovulate, or megasporangiate, or seed) cones. Male cones of pines are simple branches carrying modified leaves, the microsporophylls, which form the microsporangia where the pollen grains (male gametophytes) are formed. They are produced on lateral shoots in subterminal clusters. After the male cones shed their pollen, they dry up and abscise, leaving a needle-free zone on the branch. The morphology of the female cone is not easy to interpret. The common view is that in pines, the female cone is complex and is composed of a main branch carrying many lateral branches. Each female cone scale is a lateral branch associated with a modified leaf (a small bract adnate to the abaxial basal region of the scale). The female cones are produced on lateral branches near the apex of the main vertical shoots, singly or in small groups (Gifford and Foster 1989).

Alternation of generations with a haploid gametophyte and diploid sporophyte is characteristic of all land plants. In all seed plants (gymnosperms and angiosperms), the gametophytes are microscopic and depend on the sporophyte for their nutrition and protection. In pines, like other gymnosperms and angiosperms, the male gametophyte is the haploid pollen grain, which is usually composed of two or three cells. The female gametophyte (megagametophyte) is surrounded by the ovule and is composed of several thousand haploid cells. After fertilization of the female gamete and embryo development, the rest of the megagametophyte serves as a primary haploid endosperm (Gifford and Foster 1989), which contains food reserves for the germinating embryo.

The reproductive cycle in pines may be 1, 2, and 3 years long, depending on the species. The varia-

tion depends on the length of time that pollen remains in a resting state in the pollen chamber after fertilization (Singh 1978). The most common one, which occurs in our species of interest, takes about 2 years. At the time of pollination, in the spring, the female gamete (megaspore) is not yet developed. After pollination, the pollen grains wait in the pollen chambers of the female cones for about a year. Fertilization occurs only in the second spring. After fertilization, embryogenesis starts and continues during the summer (Gifford and Foster 1989). Many pines shed their seeds in the late summer, but others have serotinous cones and either shed only a small fraction or shed no seeds until an environmental change (hot and dry days or fire) triggers cone opening. Such serotinous cones may remain closed for decades. Of our model pines, only *P. radiata* has serotinous cones (Lanner 1998).

The female gamete develops within the archegonia. Pines usually have several archegonia in each ovule (Gifford and Foster 1989). After fertilization, two types of polyembryony occur in pines: primary and secondary. Primary polyembryony is the outcome of several fertilization events in different archegonia, forming embryos that differ genetically from each other. Later, secondary polyembryony (cleavage polyembryony) appears, in which the lower parts of the embryos develop into four genetically identical embryos. Only the most vigorous embryo survives into the seed (Willson and Burley 1983).

Several mechanisms maintain a high rate of outcrossing and low rate of selfing in pines. Pines are monoecious, that is, male and female cones are well separated within the crown. There is also a high degree of temporal gender separation in young pine trees, with pollen and seed cones being formed at different years on the same tree. Embryo competition also serves to reduce survival of selfed progeny because of the deleterious effects of genetic load (Ledig 1998; Mirov 1967; Remington and O'Malley 2000; Righter 1939; Shmida and others 2000; Willson and Burley 1983).

Abnormal flowering and homeosis (transformation of structure) are well known in pines (Bollmann and Sweet 1976; Chamberlain 1935; Dorman 1976; Lev-Yadun 1992; Mergen 1963). Developmental alterations, including homeosis, are often observed in pines after application of specific chemicals.

One of the major evolutionary changes that occurred during the evolution of land plants was the development of seed-forming reproductive organs (Gifford and Foster 1989). Certain regulatory gene families, such as MADS box genes, are involved in the development of reproductive organs in both gymnosperms (for example, *P. radiata*) (Mellerowicz and others 1998; Mouradov and others 1998, 1999; Walden and others 1998; Wang and others 1997), and angiosperms (Coen and Meyerowitz 1991; Coen and others 1990; Theissen and Saedler 1999; Yanofsky 1995). Some of these genes (certain MADS homeotic genes) are also found in ferns, but their expression is not specific to reproductive organs, as in seed plants (Hasebe and others 1998; Münster and others 1997). In Pinaceae, external application of $GA_{4/7}$ promotes cone production (Owens and Hardev 1990; Ross and Pharis 1987). The influence of $GA_{4/7}$ on expression of MADS-box genes should be investigated.

Pines are Experimentally Tractable for Culture, Propagation, and Transformation

Grafting and vegetative propagation are common methods of cloning exceptional genotypes of trees. Of our model pines, *P. radiata, P. sylvestris,* and *P. taeda* can be grafted successfully (Dorman 1976; Fowells 1965; Kozlowski and others 1991). *Pinus radiata* (Bamber and Burley 1983) and *P. taeda* (Dorman 1976) can be vegetatively propagated from cuttings.

The first successful system for micropropagation in pines was organogenesis, based on induction of adventitious shoots from cultured embryos. Cells in the epidermis and subepidermal layers give rise to meristematic centers that form shoot apicies (Mehra-Palta and others 1978; Mott and Amerson 1981; Mott and others 1977; Sommer and others 1975).

Organogenesis in loblolly pine (Amerson and others 1988) and monterey pine (Aitken-Christie and others 1988) has been intensively characterized because of commercial interest in clonal propagation of exceptional genotypes. Micropropagation through organogenesis has been superceded by somatic embryogenesis (Handley and others 1995; Li and Huang 1996; Keinonen-Mettala and others 1996), which can produce far larger numbers of plantlets per clone. Plantlets derived from cotyledons by organogenesis have an early slow growth period but are more mature compared with seedlings. Plantlets grown by organogenesis from adventitious shoots do not show any early reduction of growth (Frampton and others 1998).

Before 1986, the only evidence for DNA transformation of pines and other conifers was that of gall formation caused by *Agrobacterium* (DeCleene and De Lay 1976, 1981). Cells and tissues of many pines now are transformed readily by *Agrobacterium* (Gupta and others 1988; Levee and others 1999;

Loopstra and others 1990; Sederoff and others 1986; Stomp and others 1988, 1990; Wenck and others 1999) and by particle bombardment (Campbell and others 1992; Stomp and others 1991; Walter and others 1994). *Pinus halepensis, P. monticola, P. sylvestris,* and *P. contorta* have been transformed by *A. rhizogenes* (Magnussen and others 1994; McAfee and others 1993; Tzfira and others 1996; Yibrah and others 1996). In addition, transient expression has been used to study relative promoter activity in various tissues, including electroporated protoplasts (Lopez and others 2000), pollen (Fernando and others 2000), and differentiating xylem (Loopstra and others 1992). The first report of stable transformation and regeneration of pines was that of Walter and colleagues (1998), using particle bombardment of somatic embryos of *P. radiata.* More recently, it has been possible to transform and regenerate loblolly pine following co-cultivation with *Agrobacterium* strains carrying extra copies of virulence genes (W. Tang and R. Whetten, personal communication).

Pines Have Unusually Large Nuclear Genomes

The pine genome has 12 large metacentric pairs of chromosomes (Sax and Sax 1933). All pines are diploid. The haploid DNA content varies considerably, ranging from 21 to 31 pg (Wakamiya and others 1993). Loblolly pine has a haploid DNA content of 22 pg, roughly 20 billion base pairs, about 160 times that of *Arabidopsis thaliana* (Somerville and Somerville 1999) and about 7 times that of the human genome (Venter and others 1998).

The number of expressed genes and their gene family sizes are similar in pine and angiosperms. Most loblolly pine genes are single copy genes or members of small gene families. More than 10% of pine genes may have large numbers of family members (Kinlaw and Neale 1997; Kinlaw and others 1994; Perry and Furnier 1996). Isozyme profiles of pines show less evidence for large gene families than is apparent from Southern blots, suggesting that some members of amplified gene families may not be functional (Perry and Furnier 1996).

Gene amplification may have played some role in the evolution of large genome size in conifers (Kinlaw and Neale 1997); however, interspersed repetitive sequences may have had a major role, as appears to be the case for the grasses (SanMiguel and others 1996). Pine genomes are rich in highly repeated sequences (Kriebel 1985; Rake and others 1980), including dispersed repeated sequences likely to be retrotransposons (Doudrick and others 1995; Kossack 1989). Two retrotransposon sequence elements have distinct but highly dispersed distributions among pine genera (Islam-Faridi and others 1996; Kamm and others 1996).

Pines Have Unusual Organelle Inheritance

Organelle genomes of pines have some unusual features. The inheritance of chloroplasts and mitochondria is distinct and can be used to define maternal and paternal lineage. The chloroplasts are inherited through the pollen parent (Neale and Sederoff 1988; 1989; Neale and others 1986, 1988, 1989), whereas the mitochondrial genomes are inherited through the female gamete (Lu 1997; Neale and others 1989; Wagner and others 1987). Chloroplast DNA fragment diversity is high and has been used to distinguish populations and species (for example, Hong and others 1993; Wagner and others 1992). Pollen movement in specific populations has also been followed with organelle markers (Latta and others 1998). Mitochondrial restriction fragments also show a high degree of population differentiation (Strauss and others 1993), which has allowed description of populations before and after recent glaciation events and for evaluation of introgression (Mitton and others 2000; Senjo and others 1999; Sinclair and others 1998, 1999). RNA editing has been detected in mitochondrial genes for several gymnosperms, including *P. sylvestris* and *P. sibirica* (Lu and others 1998). Mitochondrial gene order may be highly conserved for gymnosperms (Karpinska and others 1995).

The chloroplast genomes of conifers have only one of the inverted repeats characteristic of angiosperm chloroplast genomes (Lidholm and others 1988). Otherwise, the organization of the chloroplast genome is relatively conserved between angiosperms and gymnosperms (Palmer 1991; Palmer and Stein 1986), although additional rearrangements have been identified (Strauss and others 1988). Conifers have the ability to synthesize chlorophyll in the dark. This light-independent pathway is shared with many algae and lower plants (Lidholm and Gustafsson 1991).

The core of pine genomics efforts is the discovery of specific genes by cDNA sequencing. To date, more than 18,000 pine expressed sequence tags (ESTs), selected from libraries made from different types of differentiating xylem and other tissues, have been sequenced and the information deposited in GENBANK (http://www.ncbi.nlm.nih.gov/Genbank. A significant fraction of the expressed genes are involved in cell wall biosynthesis, with many genes also involved in the mechanisms of transcription (Allona and others 1998). About half of all specific

genes can be assigned to functional categories based on sequence similarity to known genes in other genomes. Typically, an orthologous gene of known function will have approximately 60 to 80% DNA sequence conservation between pine and any angiosperm. About 10% of all pine ESTs have no reasonable sequence relationship to any known expressed genes. Studies of sequences unique to conifers and of unknown function should determine to what extent gymnosperms have genes that are not represented in angiosperms and to what extent all higher plants have essentially the same genes.

Pines Have an Unusual Advantage for Genetic Analysis

Only a few years ago, long generation times, absence of inbred lines, high levels of heterozygosity, high genetic load and high allelic diversity appeared to be formidable barriers to genetic analysis of pines (Strauss and others 1992). Instead, the high levels of heterozygosity and genetic diversity have provided large numbers of excellent genetic markers and genetic mapping of individual trees is now routine. The advantage of pines derives from the large haploid megagametophyte, which has sufficient tissue for DNA purification and polymerase chain reaction (PCR) analysis.

The megagametophyte is a direct mitotic product of one of the meiotic tetrads and therefore shows a 1:1 ratio for any locus that is heterozygous in the maternal parent. Pairwise linkage is inferred when the observed ratios differ from the expected 1:1:1:1 Mendelian ratios. The megagametophyte and the megaspore (female gamete) develop from a single meiotic product. Therefore, both the maternal contribution to the embryo and the nutritive megagametophyte tissue surrounding the embryo have the same genotype. Segregation and linkage can be quantified by analyzing multiple seeds from the same tree. The pollen contribution to the embryo can be determined by "subtracting" the contribution of the egg.

Pines Are a Model for Genomic Mapping in Forest Trees

The first linkage maps in pines were carried out using small numbers of isozyme markers (Adams and Joly 1980; Conkle 1981). Haploid analysis has now been widely applied to large numbers of PCR-based markers for genotype identification and for genetic mapping of *P. taeda, P. radiata,* and *P. sylvestris* (Devey and others 1996; Emebiri and others 1998; Hurme and Savolainen 1999; Kuang and others 1999; O'Malley and others 1996; Remington and others 1999; Yazdani and others 1995). Moderately saturated genetic maps of pines show 12 well-defined linkage groups with a total genetic map length of about 1500 cM (Gerber and Rudolphe 1994; Neale and Sederoff 1996; Remington and others 1999). This value is consistent with the observed number of chiasmata per bivalent observed in the pine genus (Saylor 1972).

Genetic maps have been obtained for many other pines (Devey and others 1995; Echt and Nelson 1997; Hicks and others 1998; Kaya and Neale 1995; Kubisiak and others 1995; Nelson and others 1993; Nelson and others 1994; Travis and others 1998). The web site Dendrome (http://dendrome.ucdavis.edu) serves as a database for genetic maps of pines and other forest trees. A high level of genetic mapping information related to wood properties, disease resistance, and growth traits has been obtained (reviewed in Neale and Sederoff 1996). DNA marker systems for restriction fragment length polymorphism (RFLP) analysis and a variety of PCR-based systems have been widely used. The best systems in use at present are amplified-fragment-length-polymorphism (AFLPs) for mapping and microsatellites for fingerprinting (Cato 1999; Echt and others 1996; Fisher and others 1998; Remington and others 1999). Genetic markers for coding sequences will be particularly useful to compare maps of other conifers (Devey and others 1994a, 1999; Harry and others 1998).

Genetic mapping using three generation outbred pedigrees of loblolly pine, which are particularly suitable for quantitative trait analysis, was developed by Neale and coworkers (Devey and others 1991, 1994b; Groover and others 1994). This approach has the advantage of maintaining large "immortal" mapping populations in the field, and consensus maps are readily created (Sewell and others 1999). Sex-related differences in total recombination frequency have been observed in *P. taeda* (Groover and others 1994) and *P. pinaster* (Plomion and O'Malley 1996). Both studies found an increased rate of recombination in the pollen parent compared with the seed parent. The increase was 26% for loblolly pine and 28% for *P. pinaster.*

Pines Provide a Model for Studies of Genetic Variation in Natural Populations

Genetic variation in pines has been studied extensively because of a high level of variation in natural populations (Hamrick and Godt 1996). Forest trees provide a rich source of material to study mutation rates because an individual tree may produce ga-

metes for a period of hundreds to thousands of years. High levels of variation are apparent in the frequency of allozymes (Adams and Joly 1980; Allendorf and others 1982; Conkle 1981; Hamrick and Godt 1996), in the high levels of DNA sequence polymorphism found in restriction fragments (Devey and others 1994a, b), and PCR-based amplified fragments, particularly random amplified polymorphic DNAs (RAPD) (O'Malley and others 1996) and AFLP markers (Remington and others 1999), and in the high genetic load (Bishir and Namkoong 1987; Franklin 1972; Remington and O'Malley 2000; Williams and Savolienen 1996).

The number of lethal equivalents for conifers is estimated to be very high compared with other organisms (Lynch and Walsh 1998). A notable exception is that of red pine, which has a low level of genetic variation (De Verno and Mosseler 1997; Moesseler and others 1992), which may reflect a genetic bottleneck in its recent past. Remington and O'Malley (2000) concluded that essentially all of the genetic load in a single loblolly pine could be accounted for by 19 lethal or semilethal loci. Similarly, inbreeding depression analyzed by mapping in *P. radiata* showed distortion from expected ratios at many sites in the genome (Kuang and others 1999).

Typically, on selfing in pines, many visible mutations are apparent (Franklin 1968; Remington and O'Malley 2000). Many mutations are albino, and morphologic variants include dwarves, color variants, and alterations in morphology that have horticultural value. A mutation in cinnamyl alcohol dehydrogenase, also discovered by selfing, dramatically affects wood color and lignin chemistry (MacKay and others 1997; MacKay and others 1999; Ralph and others 1997).

Pines Are a Model for Functional Genomics of Conifers

Microarray analysis of cDNAs from pine provides a powerful tool to investigate gene expression on a genomic scale. Changes in the relative abundance of large numbers of cDNAs may be assayed in arrays on glass slides (Schena and others 1998; Shalon and others 1996). Variation in specific mRNA abundance caused by environmental, developmental, or genetic differences can be monitored for a large fraction of a genome. In loblolly pine, large numbers of genes in differentiating xylem are differentially expressed between juvenile and mature stages and also in response to water, heat, mechanical stress, and auxin treatment (Y-H. Sun and R. Whetten, personal communication). This technology provides a rich source of data on functional genomics and molecular interactions (epistasis).

Two-dimensional gel electrophoresis has been used to identify and characterize large numbers of proteins from maritime pine (*P. pinaster*) and to examine genetic variation at the protein level. Protein polymorphisms varying in presence/absence or in mobility were detected in megagametophytes and needles (Bahrman and others 1997; Costa and Plomion 1999; Gerber and others 1993; Plomion and others 1997). Quantitative variation in specific proteins resulting from water stress (Costa and others 1998) has also been investigated. Such analysis of protein loci should be increasingly useful as gene expression data accumulate for pines.

Pines Produce Abundant and Diverse Secondary Metabolites

One of the major challenges of functional genomics is to identify the genetic factors that determine the quantity and diversity of secondary metabolites produced by pine trees. The development of new separation methods for detailed metabolic profiles promises to become of central importance (Trethewey and others 1999). Metabolic profiling is typically thought of as the characterization of the soluble components of the plant cell. However, it is equally important to characterize the chemical composition and structure of the polymers that comprise the insoluble materials of the cells, particularly for the cell walls formed in woody tissues. For example, Fourier Transform Infra Red spectroscopy (FTIR) can determine the composition of cell walls (McCann and others 1992) and nuclear magnetic resonance (NMR) can determine lignin structure (Ralph and others 1999).

New Genetic Technology Is Being Applied to Tree Improvement for Pines

Much of the interest in the genetics of pines is based on applications for tree improvement. In tree breeding, traits of interest are often difficult to characterize and have low heritabilities (Zobel and Talbert 1984). The technology of genomic mapping has led to the development of theory and methods for using genetic markers to predict performance of trees in a breeding program (marker aided selection) (Liu 1998; Neale and Williams 1991; O'Malley and Whetten 1997; Plomion and others 1996; Strauss and others 1992; Williams and Neale 1992). In principle, the methods depend on the correlation of particular quantitative phenotypes with specific regions of the genome. These methods define the relative contribution of specific chromosome regions to the total genetic variation. In a large progeny set, sub-

stantial components of the variation are associated with these genomic regions, called quantitative trait loci (QTLs). In pines, several traits of economic interest have been mapped, particularly, resistance to fusiform rust disease, resistance to white pine blister rust, resistance to pine needle gall midge, wood specific gravity, and early height growth (Devey and others 1995; Groover and others 1994; Kondo and others 2000; Plomion and others 1995a, b; Wilcox and others 1996; Wu and others 1999a, b).

Pines Have Significant Potential for Directed Genetic Modification

Central to the development of a model system is the potential for genetic engineering. Wood is a leading industrial material and the potential to modify wood properties places genetic engineering of wood properties at an interface between molecular genetics and material science with the prospects of new or improved materials based on the properties of wood (Whetten and Sederoff 1991). Forest trees are generally in the earliest stages of domestication, and the major genetic changes that characterize our domesticated plants and animals have yet to be made for forest trees. In addition, small improvements in wood properties leading to more efficient pulping or processing would have significant value because of the large scale of the wood and paper industry. Improved growth rates could also decrease the demand on the land and reduce our dependence on fossil fuels, because wood is a renewable energy resource. Results from modification of lignin through transgenic plants or mutations that suppress the expression of enzymes of the lignin biosynthetic pathway indicate that the chemistry of the wood cell wall can be dramatically altered by genetic modification (Hu and others 1999; Ralph and others 1997; Sederoff and others 1999).

Weaknesses of the System

The limitations of pines as model systems arise from their large size, the very large genome, their long generation time and the time needed to follow certain developmental processes or to study mature traits. Cell and tissue culture systems, although greatly improved, are still difficult and often genotype dependent. Production of transgenic plants is slow. Many aspects of pine biology must be studied outdoors in different locations and under widely different climatic conditions. No single pine species can grow in all the environmental conditions prevailing in the tropical, subtropical, and temperate regions of the world. The large genome size of pines precludes the possibility of complete sequence with available technology. It is now possible to sequence a human genome in only a few years. Current sequencing technology would require 20 years to determine the complete sequence of a loblolly pine genome.

What Pines Need as Model Systems

In the future, we may expect major technical advances through application of genomics, with the identification of function for large numbers of expressed pine genes. A great deal of fundamental information will come from comparison of pines with model angiosperms. It will still be necessary to carry out extensive work on the pines to provide sufficient information about gymnosperms, the perennial growth habit, extended longevity, and the diversity and differentiation of wood properties.

Significant advances in forestry may be expected from increased integration of genomics, quantitative genetics, and tree breeding. Improved methods are being developed to correlate linkage associations of specific phenotypes for traits of value with linked DNA markers in established pedigrees and mapping pine populations. Such an approach will be much more valuable if it becomes possible to make such associations in natural populations because that would bypass the long generation times. Association of DNA markers and phenotypes in populations may allow identification of candidate genes identified by studies of location and function of expressed genes.

When large sets of data for functional genomics are available, the biologic aspects of many complex multigene functions will be studied in far greater detail. Even if genomic advances are not sufficient to understand many complex biological processes, they will surely lead the way for such understanding in the future.

What Is Needed to Realize the Potential of the System?

Large numbers expressed pine genes should be identified and characterized by sequence, expression patterns, and the genes should be located on a genetic map. Improved methods for high throughput mapping and functional *in situ* expression (Koltai and Bird 2000) could contribute significantly to attaining this goal. It will be important to apply "genetic knockout" analysis of specific genes to pines. These variants may be generated by transgenic plant technology, and natural variants may be found and analyzed. Because of the high rate of natural variation, null mutations may be detected in progeny from selfed trees at high frequency. Metabolic profiling

and detailed chemical characterization of cell walls will provide a great deal of information about the metabolism, biosynthesis, and structure of the wood cell wall—the major product of pines.

The long-term application of the technology of genomics and genetic engineering in pine is the creation of trees modified in growth traits for early establishment of seedlings in the field, rapid growth, nutritional efficiency, resistance to pest and pathogens, and the modification of wood properties. Altered wood properties may consist of increased uniformity, greater strength, or improved pulping properties. The result would be a diversity of specialized pine trees that have shorter rotation times and superior wood properties. In addition, the ability to monitor gene expression on a genomic scale should become a powerful tool for molecular studies of adaptation and evolution in natural systems. The effects of specific genes and the interactions of large numbers of genes can be monitored to provide an understanding of the nature and extent of epistasis underlying the role of specific gene complexes of forest trees in adaptation to specific ecologic systems. In sum, there is great scientific and economic value in the development of pines as model systems.

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