PLANT BIOTECHNOLOGY

Moving Forest Trees Into the Modern Genetics Era

For genetic engineers, trees are mostly virgin territory. With their large sizes, slow growth, and huge genomes—up to 10 times bigger than those of humans—trees, especially forest species, have been poor prospects for the gene transfer methods that are proving so useful for improving small crop plants like the tomato or cannola. "Tree breeding is like reinventing agriculture," says molecular biologist Ronald Sederoff of North Carolina State University (NCSU) in Raleigh, whose own work focuses on trees. "Maize and wheat have gone through 5 to 10 thousand generations of cultivation and selective breeding, while the most advanced forest tree-breeding programs are in their third generation." The next generations of genetically improved forest trees could come a lot faster, however.

Within the past 4 to 5 years, advances in gene mapping techniques have provided researchers with new tools for rapid identification of trees with desired traits, such as fast growth and resistance to disease or cold temperatures, that can be used to breed improved species by conventional means. What's more, the genetic maps this work is producing should ultimately allow isolation of tree genes conveying desirable traits that could be genetically engineered directly into target tree species. Indeed, the feasibility of the gene transfer approach to tree improvement has already been demonstrated, although with genes from nontree species (see box).

NCSU FORESTRY

Pine patch. As the United States' most widely planted commercial tree, the loblolly pine is a prime target for genetic improvement.

The practical results of such research might include trees that grow faster, producing more paper at reduced cost. The harvest might also include wood that is a superior fuel, or orchard varieties more resistant to disease, drought, and cold. Indeed, plant molecular biologist Marc Van Montagu of the University of Ghent in Belgium describes the genetic engineering of trees as "very promising" for commercial purposes.

Although most genetic research on plants has been done on small, short-lived species, trees haven't been entirely neglected. In fact, the first genetically engineered tree—a poplar carrying a gene for herbicide resistance—was produced nearly a decade ago, with the same gene transfer methods developed for annual plants. But while this early experiment proved that trees could be genetically engineered, little more was done.

One problem was that many of the traits that researchers wanted to introduce into trees are themselves encoded by tree genes, and at the time there was no efficient way to track down those genes. The size of the genomes and, especially, the long generation time of trees—it takes them 10 to 20 years to reach sexual maturity—made it tough to perform the classical genetic studies, involving repeated backcrosses, commonly used to localize genes. As plant molecular biologist David Neale of the U.S. Department of Agriculture's (USDA's) Institute of Forest Genetics in Berkeley, California, puts it, "Trees are uncooperative experimental organisms."

That situation began changing about 5 years ago, however, with the development of new techniques for identifying "markers," DNA sequences that vary from one individual to the next. Markers can help locate desired genes, because any gene consistently inherited with a marker must lie near it on the same chromosome.

One way to find markers in trees, the random amplified polymorphic DNA (RAPD) method, was devised in 1990 by Scott Tingey's team in the Agricultural Products Division of DuPont in Wilmington, Delaware. Researchers using this method first make "tenmers," random 10-nucleotide-long DNA sequences that serve as primers for enzymatic amplification of segments of DNA from the target species.

To see whether these randomly amplified sequences have the necessary sequence variation to serve as markers, they have to be screened, a process that is greatly aided by a particular feature of the seeds of conifer trees. In the seeds, the tree embryo is surrounded by tissue that is a product of the meiotic divisions that form the female germ cells (the megagametophytes). As a result, the megagametophyte tissue carries only maternal genes and is haploid, containing one set of chromosomes instead of the two found in ordinary somatic cells.

A researcher can then determine which of the amplified RAPD sequences qualify as markers by analyzing for them in megagametophyte tissue from several seeds, looking for any sequences that are found in about half the seeds. This indicates that the two original partner chromosomes carry different variants of the sequence, only one of which was amplified by RAPD. Such sequences can thus serve as markers. And while mapping requires many markers, finding them is easy, Sederoff says: "You can use hundreds of thousands of different tenmers, and each amplifies a different set of fragments, generating virtually unlimited sets of markers."

Once the markers are in hand, they can readily be mapped—a task that in other species requires looking at how markers are inherited. In conifers, it's accomplished by looking for those markers that are consistently found together in the megagametophyte tissue of the same seeds, an indication that they are located on the same chromosome. In this way, it's possible to build up a genetic map very rapidly. A variation of the RAPD technique can also be used to identify markers in nonconifers, the flower-bearing angiosperms.

Indeed, markers generated by the RAPD method quickly proved their mettle. In 1992, a team led by John Carlson of the University of British Columbia, in Vancouver, reported using the technique to produce a genetic linkage map of the spruce, and at about the same time Sederoff's group at NCSU used RAPD to produce a genetic map of the loblolly pine, the United States' most widely planted commercial tree.

And RAPD markers are not the only ones in the tree mappers' tool kit. A second technique, developed about 3 years ago by Marc Zabeau of Keygene, a biotech company in Wageningen, the Netherlands, is beginning to aid their efforts, although it can only be applied to those few forest tree species that have established breeding programs and documented multi-generational tree lines available for analysis.

Zabeau's method, called amplified fragment length polymorphism (AFLP), cuts the genomic DNA into pieces with restriction enzymes and then amplifies select bits. Unlike the RAPD technique, which uses random primers for the amplification, however, AFLP uses primers that have been custom-designed to vary in length by one to a few bases. Because the binding between the primers and the DNA pieces they pick out for amplification is very specific, the primers can distinguish between pieces that differ by as little as one or two bases.

And while the AFLP work isn't as far

Genetic Engineering Turns to Trees

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Early bloomer. The aspen shoot with the *LFY* transgene *(left)* has flowers *(arrows)*, while the control aspen may have to wait 10 years.

For many of the goals of tree geneticists—improving fruit or fiber yield, for example—there's no substitute for genes from the trees themselves. But even as the search for potentially valuable tree genes goes on (see main text), plant biotechnologists are moving ahead, engineering trees with genes from nontree species and performing other genetic manipulations to improve their quality.

In some cases, this has involved introducing foreign genes for pest or herbicide resistance into trees. Take work being done by the groups of Steven Strauss at Oregon State University (OSU) in Corvallis and of Brent McCown and David Ellis at the University of Wisconsin, Madison. To try to produce trees that are better protected against defoliating insects, the OSU team introduced the gene for a potent insecticidal protein from the bacterium *Bacillus thuringiensis* (*Bt*) into poplar trees, while the Madison workers have done the same for poplars and white spruce trees. The transfers worked: "The trees were killers" of insects feeding on their leaves, McCown says.

And the *Bt* gene is not the only one being introduced into trees in hopes of making them more resistant to insects. Gilles Pilate of the Forest Tree Breeding Station Laboratory in Ardon, France, and his colleagues have genetically engineered poplars to express an inhibitor of a protein-cutting enzyme needed for insect digestion. When fed foliage from the transformed trees, beetles of the species *Chrysomela tremulae* had a 40% mortality rate, well above that of insects eating leaves from conventional poplars.

In another instance, gene transfer into trees may be beneficial to tree breeders, if not to the trees. Last year, Detlef Weigel of the Salk Institute for Biological Sciences in La Jolla, California, and Ove Nilsson of the Swedish University of Agricultural Sciences in Umea transferred the *LEAFY* (*LFY*) gene, which controls flower development in *Arabidopsis thaliana*, a small, rapidly growing plant, into the European aspen. Normally, aspen takes a decade or two to reach sexual maturity and flower, but plants expressing *LFY* began flowering during the first year of growth, Weigel says. As a result, tree breeders might get a boost if other tree species can be induced to express the *LFY* gene. "If you can get a tree to flower within a limited period of time, then you can accelerate generations of breeding by a large factor," says Ronald Sederoff of North Carolina State University.

In addition to attempting to improve trees by introducing foreign genes, researchers are also taking another approach: using "antisense constructs," nucleic acids which bind to the genes themselves or to the messenger RNAs they produce, to inhibit formation of the gene products. One major target of this strategy is the genes that make the enzymes needed for synthesizing lignin, a cementlike polymeric compound that must be removed before wood is converted to paper. The hope is that by inhibiting production of the enzymes, the lignin content of the trees can be reduced, thereby helping bring down both the costs and pollution associated with paper production.

Early efforts by Wout Boerjan's group at the University of Ghent, Belgium, have shown that antisense constructs do inhibit production of two enzymes needed for lignin production, although the amount of lignin made didn't go down correspondingly. Now the Ghent team is targeting two other enzymes in the lignin synthesis pathway. If these and the other genetic manipulations of trees now being attempted pan out, tree biotechnologists may find that their field has become a growth area in more ways than one.

–A.S.M.

along as that with RAPD markers, the newer technique is already proving its mettle. Working with fast-growing hybrid poplars, which have been cultivated in tree plantations, Maria Teresa Cervera of the Van Montagu team has shown, for example, that the inheritance of AFLP markers associated with resistance to a fungal rust disease can be traced through at least two generations.

One goal now is to use these new mapping procedures to pick out the trees that breeders want to propagate because they carry genes for desirable traits. Until very recently, breeders could tell if a tree has a gene only by growing it to see if it has the trait the gene confers. Not only does that take a long time, but the results can be confounded by variations in temperature, humidity, and other environmental factors that also influence how well a tree grows or its susceptibility to disease. "Knowing which trees to propagate is a major problem in tree biotechnology," Sederoff says.

But once RAPD or AFLP markers have been linked to a trait by screening to see if they are routinely present in trees that have the desired property, researchers can find trees that carry the gene simply by screening for the markers. Because the screening can be done on very young trees or even on embryonic tissue, it should speed up selective breeding, making it possible to apply this venerable technique to more tree species. "A dense map of markers will be very useful for tree breeders," says Outi Savolainen of the University of Oulu in Finland, whose team has identified markers for cold hardiness in Scots pine and is using them to identify trees that can thrive near the Arctic Circle.

Ultimately, though, researchers want to use the markers to pin down the location of potentially valuable genes so that they can be cloned, much as human disease genes are being isolated by these "positional cloning" methods. Improved strains of the same or other tree species could then be created by genetically engineering the tree genes into individuals currently lacking them. Two groups have recently taken a first step toward that goal by using RAPD markers to map the locations of disease-resistance genes in trees.

Last March, USDA's Neale and his colleagues at the Australian Commonwealth Scientific and Industrial Research Organization reported in the *Proceedings of the National Academy of Sciences* that they had linked several RAPD markers to a gene in sugar pine trees that confers resistance to a fungal disease called white pine blister rust, which causes catastrophic epidemics. And in unpublished work, Sederoff and his colleagues at NCSU, the USDA Forest Service in Athens, Georgia, and the New Zealand Forest Research Institute in Rotorua have located a gene that confers resistance in loblolly pine to fusiform rust disease, another major fungal pathogen.

But even though the two teams have narrowed down the locations of the genes they are looking for, they still have to search a lot of DNA to pin down and clone their targets. Indeed, the researchers estimate that it could take 5 or more years to track down the disease-resistance genes. But then, by the standards of trees—and past efforts to improve them—5 years is a brisk pace.

–Anne Simon Moffat