world should also prove useful in addressing questions related to adaptation to warming temperatures.

It is well known that trees grow at different rates and that they are also variable in how they allocate carbon to various above- and below-ground components. This information could be used to make genetic selections for trees to optimize carbon sequestration. Selections of trees with rapid growth rates, strong allocation to root systems, and inherent resistance to decay could be done for various environmental conditions in the major tree-growing regions of the world. Likely, such selections will include use of exotic species, hybrids of local and exotic species, or genetically engineered trees with altered carbon allocation patterns.

In contrast to long-term evolutionary trends for which local populations are well adapted, the rapid change in stresses of anthropogenic origin suggests that genetic management of forests will be essential. Methods to link tree breeding for utility benefits with gene conservation to facilitate sustainable forestry should be given a high priority. The importance of maintaining high levels of genetic diversity in breeding populations and in plantations cannot be overstated.

Recent development in understanding mechanisms of stress tolerance suggest a commonality of oxidative stress from diverse factors such as air pollutants, herbicides, temperature extremes, toxic salts, and drought. This finding may lead to an increased understanding of the antioxidant tolerance mechanisms and, eventually, to the possibility of selecting for stress tolerance. This could be particularly valuable for developing forest trees for unpredictable future stresses.

Air pollution, climate change, forest trees, natural selection, biodiversity, adaptation, trembling aspen, red pine, silver fir, tree breeding.

See also: **Biodiversity**: Plant Diversity in Forests. **Environment**: Environmental Impacts; Impacts of Air Pollution on Forest Ecosystems; Impacts of Elevated CO_2 and Climate Change. **Health and Protection**: Diagnosis, Monitoring and Evaluation. **Site-Specific Silviculture**: Silviculture in Polluted Areas. **Tree Physiology**: Forests, Tree Physiology and Climate.

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Molecular Biology of Forest Trees

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Introduction

Transformation and Regeneration

In order to genetically engineer a plant, one must be able to insert a gene into the genome of an individual plant cell and then cause that cell to differentiate into a whole plant. The former process is referred to as transformation; the latter, regeneration.

The most common way of transforming cells exploits the ability of *Agrobacterium tumefaciens*, the causative agent of a common plant disease known as 'crown gall.' *Agrobacterium* contains a closed-circular piece of double-stranded DNA called the tumor-inducing (Ti) plasmid. During infection, *Agrobacterium* inserts a segment of the Ti plasmid, called T-DNA (transferred DNA), into the plant's nuclear genome. This T-DNA contains genes encoding enzymes that catalyze the synthesis of plant growth regulators (cytokinin and auxin) which

together control cell proliferation. This results in the formation of a tumor, within which the bacterium resides. The T-DNA also contains genes encoding enzymes that catalyze the synthesis of unique amino acids that the plant cannot utilize, and that serve as a carbon source for the bacterium.

Agrobacterium does not select specific genes to be shuttled into the plant. The T-DNA is defined by specific border sequences; thus, any genes located between these borders will be transferred. Plant genetic engineers isolate the Ti plasmid, excise the genes responsible for pathogenesis, and replace them with genes of interest. A minimum of two genes generally is inserted between the T-DNA borders: the target gene (e.g., insect or disease resistance) and a selectable marker. After the modified plasmid is reinserted into *Agrobacterium*, a piece of plant tissue (explant) is co-cultivated in a suspension of bacterial cells containing this modified Ti plasmid. However, not all of the explant cells will be transformed by the bacterium.

Selection helps determine how successful transformation was. The most commonly used selectable marker gene is *NPTII*, which imparts resistance to kanamycin. Untransformed plant cells ordinarily die when exposed to this antibiotic. When co-cultivated explants are plated on a solid medium containing kanamycin, only cells transformed with *NPTII* survive. Because the selectable marker gene is directly linked to the gene of interest (transgene), it too should be present in the transformed cell.

Another DNA delivery system, biolistics, involves coating microscopic beads (usually gold or tungsten) with DNA. These beads are propelled at an explant, usually with a burst of compressed air as the driving force. Once inside the cell, DNA that sloughs off the bead can recombine with a plant chromosome. Biolistics is often less efficient than *Agrobacterium*-mediated transformation because of cellular damage from the impact of the beads, digestion of the transgene by cytosolic enzymes (nucleases), and the need for recombination. The selection process for cells transformed biolistically is as that described above.

Transformed cells that survive selection are used to regenerate a whole plant. The two main routes for *in vitro* regeneration are embryogenesis and organogenesis. In the former, cells are coaxed to differentiate into an embryo, similar to what is contained within a plant's seed. Organogenesis, on the other hand, is the process by which cells differentiate directly into specific organ types (e.g., shoots and roots). Both means of regeneration are done by successive transfers of the co-cultivated explants to media containing the proper type and concentration of plant growth regulators, mainly cytokinin and auxin. Varying the cytokinin : auxin ratio will result in callus formation, shoot organogenesis, or root initiation.

Recombinant DNA Techniques

The expression of a gene's coding sequence (i.e., transcription and processing to produce mRNA and translation of mRNA into protein) is carefully regulated by adjacent control sequences. Promoters are upstream elements that direct the timing, location, and extent of a gene's expression. Constitutive promoters allow for high levels of expression, in (nearly) all tissues, all of the time. The most commonly used constitutive promoter is derived from the cauliflower mosaic virus (CaMV) *35S* gene. Other promoters can be activated through treatment with a specific inductive agent, or may allow for tissue- and/or temporal-predominant expression. Enhancers are the portion of a promoter that can elevate a gene's expression level, and can act in *trans*. There are also downstream elements, terminators, which signal the end of transcription.

Once a gene is transcribed, its message undergoes processing. In some cases interspersed sequences, called introns, are cut out of the mRNA at well-defined sites. The remaining sequences, termed exons, are then spliced together.

It is possible to enzymatically reverse-transcribe single-stranded mRNA to reproduce the double-stranded DNA from which the message was derived. This product is called complementary DNA (cDNA). Complementary DNA does not contain introns that may have been present in the corresponding genomic DNA. A cDNA library is a collection of sequences from only those genes that were being actively transcribed at the time the mRNA was extracted. Partial sequence information derived from the cDNAs in a given library is called an expressed sequence tag (EST) library.

Restriction enzymes are proteins that catalyze the cleavage of DNA at very specific recognition sites, usually about four to eight base pairs in length. Another enzyme, called ligase, fuses the ends of DNA that have been cut by the same restriction enzyme. This pair of enzyme types allows molecular biologists to mix and match various coding sequences and promoters.

Reporter genes allow one to visualize a promoter's expression pattern. The most common reporter gene, β-glucuronidase (GUS), encodes an enzyme that catalyzes the conversion of a colorless substrate to an insoluble, blue-colored product that precipitates in cells expressing the gene encoding it.

Polymerase chain reaction (PCR) is a technique that allows for amplification of a specific piece of

DNA. Short pieces of DNA (usually about 15 to 30 nucleotides in length), referred to as primers, are designed to complement sites on opposite strands of the target DNA. These primers are mixed with a DNA sample containing the fragment to be amplified, along with a complete set of nucleotides (i.e., dATP, dCTP, dGTP, and dTTP) and thermostable DNA polymerase. The mixture is heated to 94°C for approximately 1 min to separate the two strands of DNA (denature). Subsequent cooling of the mixture allows complementary strands to anneal to each other; the optimal annealing temperature depends on the length and composition of the primer (usually about 55–60°C). Because the primer DNA is of a much higher concentration than the template, it is much more likely to find its partner. The mixture is then heated to a temperature that is optimal for the polymerase to extend the primers using the genomic DNA as a template (72°C). Successive rounds of denaturation, annealing, and elongation result in a geometric increase (up to 4×10^6 times in 25 cycles) in the accumulation of product. Reaction mixtures are contained in tubes that are inserted in the aluminum block of a thermocycler. This machine can rapidly heat and cool liquid that is circulated through the interior of the block, and has a microprocessor that can be programmed to maintain different temperatures for various lengths of time.

Nucleic acids (e.g., restricted genomic DNA, PCR-amplified product, RNA, etc.) can be separated via electrophoresis and visualized. A dilute ($\sim$0.8%), heated solution of agarose (a polysaccharide) is poured into a form and allowed to solidify (as a gel). A 'comb' is inserted into the solution before it cools to create depressions (wells) in the gel, where DNA samples can be loaded. A strong electrical current is passed through the gel matrix, allowing the nucleic acids (which are charged molecules) to be separated based on their molecular weights. A standard, containing a mixture of nucleic acid fragments of known size, is run in a parallel lane on the gel, for estimating the size of nucleic acid fragments contained within the sample. To visualize the separated DNA fragments, the gel is stained with a dye, such as ethidium bromide, which binds to nucleic acids and fluoresces an orange color under ultraviolet light.

Platforms for Studying Tree Biology

Marker-Aided Selection

Marker-aided selection (MAS) involves the identification of individuals based on the presence of DNA markers in offspring derived from parents whose genomes have already been mapped. DNA markers are usually random nucleotide sequences that do not encode a functional gene; they are frequently amplified via PCR and are visualized on a gel. The position of the markers on a chromosome is mapped by determining the frequency of their mutual recombination when haploid gametes are formed in a given individual. To accomplish this, one needs a pedigree (a population in which gamete contribution from specific individuals can be traced to their offspring). Target genetic traits, that show extremes in variation, can be identified in a segregating population at an early stage in plant growth, based on linked markers. Traits of interest that are measured on a quantitative (linear) scale are referred to as QTLs (quantitative trait loci). These traits are typically affected by more than one gene. Examples include wood density, stem form, and frost resistance.

The potential impact of MAS on traditional breeding is tremendous. Once breeders know which bands on the gels indicate extremes for the traits of interest, screening can be conducted at a very early age, and selected individuals can be clonally propagated. Conducting this work in a laboratory obviates the need for expensive field trials, and forest managers will realize the additional genetic gain soon after crossing the selections from the previous generation. Sexual reproduction following MAS is possible, but gains will be fewer and greatly delayed relative to the clonal propagation. This is because juvenile selections must reach maturity, and the planting stock will not be genetically identical to the parents. However, wide-scale adoption of MAS for tree improvement has not been realized. Various problems impede the application of MAS, including the lack of cost-effective, high-throughput marker systems and lack of linkage disequilibrium. The latter is said to occur when the observed frequencies of haplotypes (a set of closely linked genetic markers present on one chromosome, which tend to be inherited together) in a population do not agree with haplotype frequencies predicted by multiplying the frequency of individual genetic markers in each haplotype.

Gene-Tagging Methods

Because of the ease with which it can be transformed, poplar (*Populus*) is a convenient model system for discovering tree genes of potential commercial value. Using gene tagging, a new gene or regulatory element is inserted into the genome as a probe for determining the function or expression pattern of genes adjacent to the insertion site. With gene-trap tagging, a reporter gene (e.g., GUS) is used to visualize the expression pattern of a nearby gene (**Figure 1**). In another tagging method, activation tagging, a strong

Figure 1 Staining of a poplar gene-trap line. The blue color demonstrates that GUS expression was limited to the vascular tissue in this leaf. Photograph supplied by Andrew Groover, Institute of Forest Genetics, US Forestry Service, Davis, CA, USA.

enhancer that is effective some distance from a native promoter is randomly inserted in the genome. Elevated expression of the nearby gene may result in an aberrant phenotype. Alterations that yield desired phenotypic changes, such as early flowering, modifications in crown form, or root development, are then analyzed for the causative gene (**Figure 2**). Overexpression of some native genes (e.g., those affecting wood quality) may not give rise to a visually apparent change. In this case, other, high-throughput analyses are needed for screening a population of transgenics (see below).

Both gene-trapping and activation tagging are a form of 'gene discovery' because the genes identified may be functionally unknown. Nevertheless, the unique nature of the inserted sequences (tags) permits the affected gene to be easily identified and isolated.

Poplar Genome Sequence and Informatics

The US Department of Energy has committed $28 million to producing a 6× draft sequence (on average, every gene is sequenced six times) for the entire *Populus* genome by the end of 2003. As a result,

Figure 2 A GA 2-oxidase (GA2ox) mutant isolated via activation tagging. The GA2ox gene encodes an enzyme that degrades biologically active forms of gibberellic acid, a plant growth regulator involved in controlling various aspects of plant growth and development (e.g., seed germination, flower initiation, fruit development, stem elongation, wood formation, leaf expansion). Mutant (left) and wild-type (right) plants are the same age and were grown under the same conditions. Photograph supplied by Victor Busov, Forest Science Department, Oregon State University, Corvallis, OR, USA.

poplar is only the third plant species for which the entire genome sequence is available.

To maximize its utility, a genomic sequence must be annotated. Genome annotation, one aspect of the rapidly evolving field of informatics, has two components. The first is structural, involving the identification of hypothetical genes, termed open reading frames (ORFs), in the DNA sequence using computational gene-discovery algorithms. The second component focuses on assigning function to the predicted genes by searching databases for genes of known function that have similar sequences. For complex eukaryotic genomes, the main problem lies in the structural component of annotation. In eukaryotic genomes, a gene is defined as a locus of cotranscribed exons, which may give rise to several splice variants and, hence, multiple protein products with multiple functions. The structural identification of genes depends heavily on the use of homologous cDNA/EST or protein sequences. Algorithms for coding-sequence recognition exhibit performance trade-offs between increasing sensitivity (ability to detect true positives) and decreasing selectivity (ability to exclude false positives). The identification of intron–exon boundaries and splice sites is of further importance. Genes not represented by homologous DNA or protein sequences must be identified by *de novo* methods, which remains a serious impediment to genome annotation (described below).

Transformation to Confirm Gene Functionality

Transformation is an important tool for analyzing gene functionality. Through constitutive or conditional up- or down-regulation (knock-in/knock-out, KI/KO) of a target gene, important information about its function and downstream targets can be obtained. To date, tree genetic engineering has largely been performed using strong constitutive promoters, the aim being to obtain maximum levels of expression and, consequently, maximal effect. Such a strategy may be useful for imparting certain commercial traits (e.g., insect resistance or herbicide tolerance), but is not practical when the goal is to alter the expression of an endogenous gene. In fact, numerous cases of gene silencing have resulted from this approach. Moreover, altering the expression of key genes, such as transcription factors or other regulators, may have lethal or at least strong negative effects on plant development. In the future, an important application of genetic engineering will be large-scale evaluation of gene function via KI/KO strategies. Without a reliable system for conditional transgene expression, it may be impossible to produce transgenic plants with altered expression of numerous genes vital to growth and development. It is imperative for the inducer to be highly specific to the target promoter and to lack phytotoxicity. The target promoter must also be tightly regulated (i.e., no 'leaky' expression), with high-level expression being conferred upon induction.

High-Throughput Analyses

Gene expression Microarray technology can be used to study the simultaneous expression patterns of thousands of genes (**Figure 3**). Here, unique sequences of DNA (either oligonucleotides or cDNA) sequences are anchored to a glass slide in neatly arrayed microscopic spots (the probe). Spotting is done using very fine needles and robotics. Messenger RNA (the target) is isolated from both control and treated plants; each RNA sample is labeled with a different color fluorescent dye. The labeled RNA is allowed to hybridize to its complementary single-stranded DNA probe before the microarray is scanned. A laser then excites each fluorescent dye at a specific wavelength, and emissions are captured digitally. The fluorescent images for the control and treated samples are superimposed and the resulting color of each spot reveals whether a given gene is differentially expressed in the two samples.

Chemical characterization If the forward genomics approaches described above do not result in a visual phenotype, one must have a way to quickly screen large populations of independent transgenic lines. Techniques have been developed for using near-infrared (NIR) spectra to obtain quantitative measurements of lignin, cellulose, other carbohydrates, and extractives from plants. The accuracy and precision of these techniques is equal to conventional chemical methods. Similar success has been reported with descriptions of physical properties (e.g., density, microfibril angle, modulus of rupture, and tensile stress), making it possible to use NIR data to infer mechanical properties of wood. Samples showing atypical NIR phenotypes can be further subjected to mass spectral analyses for more detailed insights into the biosynthetic networks that have been perturbed.

Applied Technology

Recent Progress

Trees have already been transformed with genes that impart a variety of commercially useful traits, including insect resistance (**Figures 4** and **5**), herbicide tolerance, modified lignin and cellulose, altered metabolism, phytoremediation, and hormone biosynthesis.

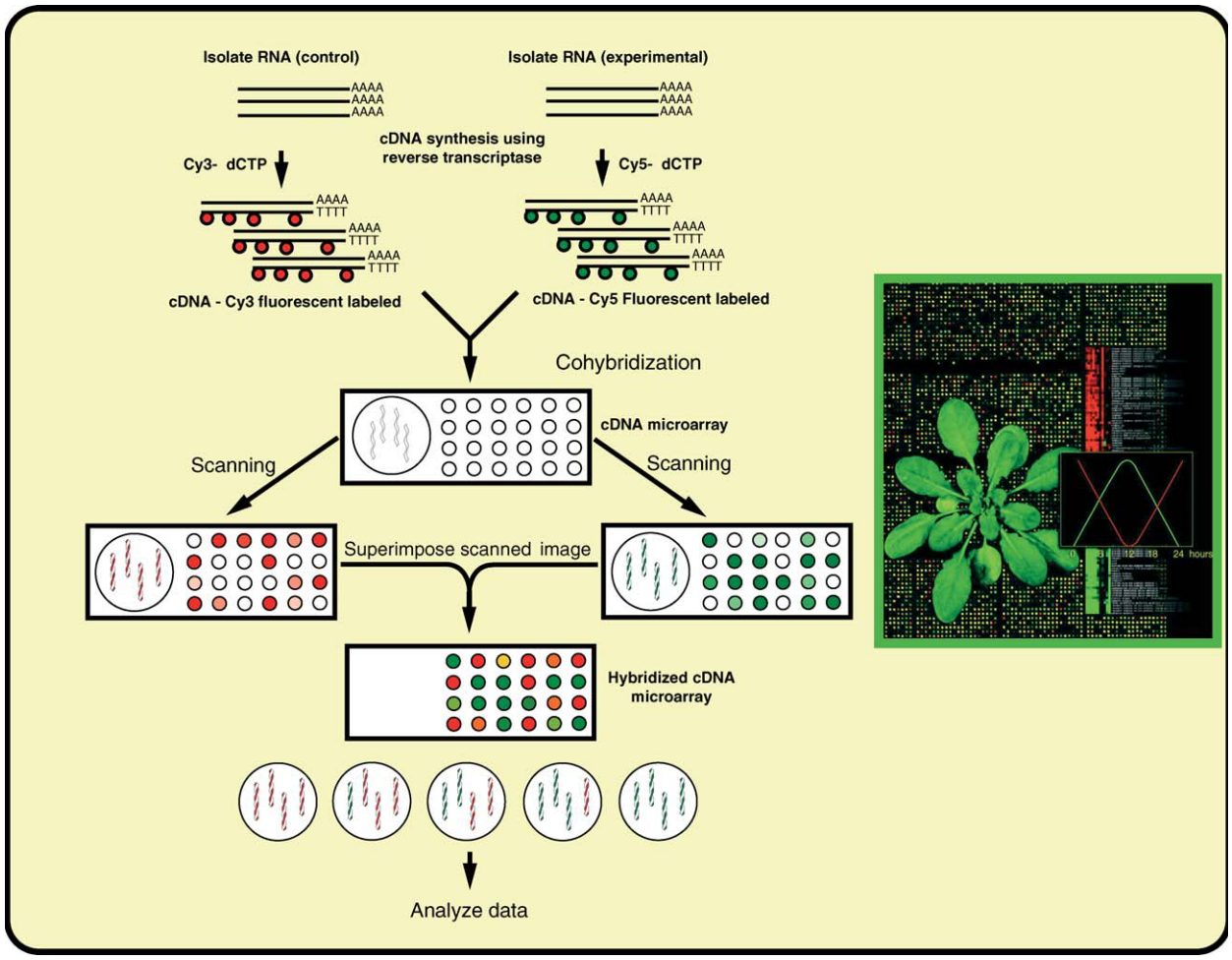

Figure 3 Experimental approach for labeling, hybridization, and scanning cDNA microarray. RNA is isolated (from control and experimental conditions) and labeled separately using two distinct fluorescent dyes (Cy3-dCTP and Cy5-dCTP) using reverse transcriptase. Both the labeled samples are cohybridized to the microarray. After hybridization and washing, the microarray is scanned using a confocal laser scanner at specific wavelengths, 543 nm (Cy3-dCTP) and 633 nm (Cy5-dCTP), respectively, for the two fluorescent dyes. The two fluorescent images are superimposed, and the data is analyzed for gene expression, using bioinformatics and image processing software. Fluorescent spots that are either red or green indicate the gene represented in the spot is expressed under one condition but not in the other. Spots carrying yellow/purple grades indicate that the gene represented in the spot is differentially expressed between the control and experimental conditions. Reproduced with permission from Rishi AS, Nelson ND, and Goyal A (2002) DNA microarrays: gene expression profiling in plants. *Reviews in Plant Biochemistry and Biotechnology* 1: 81–100. Arabidopsis Microarray insert reproduced with permission from Wisman and Ohlrogge (2000) *Plant Physiology* 124: 1468–1471.

Plants are now being used as bioreactors to produce recombinant proteins for commercial purposes in a rapidly emerging field of biotechnology known as 'molecular farming' (or 'biopharming'). However, recent incidents (e.g., StarLink and Prodigene) have stirred public uneasiness regarding the potential for contaminating the food supply with genetically modified crops that are expressing biologically active molecules. Therefore, the use of non-food crops, such as poplar or other tree species, may alleviate public and regulatory concerns about utilizing plants as 'factories' to produce anything from biodegradable plastics and industrial enzymes to antibodies and other pharmaceuticals. It is possible to effect the production of these compounds in transgenic trees, as a way of adding value to the crop. Transgenes can be placed under the control of an inducible, leaf-specific promoter so that there is no metabolic drag during the life of the plant, and so the transgene is not expressed in the bole of the tree.

Public Concern

Genetic engineering has been used to introduce novel, commercially valuable traits into a variety of agronomic crops. Although the potential benefit of these traits has also been demonstrated in transgenic trees, no such trees are currently being grown in the USA for commercial purposes.

Figure 4 Field-grown insect-resistant poplars. The tree depicted in the left-hand panel was transformed with the *Cry3A* gene from *Bacillus thuringiensis*. The tree on the right is a nontransgenic control. Damage is the result of feeding by larvae of the cottonwood leaf beetle (*Chrysomela scripta*).

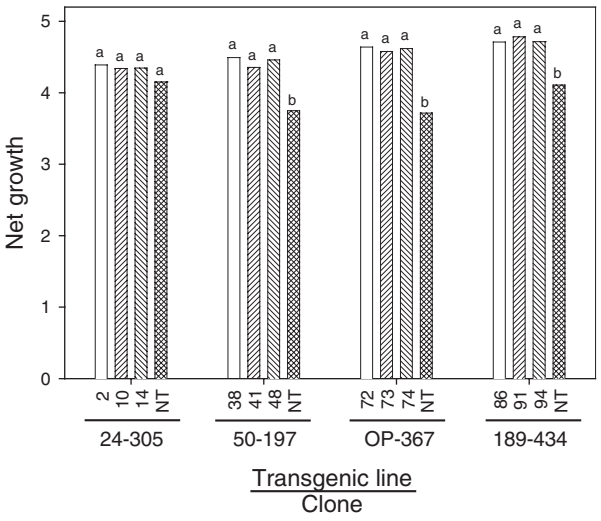

Figure 5 Growth of insect-resistant transgenic poplar trees. The first three bars in each cluster represent mean growth of 10 independent transgenic lines containing the *Cry3A* gene; black bars are for nontransformed controls (NT). Bars labeled with the same letter are not significantly different from each other. On average, nontransgenics grew 24% slower than the transgenic lines. Slower growth is presumably the result of having less photosynthetic capacity, as a result of insect feeding.

Agronomic crops generally are herbaceous annuals that are highly domesticated and have few, if any, wild relatives. Most tree plantations are established in close proximity to wild or feral relatives, increasing the probability of transgene spread. Thus, a major concern over the use of transgenic trees is the potential for extensive transgene dispersal through pollen and seeds. Because the issues surrounding the commercial deployment of transgenic woody perennials are more complex, federal regulatory agencies are now deciding what additional safeguards need to be put in place. Key areas of concern include: increased invasiveness, horizontal transfer, and development of resistance by the pest to the transgene product.

Flowering Control

Before transgenic trees can be grown for commercial purposes it is imperative to have a system for mitigating the spread of transgenes to interfertile wild relatives. Sterility is the most effective way of accomplishing this objective, as well as maintaining rapid growth. Normally, after trees undergo the transition to maturity, photosynthate is diverted away from vegetative growth and used to produce reproductive structures. Blocking flowering will likely result in preserving juvenile growth rates and preventing the formation of unwanted reproductive structures (e.g., seed pods, cotton, pollen, etc.). Finally, sterility will help curb genetic pollution. Trees such as poplar, which can be vegetatively propagated, are often grown in intensively managed plantations. In some cases, a single genotype is planted across thousands of acres. Because these trees are clonally propagated, all of their cells, including their pollen, contain exactly the same DNA. This monotypic pollen can travel considerable distances and fertilize flowers on compatible wild trees, which could affect genetic diversity in the wild. Flowering control has the potential to reduce the likelihood of this occurring.

Methods for Engineering Reproductive Sterility

One common way to engineer sterility is to ablate cells by expressing a cytotoxin gene in a tissue-specific

manner. Floral promoters can be fused to one of a variety of cytotoxin genes that lead to rapid and early death of the cells within which the gene expressed. One of the more popular ways to engineer sterility in herbaceous plants employs an RNase gene, the product of which degrades messenger RNA.

A second way to genetically engineer flowering control is through the use of dominant negative mutations (DNMs). DNM genes encode mutant proteins that suppress the activity of coexisting wild-type proteins. Inhibition can occur by a variety of means, including formation of an inactive heterodimer, sequestration of protein cofactors, sequestration of metabolites, or stable binding to a DNA regulatory motif. Overexpression of floral regulatory genes that encode proteins with altered amino acid in the highly conserved domains can result in mutant floral phenotypes. Similar changes can also eliminate the encoded protein's ability to bind DNA. A potentially powerful alternative approach is to introduce a transgene encoding what is called a 'zinc finger' protein, which is specifically designed to block transcription of the target gene.

A third technique to control flowering involves gene silencing. In a variety of eukaryotic organisms, double-stranded RNA is an inducer of homology-dependent gene silencing; use of double-stranded RNA to induce silencing has been termed RNA interference. Studies in plants have shown that strong silencing can be achieved by introducing a transgene containing an inverted repeat of a sequence that corresponds to part of the transcribed region of the endogenous gene targeted for silencing. Such transgenes induce posttranscriptional gene silencing (PTGS) by triggering RNA degradation. Although this approach appears to provide a reliable means for engineering stable suppression of gene activity in plants, whether PTGS will be effective practically is uncertain, due in part to the ability of plant viruses to suppress PTGS. An alternative is to use a transgene containing an inverted repeat of a target gene's promoter region; this has been shown to induce *de novo* DNA methylation and transcriptional gene silencing (TGS). Unlike PTGS, TGS is not susceptible to viral suppression; however, it is unclear whether all endogenous plant promoters can be silenced by this method.

All of these approaches rely on the use of genes that control floral development, either through the use of floral-specific promoters or coding sequences with high homology to native genes that are targeted for suppression/silencing. In addition, flowering-time genes provide a means of advancing or retarding the onset of reproductive growth. The former can facilitate more rapid progress through conventional breeding; the latter can, depending on the rotation of the crop, serve as a transgene confinement strategy.

The Need for Transgene Stability

Stability of transgene expression is especially important for trees, which undergo numerous dormancy cycles and are often exposed to extreme environmental changes during their long lives. Unstable expression has taken on greater significance given recent reports that environmental changes and dormancy can trigger transgene silencing. Sexual reproduction may also affect the stability of transgene expression. To date, little evidence exists for somaclonal variation or transgene instability in poplar, but these characteristics will need to be evaluated over several years and in a variety of settings for any transgenic tree that is to be commercialized.

See also: **Genetics and Genetic Resources**: Cytogenetics of Forest Tree Species; Genetic Systems of Forest Trees. **Tree Breeding, Practices**: Breeding for Disease and Insect Resistance. **Tree Breeding, Principles**: A Historical Overview of Forest Tree Improvement; Breeding Theory and Genetic Testing; Forest Genetics and Tree Breeding; Current and Future Signposts.

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