

## Review

# Forestry's fertile crescent: the application of biotechnology to forest trees

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## Summary

Relative to crop plants, the domestication of forest trees is still in its infancy. For example, the domestication of many crop plants was initiated some 10 000 years ago in the so-called 'Fertile Crescent' of the Middle East. By contrast, the domestication of forest trees for the purposes of producing more fibre began in earnest in the last half century. The application of biotechnology to forest trees offers a great potential to hasten the pace of tree improvement for desirable end uses. This review outlines some of the progress that has been made in the application of biotechnology to forest trees, and considers the prospects for biotechnologically based tree improvement in the future.

**Keywords:** domestication, genetic engineering, forestry, tree, wood.

## The importance of forestry

It is difficult to overstate the importance of wood. For the majority of people in the world, wood is crucial for their sustenance – for heating their homes and cooking their food. In 1999, well over half of the global total roundwood production of 3335 million m<sup>3</sup> was used as woodfuel (FAO, 2001). Of this, about 90% was produced and consumed in developing countries, for the purposes of heating and cooking (FAO, 2001). The consumption of wood products is increasing in concert with population growth and economic development. It is expected that the demand for more biomass from trees will increase in the future – both to sequester carbon dioxide already in the atmosphere (Scholes and Noble, 2001), and to meet the demand for renewable energy alternatives to fossil fuels.

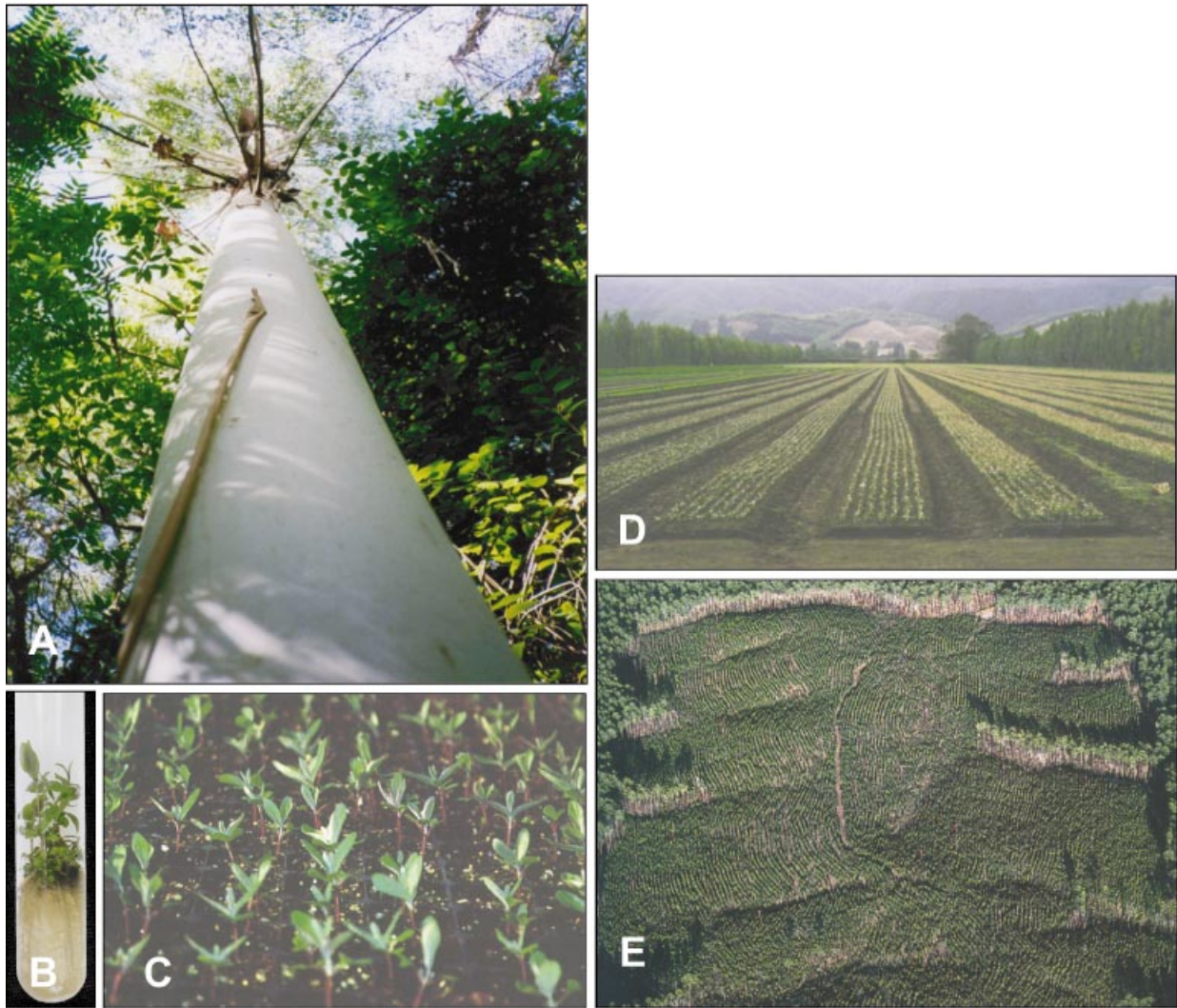
Simultaneous with the demand for forest products, there is a great demand to conserve forest ecosystems, for both their ecological and aesthetic values. Efforts around the world are aimed at achieving sustainable forest management, an approach that balances economic, environmental and social objectives (FAO, 2001). One way of meeting the increasing demand for forest products, while conserving forest ecosystems, is to increase the productivity of forest trees on managed plantations, to ensure that ecologically

and socially valuable old-growth forests are under less pressure from harvesting.

## Tree domestication

While there has been an extensive history for the domestication of food crops, the domestication of forest tree species has only recently begun (Libby, 1973). The history of the domestication of a wide variety of food crops, including wheat, chick pea, and the fibre crop flax began around 10 000 years ago, in the 'Fertile Crescent' of South-west Asia, an area found in present-day Turkey and Syria comprising the upper reaches of the Tigris and Euphrates Rivers (Diamond, 1997a,b; Lev-Yadun *et al.*, 2000). While some evidence suggests that trees were cultivated alongside food crops in traditional agroforestry, at the same time that food crops were being domesticated (Chepstow-Lusty *et al.*, 1998), trees themselves were not domesticated except for the partial domestication of some fruit trees. Until relatively recently, there has not been a 'Fertile Crescent' for forest trees. But this situation is changing.

Tree improvement, also referred to as domestication, began in earnest about half a century ago with conventional breeding (Libby, 1973; Zobel and Talbert, 1984). Forest tree breeding efforts have focused on the most economically



**Figure 1** Current approaches to forest tree domestication frequently involve artificial selection followed by clonal propagation. Conventional forest tree domestication has involved the breeding and selection of superior individuals for use in tree improvement programmes, such as this example of *Eucalyptus globulus* found in Australia (A). This has generally been followed by clonal propagation via vegetative means (B), such as rooted cuttings, as in the case of this eucalyptus plantlet, or somatic embryogenesis. Clonally propagules are prepared for deployment in the greenhouse (C) and/or the field (D), and are finally deployed in plantations (E) as seen in these examples for eucalyptus (C and D) and pine (E) near Rotorua in New Zealand.

valuable and fastest growing species (see Appendix). Conventional tree breeding has been hampered for a number of reasons, almost all of which are related to the biology of forest trees. For example, trees are very long lived and very slow to mature. Conventional forest tree breeding is also limited by the mating system of forest trees. Many of the most economically important forest tree species are out-crossing genera. Due to the extent of heterozygosity in these species, many recessive deleterious alleles are retained within populations – causing a high ‘genetic load’ and extensive inbreeding depression (Lynch and Walsh, 1998; Williams and Savolainen, 1996). This restricts the use of conventional breeding practices, such as selfing and backcrossing

(Williams and Savolainen, 1996), and makes it very difficult to fix desirable alleles in a particular genetic background. Thus, fixing a naturally rare, recessive allele of commercial value – such as one for modified crown structure, sterility, or wood chemistry – is extremely difficult and time consuming. Consequently, once superior individuals (clones) have been identified, it is desirable to deploy them in a commercial context using a method of deployment that avoids the breeding cycle as much as possible. As a result, many tree improvement efforts have made use of clonal (i.e. vegetative) propagation to deploy superior individuals when possible (Figure 1). This method captures all of the genetic effects (dominance, additive and epistatic) and allows the deployment of superior

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genotypes while circumventing the need to proceed through a breeding cycle. Nevertheless, this method still requires the generation and identification of superior genotypes, which is both a time consuming and difficult process.

Molecular genetics offers a means of circumventing this problem. Molecular markers for target alleles in candidate genes, even if rare, when combined with intensive selection could allow subpopulations to be developed with rare homozygous genotypes. Even more powerfully, genetic engineering, by proving dominant forms of alleles for specific genes, can allow a novel trait to be imparted to any genotype in a single generation with little or no alteration to its other genetic properties. Thus, if there is to be a Fertile Crescent in forestry, it is to be found in the laboratories that operate at the interface of basic plant molecular genetics and forestry.

### Domestication for wood properties

The ultimate aim of all domestication efforts is to improve the productivity of the crop for desired end uses. In the case of forest trees, increases in the volume of wood produced, or enhancements in the properties of that wood, are obvious targets for crop domestication. In several instances genetic transformation has been used to modify wood quantity or quality in trees. Most of these studies have focused on altering the chemical composition of cell walls in order to impact down-stream processing for desired end-uses. Most of these studies have focused on altering the quantity or quality of lignins in the wood.

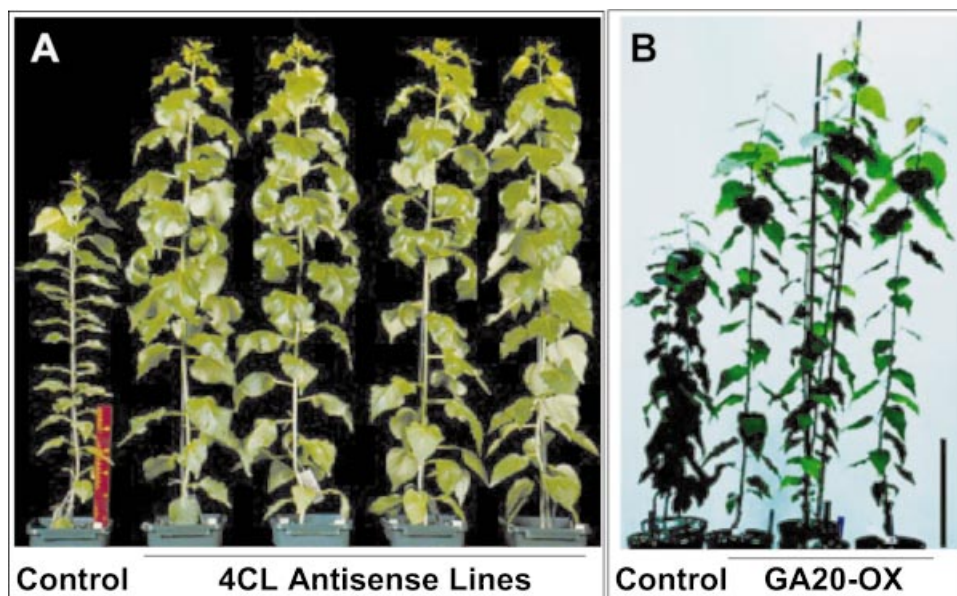
Lignins are three-dimensional phenolic polymers that are embedded in the cell walls of specialized secondary cell walls in specific cell types. Xylem cells, which make up wood, have highly lignified cell walls. Lignin functions as an inter- and intramolecular glue, which cross-links various cell wall components and embeds the cellulose microfibrils within a dense matrix (reviewed in Campbell and Sederoff, 1996).

In the manufacture of pulp and paper, cellulose microfibrils are the component of the cell wall that is desired, requiring that cellulose microfibrils be liberated from the lignin matrix. Furthermore, residual lignin components are susceptible to oxidation and will cause a yellowing of the resultant pulp or paper if not thoroughly removed or bleached. Thus, the costly portion of the pulp and paper making process, from both an economic and environmental perspective, is attributable to the removal of lignins. Therefore, it is highly desirable to develop means by which lignin content is decreased, or make lignins more extractable, while maintaining basic

structural integrity and the resistance of wood to herbivores and pathogens.

Changes in the chemical composition of lignins can make them more extractable (Chiang *et al.*, 1988). The phenol groups in lignins differ in their extent of hydroxylation and methoxylation. In general both gymnosperm and angiosperm lignins contain guaiacyl (G) units; whereas only angiosperms have additional syringyl (S) units (Campbell and Sederoff, 1996). Syringyl units have an additional methoxyl group in comparison to guaiacyl units. In nature, within angiosperms there is a significant amount of variation in the ratio of G to S units (Campbell and Sederoff, 1996). Pulping analyses revealed that lignins were more readily removed from plants with higher S:G ratios (Chiang and Funaoka, 1990; Chiang *et al.*, 1988). Therefore, one strategy to increase the extractability of lignins is to engineer an increase in the S:G ratio in wood. This has been achieved by over-expressing a rate-limiting enzyme involved in the biosynthesis of S units in transgenic poplar plants, under the control of a promoter that directed strong xylem expression of the gene (Franke *et al.*, 2000). This increases the flux of lignin monomers into S units, at the expense of making G units. In some transgenic poplar plants, this resulted in a change in the quantity of S units from the wild-type level of 55 mol% to 85 mol%, effectively converting most G units to S units (Franke *et al.*, 2000). As predicted, the lignins from these plants were more readily removed (Franke *et al.*, 2000). These plants could be quite useful in the pulping process, resulting in the removal of more lignins under less harsh conditions.

Antisense suppression of genes encoding lignin biosynthetic enzymes has also been effective at reducing lignin quantity. The monomeric precursors to lignin polymers are derived from the biosynthetic activity of general phenylpropanoid metabolism. Antisense suppression of one of the enzymes of general phenylpropanoid metabolism, hydroxycinnamate : CoA ligase (4CL), resulted in a decrease in lignin content in transgenic aspen plants (Hu *et al.*, 1999). Down-regulation of a specific member of the 4CL gene family, which appeared to be involved in directing phenylpropanoids towards lignin biosynthesis, appeared to result in a decrease in lignin biosynthesis without impacting other major branches of phenylpropanoid biosynthesis, such as flavonoid biosynthesis (Hu *et al.*, 1999). Furthermore, due to the decrease in the percentage of lignin in the cell walls, the percentage of cellulose in the cell walls was higher (Hu *et al.*, 1999). Thus, per unit of biomass, the cellulose : lignin ratio is higher than in wild-type plants. Transgenic aspens with decreased 4CL activity also appeared to grow considerably faster than their wild-type counterparts under greenhouse



**Figure 2** Two means of achieving increased growth in forest trees. (A) Hybrid poplar plants were genetically engineered with an antisense construct that decreased the activity of a lignin biosynthetic enzyme, hydroxycinnamate : CoA ligase (4CL). In addition to having less lignin, transgenic poplar lines containing this construct (4CL Antisense Lines) were significantly larger than the corresponding control (Hu *et al.*, 1999) (Figure kindly provided courtesy of Prof. Vincent Chiang with permission from the Nature Publishing Group, Copyright 1999 <<http://www.nature.com/biotech>>.) (B) Hybrid poplar plants were transformed with a construct that over-expressed the gibberellin (GA) biosynthetic enzyme, GA 20-oxidase. In addition to having higher levels of bioactive GAs, plants over-expressing GA 20-oxidase (GA20-OX), were taller and had longer wood fibres than the corresponding controls (Eriksson *et al.*, 2000). (Figure kindly provided courtesy of Dr Thomas Moritz with permission from the Nature Publishing Group, Copyright 2000 <<http://www.nature.com/biotech>>.)

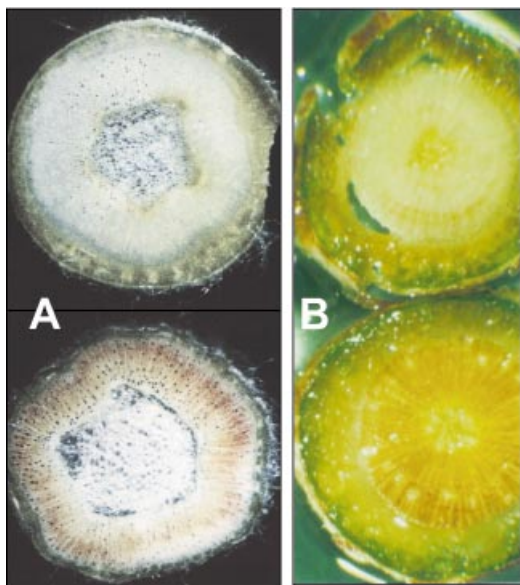
conditions (Hu *et al.*, 1999) (Figure 2). Because of its potential commercial importance, modulation of the 4CL gene is being studied in field trials and is likely to be pursued in a number of other tree species.

The monomeric precursors of lignins are channelled from general phenylpropanoid metabolism into the biosynthesis of lignins proper by the activity of a group of enzymes that includes hydrocinnamyl alcohol dehydrogenase (CAD). CAD is a relatively 'late' step in the lignin biosynthetic pathway, as the CAD substrate, as well as the product, can both be incorporated into lignin polymers. Antisense suppression of CAD activity in transgenic aspen resulted in an increase in the extractability of lignins from these plants (Baucher *et al.*, 1996). It is believed that the misincorporation of alternate lignin precursors into the cell wall enhances lignin extractability (Baucher *et al.*, 1996). The presence of these alternative precursors causes the plants that have suppressed CAD activity to have red coloured wood (Figure 3). Transgenic aspens with antisense suppressed CAD de-lignify much more readily and are better feedstocks for the pulping process (Baucher *et al.*, 1996). Importantly, recently published and extremely comprehensive field trial results using transgenic poplars harbouring the antisense CAD construct showed that the benefits to be gained from these transgenic trees could be

realized under field conditions, with no apparent environmental impact (Pilate *et al.*, 2002).

Interestingly, a naturally occurring variant of *Pinus taeda* harbours a null mutation in the *CAD* locus, which knocks out CAD enzymatic activity (MacKay *et al.*, 1997). Pine trees that are homozygous for the *cad* null allele are very similar to the CAD antisense aspens in a number of ways, including the colour of the wood (Figure 3), the wood chemistry, and the fact that lignins are more extractable from these plants (MacKay *et al.*, 1999). Even plants that are heterozygous for the *cad* null allele have enhanced pulping characteristics, and exhibit no other visible phenotype (MacKay *et al.*, 1997). These heterozygotes are thought to be ideal goals for tree improvement programmes, and interestingly already exist in large numbers in *Pinus taeda* improvement programmes due to phenotypic selection. This is because a tree harbouring the null allele (clone 7-56) was identified as a good general combiner and thus used extensively as a seed parent (Wu *et al.*, 1999). Serendipitously, 7-56 was later found to harbour the *cad* null allele. These findings demonstrate that natural allelic variation for lignin characteristics exist within natural gene pools. A mutant screen for null alleles at other loci that affect wood properties might also prove beneficial. Heterozygous or homozygous trees could then be



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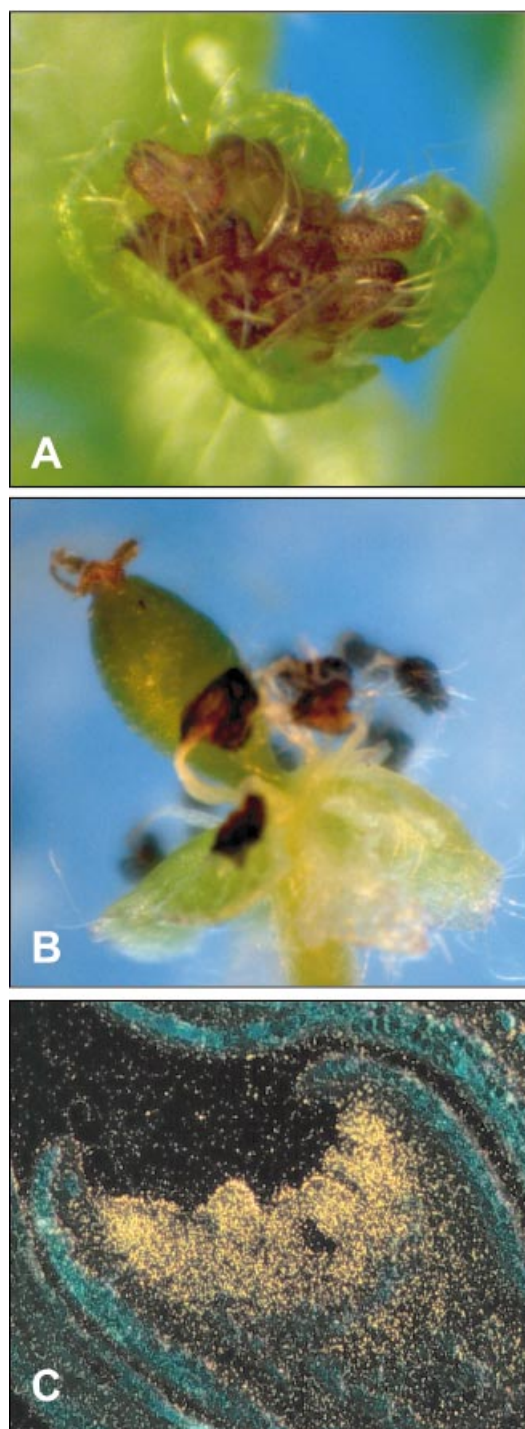
**Figure 3** Two methods of achieving increased lignin extractability in forest trees. The left hand panel (A) compares a stem cross section from a wild-type hybrid poplar (top) with that from a transgenic hybrid poplar that has been genetically engineered using an antisense construct to suppress the activity of the lignin biosynthetic enzyme hydroxycinnamyl alcohol dehydrogenase (CAD) (bottom). In addition to having a lignin that was more extractable, the CAD suppressed poplars produced a characteristic red wood (Baucher *et al.*, 1996). (Figure kindly provided courtesy of Dr Wout Boerjan and the American Society of Plant Biologists. <<http://www.plantphysiol.org/>>.) In the right hand panel (B), a stem cross-section from wild-type loblolly pine (top) is compared with that from a loblolly pine tree that has a mutant CAD allele such that it is a null for CAD activity (bottom). Again, as is the case for plants with antisense-suppressed CAD activity, the CAD null mutant also had more extractable lignin and produced the characteristic red wood (MacKay *et al.*, 1997). (Figure kindly provided courtesy of National Academy of Sciences, USA. Copyright 1997 <<http://www.pnas.org/>>.)

vegetatively propagated or the allele introgressed via marker aided breeding.

Genetic engineering has also been used to enhance wood properties other than those related specifically to wood

**Figure 4** Precocious and hermaphroditic poplar flowers induced by the over-expression of *LEAFY* and a poplar orthologue (*PTAG2*) of *AGAMOUS* on poplar plants in the greenhouse several months after transformation. Wild-type poplar flowers normally consist of only two whorls, an inner whorl of either stamens or carpels, and an outer whorl believed to be derived from sepals and/or petals termed the perianth cup. (A) A nearly normal male flower typically induced by over-expression of *LEAFY* in male poplar genotype INRA 353-38 (*Populus tremula* × *P. tremuloides*). (B) A hermaphroditic flower, containing both carpels and stamens, induced by over-expression of both *LEAFY* and *PTAG2* in the normally male poplar genotype INRA 353-38. (C) *In situ* hybridization showing *PTAG2* expression in a developing wild-type male flower. *PTAG2* expression is detected through out the inner whorl, where stamen primordia have formed, but not in the developing perianth cup (Brunner *et al.*, 2000)

chemistry. The biosynthesis of active gibberellins (GAs) in transgenic poplar was altered by over-expressing the gene encoding GA 20-oxidase, a rate limiting step in GA biosynthesis (Eriksson *et al.*, 2000). This resulted in an increase in the growth of the plants (Figure 2), and a corresponding increase in fibre length in the wood (Eriksson *et al.*, 2000). Fibre length is a trait of major industrial significance because



it is directly related to the strength of pulp and paper products (Eriksson *et al.*, 2000). While these transgenic poplar plants were impaired in other processes, such as initial root formation, they were still able to root later in development (Eriksson *et al.*, 2000). More specific promoters are likely to be capable of avoiding most pleiotropic effects.

Identification of the genes that control wood formation will be a prerequisite for future genetic engineering strategies aimed at modifying the rate of wood biogenesis or the properties of the wood itself. To this end, the characterization of the 'wood transcriptome' is underway for several forest tree species. Publicly funded, expressed sequence tag (EST) sequencing projects have been reported for poplar species (Sterky *et al.*, 1998) and loblolly pine (Allona *et al.*, 1998). The initial reports indicated that between 1000 and 3000 ESTs had been generated by randomly sequencing clones from cDNA libraries constructed from mRNA isolated from the differentiating xylem from these species. Currently more than 100 000 ESTs have been generated for *Populus* (<<http://Poppel.fys-bot.umu.se>> <<http://mycor.nancy.inra.fr/PoplarDB.html>> and approximately 70 000 ESTs have been generated for *Pinus taeda* (<<http://www.cbc.umn.edu/ResearchProjects/Pine/DOE.pine/index.html>> and new public efforts have been initiated for maritime pine (*Pinus pinaster*, <<http://www.pierroton.inra.fr/Lignome/Posters/lignomeforet.jpg>>), oak (*Quercus* sp., <<http://www.pierroton.inra.fr/Lignome/Posters/lignomeforet.jpg>>) and *Eucalyptus grandis*. (<<http://www4.ncsu.edu/unity/users/a/aamyburg/www/Eucalyptus/ESTs.htm>>.)

To date, the most comprehensive analysis of transcriptome activity during wood formation has focused on poplar. Combining an elegant method which was devised to uniformly amplify small amounts of RNA (Hertzberg *et al.*, 2001) with an intricate approach devised to collect individual differentiation zones from differentiating wood, a transcriptional 'roadmap' for wood formation has been established on the basis of microarray analysis in poplar (Hertzberg *et al.*, 2001). One of the most interesting aspects of this work was that the expression of specific gene family members at a given stage in wood formation could be readily discriminated. For example, very strong correlations were found between the expression of gene family members within the MYB family of transcription factors and particular stages in the wood formation process (Hertzberg *et al.*, 2001). Additionally, even when multiple and distinct genes encoded different lignin biosynthetic enzymes, it was possible to determine which specific genes were most abundantly expressed during the elaboration of lignins at different stages of wood formation. This type of correlative approach allows one to hypothesize about the involvement of a particular gene in the process of wood

formation. Furthermore, it suggests which genes might be good candidates for the directed manipulation of different stages of wood formation.

The postgenomic era has a lot to offer researchers operating in 'forestry's fertile crescent'. For example, *Arabidopsis* mutants, which are impaired in aspects of xylem formation and/or cell wall biosynthesis, allow the rapid identification of the underlying genes (e.g. Taylor *et al.*, 1999). Wood is simply secondary xylem, and many of the features of wood are imparted by the cell walls of these cells. The identification of *Arabidopsis* genes involved in these processes will suggest which candidate genes may be important in traits related to wood quality or quantity in trees. For example, experiments could be undertaken to genetically modify the expression of the orthologous gene in a tree species to determine its effect on wood formation. Alternatively, one could examine whether allelic variation at the orthologous locus in a tree contributed to the natural variation observed in wood-related traits. Analogous approaches could be used to identify genes involved in stress resistance, growth, reproductive development and other traits that are important for tree breeding.

Moreover, *Arabidopsis* can be used as a 'proxy tree'. *Arabidopsis* has the capacity to form secondary xylem (Busse and Evert, 1999; Dolan and Roberts, 1995; Kondratieva-Melville and Vodolazsky, 1982; Lev-Yadun, 1994; Zhao *et al.*, 2000). Therefore, *Arabidopsis* could be used in both mutant analyses and genetic engineering experiments to identify and validate genes that may be important in wood formation in trees, thereby circumventing the need to carry out costly, time and space consuming pilot experiments with tree species. Thus, postgenome *Arabidopsis* research offers the opportunity to ratchet off the tools and results obtained using *Arabidopsis*, and hasten the rate of progress toward the domestication of tree species.

### Domesticating tree reproduction

The long delay in the onset of flowering in forest trees, which lasts up to 30 or 40 years in some species, and the numerous vegetative characteristics that change with maturation, have long been of interest from both fundamental biological and tree breeding perspectives (reviewed in Greenwood, 1995). Protracted juvenile periods effectively preclude a number of breeding options for trees because of the time and space required (due to their large size at flowering). If genes could be identified and manipulated to enable flowering to be induced at will, inbreeding as a means for identifying and fixing beneficial recessive mutations, and introgression/backcrossing as a means to increase the frequency of rare

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alleles in breeding populations – standard practices in agronomic crop breeding – could become realities. The importance of inbreeding for accelerating domestication was recently highlighted by Allard (1999), who pointed to the large increases in productivity in maize that resulted from its conversion from an open-pollinated/outcrossing mode of breeding to one based on inbreeding/hybridization.

However, the converse – the ability to prevent the floral transition – is also desirable in trees for other reasons. Forest trees grown under intensive culture flower earlier than in the wild and produce large quantities of pollen and seed. A domesticated forest tree would flower late or not at all so that additional resources for vegetative growth are available (Bradshaw and Strauss, 2001). Moreover, flowering is a major constraint to the use of genetic engineering in tree improvement. Because most forest trees have an abundance of wild or feral relatives, outcross, and display long-distance gene flow via pollen and sometimes seed, there is likely to be considerable activist and public concern about large-scale use of genetically engineered trees. It is likely that US regulatory agencies will require a high level of transgene containment before most commercial applications of transgenic forest trees are approved (e.g. CEQ, 2000). Prevention of flowering would avoid, or greatly reduce, the public relations and ecological complications associated with genetically modified trees, allowing their full utilization for improving plantation productivity (reviewed in Strauss *et al.*, 1995). Fortunately, the 'tools' for successfully preventing functional flowering are available for several tree species, and those for manipulating the onset of flowering are on the horizon.

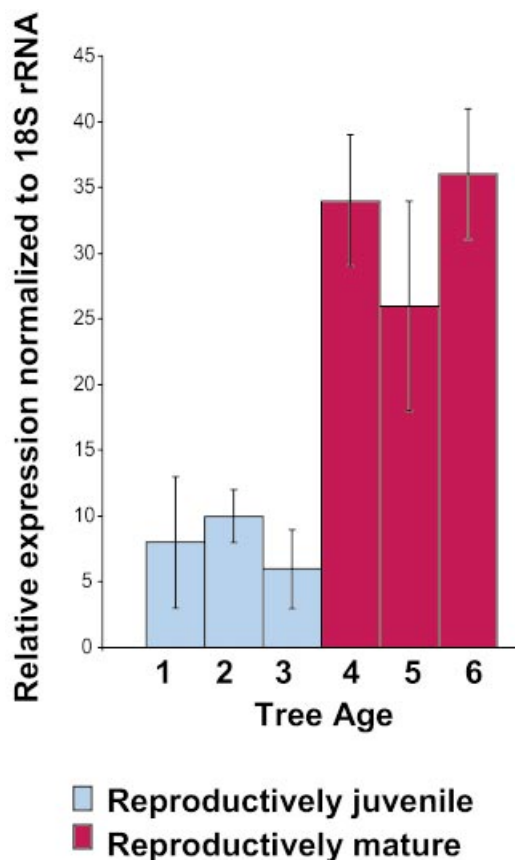
Studies in a diversity of plants, particularly *Arabidopsis* and *Antirrhinum*, indicate that the genes and mechanisms controlling floral meristem and organ development are generally conserved among angiosperms (recently reviewed in Gutierrez-Cortines and Davies, 2000; Pidkowich *et al.*, 1999). Furthermore, this conservation appears to extend to a significant degree to conifers. Based on RNA expression studies, and phenotypes induced by ectopic expression in *Arabidopsis*, spruce homologues of the C-class organ identity gene *AGAMOUS* (*AG*) appear to share a similar function in specifying female and male reproductive organs (Rutledge *et al.*, 1998; Tandre *et al.*, 1998). Similar studies also suggested that pine orthologues of the floral meristem identity gene *LEAFY* (*LFY*) may have functions which are largely equivalent to that of their angiosperm counterparts (Mellerowicz *et al.*, 1998; Mouradov *et al.*, 1998).

Inhibition of floral regulatory genes by mutation or post-transcriptional gene silencing (PTGS) has resulted in male, female and bisexual reproductive sterility in annual plants.

Thus, there is considerable evidence to support that silencing of tree homologues, especially when inverted repeat transgenes are used to induce PTGS (e.g. Chuang and Meyerowitz, 2000; Smith *et al.*, 2000), will be an effective and efficient approach to engineering sterility in trees. To overcome genetic redundancy in function, silencing of more than one gene may be needed to achieve complete sterility. Because the expression of these genes is often limited to floral tissues, use of their promoters to drive the expression of a cytotoxic gene is another promising approach, and combined with RNAi methods, could provide a genetic 'backup' mechanism so that breakdowns in sterility during large-scale deployment are extremely rare (Strauss *et al.*, 1995).

The major obstacle to engineering sterility in forest trees is simply demonstrating that a tree is reliably sterile under field conditions, and for many species, inefficient transformation, regeneration and field-testing capabilities are serious impediments. Transgenic trees with sterility constructs have been generated and some established in field tests, but the results cannot be observed until the trees reach maturity. These studies usually need to employ trees that lack nearby wild relatives, or provide other containment procedures (e.g. physical isolation or biological buffer zones), so that gene dispersal into wild populations is minimal. This points to another reason why the ability to induce early flowering is important: to speed the development and verification of sterility transgenes, preferably while in the greenhouse.

The finding that over-expression of *LFY* induced the formation of flowers in transgenic poplar shortly after transformation generated much excitement, and indicated that flowering in trees could be usefully manipulated (Weigel and Nilsson, 1995). Although these flowers are not entirely normal, and additional studies showed that *LFY*'s ability to induce early flowering in poplar was highly dependent on genotype (Rottmann *et al.*, 2000), co-transformation with *35S::LFY* can be useful for studying sterility transgenes and floral gene function. For example, using *35S::LFY*, the effects of over-expression of a poplar *AG* orthologue (Brunner *et al.*, 2000) on floral phenotype could be observed about 1 year following transformation (A. Brunner, unpublished data: Figure 5). It gave the surprising result that this simple genetic alteration could change the normally unisexual poplar flower to a hermaphroditic one. Recently, over-expression of either *LFY* or *APETALA1* was shown to accelerate normal flowering and fruit production in a citrus cultivar (Pena *et al.*, 2001). Clearly, more research is needed on the control of the floral transition in trees in order to develop methods for manipulating flowering time that are of practical use and widely applicable to various genotypes and species.



**Figure 5** Variation in vegetative gene expression of the poplar *LEAFY* orthologue (*PTLF*) is correlated with reproductive competence in preliminary studies. Expression was measured in vegetative buds (i.e. meristem and leaf primordia) that were initiated during the growing season of collection (collected in mid-summer) from a male poplar clone. Relative quantitative expression was determined by real-time, reverse transcriptase-PCR. Bars show one SD based on three replicate measurements within a single experiment.

The ideal sterility method from a tree domestication perspective would be to prevent the transition to flowering entirely, rather than to disrupt flower development. Many of the vegetative traits that change with maturation are also highly relevant to tree domestication. These include a progressive loss of rooting and *in vitro* regeneration abilities, changes in branching, and the transition from juvenile to mature wood characteristics. The mechanistic relationships between changes in vegetative features and reproductive competence are unknown, but it appears that at least a subset of vegetative traits and the acquisition of reproductive competence are regulated independently. For example, the transition from juvenile to adult leaves and the onset of flowering are under independent quantitative genetic control in *Eucalyptus globulus* (Jordan *et al.*, 1999). It is also widely observed that age-related changes in various measures of vegetative maturation occur at different rates. Independence among

vegetative phase changes, and these and the floral transition, enhances the potential for heterochronic evolution, and may be particularly advantageous to plants with long life cycles. It is also desirable from the standpoint of tree domestication. Domesticated trees could be engineered to exhibit accelerated maturation for some traits, such as wood characteristics, while remaining reproductively juvenile.

In most plants, the floral transition is influenced by environmental cues such as photoperiod and vernalization, as well as by endogenous signals related to plant age. Genetic networks that interact to control flowering are rapidly being unravelled in *Arabidopsis*, and at least 80 loci that affect flowering time have been identified (reviewed in Simpson *et al.*, 1999). One approach to studying phase change in trees is to build on this work by studying tree homologues of genes that are known to regulate flowering time and inflorescence meristem differentiation in *Arabidopsis* and other annual plants. For example, ectopic expression of *TERMINAL FLOWER1 (TFL1)* gene from *Arabidopsis* greatly delays the development of floral meristems, resulting in a highly branched inflorescence (Ratcliffe *et al.*, 1998). Over-expression of either of two *Eucalyptus* homologues to *TFL1* in transgenic *Arabidopsis* prevented the initiation of any flowers, giving rise to a much larger and long-viable plant than normal (Collins and Campbell, manuscript in preparation). If the same phenotype is produced after testing in transgenic trees, and does not impair vegetative development, it could provide a means to prevent floral meristems, and thus flowers, from forming entirely.

Genomic catalogues of forest trees are providing many new options for study. For example, by analysis of the Swedish poplar EST database <<http://Poppel.fysbot.umu.se>> complemented by direct cloning, we have identified 20 putative poplar orthologues of flowering time genes described in annual plants. Because poplars possess a number of characteristics that enable their efficient functional analysis, we can move directly to analyse the roles of these genes. First, poplar's amenability to transformation enables study via over-expression and transgene-induced PTGS. Second, poplars are adapted to vegetative spread as part of their life-cycle, and vegetative propagules display many of the same juvenile characteristics as seed-derived plants. Thus, a series of ramets that have been through a successive number of growing seasons provides a continuous 'age' gradient of a single genotype. This facilitates an intensive study of quantitative and cell-specific changes in gene expression in relation to phase transition.

By studying gene expression in diverse poplar tissues collected during the juvenile to mature transition, and throughout



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the seasonal cycle, we have been studying expression changes in relation to the onset of flowering. For example, in preliminary studies we found that vegetative expression levels of the poplar *LFY* orthologue (*PTLF*) differed significantly between juvenile and mature ramets in newly differentiated vegetative buds (Figure 5). Expanding this approach to study of a large number of genes via EST microarray hybridization might identify many additional genes and networks of regulatory interactions that take part in regulating vegetative to floral phase transition.

There has been very limited progress for many years in understanding molecular regulation of the prolonged maturation of trees. Although the identification of markers of maturation continues, and has even accelerated of late thanks to a large European collaborative project ('Development, validation and application of molecular, morphological and physiological markers for juvenile and mature state characterization in woody plant species', <<http://www.neiker.net/phasedb/>>), the identification of genes that can be used to *control* maturation is likely to depend on detailed study of key regulatory genes first identified in *Arabidopsis* or other model annual plants. In poplar, with a number of genomic tools available – most notably facile transformation, genetically controlled developmental gradients, large EST/microarray analysis capabilities, and a complete genome sequence in the near future <<http://www.ornl.gov/ipgc/>> – the field is now poised to make major contributions toward the resolution of this long-standing problem. This, in turn, promises to lead to the development of major new transgenic strategies for domestication.

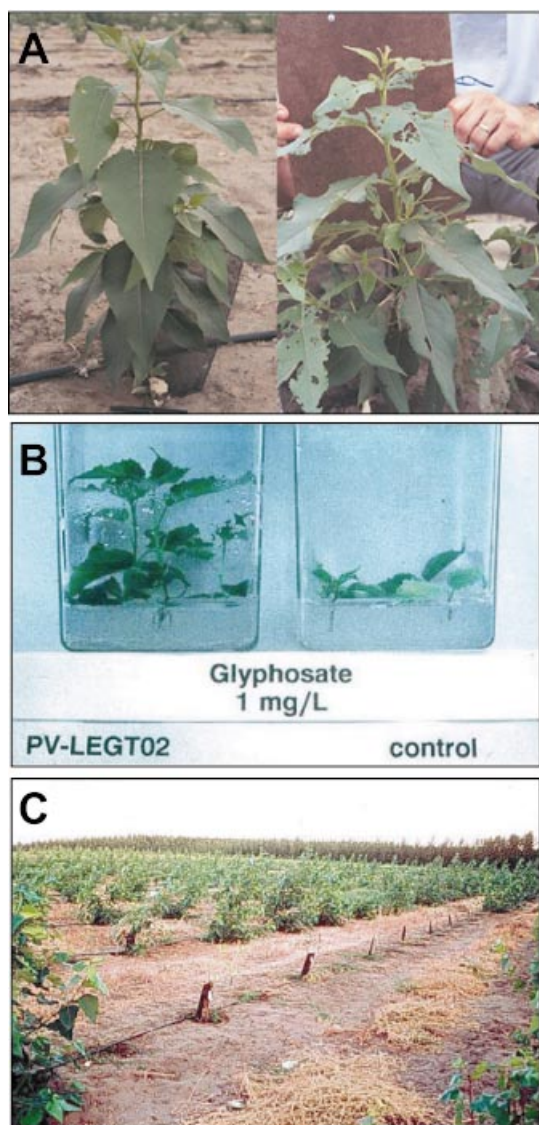
### Herbivore and pathogen resistance

By virtue of selecting and deploying superior individuals and families, domestication inevitably involves a narrowing of genetic diversity. One of the consequences of this is that the domesticated population will not possess all of the alleles that are present in wild progenitors that confer resistance to herbivores and pathogens. Furthermore, as the domesticated population grows larger, and the more uniformly it is deployed on the landscape, there is selection pressure on herbivores and pathogens to overcome any resistance mechanisms that the domesticated population may possess. This is particularly true for forest trees because genotypes remain in the environment for a period of time that usually encompasses many more generations of the herbivore or pathogen. Consequently, a major aim of breeding programmes is to infuse alleles that confer robust and durable herbivore and pathogen resistance. In food crops, this has involved both introgression

of resistance alleles from wild populations into domesticated populations, and, in recent years, the introduction of novel resistance mechanisms through genetic engineering. Forest tree improvement is expected to be no different.

To date, there have been no examples of the introduction of pathogen resistance into forest tree populations using genetic engineering. Nevertheless, an excellent example of what can be accomplished for forest trees has been provided in the case of papaya. Papaya is a horticultural tree valued for its fruit production. Several years ago, the papaya industry in Hawaii was facing economic disaster on account of the susceptibility of domesticated papaya to the viral pathogen, papaya ringspot virus (PRSV). By over-expressing viral coat protein, a well established method for herbaceous annual plants, viral resistance was successfully engineered into papaya (Tennant *et al.*, 1994). These plants have now been used commercially and have demonstrated strong and stable field resistance. To date, very little is known about the effects that viruses have on forest tree productivity, but it is believed that at least a portion of the cases of forest decline may be attributable, in part, to viral activity (Neinhaus, 1985).

The production of insect resistant plants via genetic engineering has generally taken one of two approaches. The first approach makes use the Bt toxin derived from *Bacillus thuringiensis*. This toxin damages the digestive mechanisms of the larvae that feed upon it. The toxin specifically affects insects belonging to the lepidopteran, dipteran and coleopteran orders of insects, which include a number of major herbivores of forest tree species. The Bt toxin gene was first used to transform hybrid poplars by McCown *et al.* (1991) via direct (biolistics) gene transfer. The introduction of the Bt toxin gene resulted in a significant reduction in forest tent caterpillar (*Malacosoma disstria*) survival and growth rates of the gypsy moth larvae (*Lymantria dispar*). Ellis *et al.* (1993) used the same approach to produce insect resistant white spruce (*Picea glauca*). The transgenic spruce displayed resistance to the spruce budworm (*Chorisonera fumiferuna*). European larch with improved resistance to common defoliator larch casebearer (*Coleophora laricella* (Hbn)) was produced by *Agrobacterium*-mediated transformation of the Bt toxin gene (Shin *et al.*, 1994). Wang *et al.* (1996) used *Agrobacterium*-mediated transformation to introduce truncated Bt  $\delta$ -endotoxin DNA fragments into poplar (*Populus nigra*) in order to confer resistance to the gypsy moth and the poplar looper (*Apocheima cineraius* Erscheff). In China these two insects accounted for 40% of damage by insects in 1989. Several resistant clones were selected for large-scale field evaluation. Meilan *et al.* (2000) demonstrated high levels of field resistance to defoliation by the cottonwood leaf beetle



**Figure 6** Transgenic approaches to herbivore resistance or herbicide resistance in forest trees. (A) The transgenic tree on the left shows that expression of *Bt* toxin in cottonwood leads to high levels of field resistance to defoliation by the cottonwood leaf beetle, relative to the control tree shown on the right. (B) Trees of the genus *Populus* can be genetically engineered to be significantly more resistant to the herbicide glyphosate (left) than corresponding controls (right). This resistance can be used in the field (C), where glyphosate is used to suppress competition from weeds, such that transgenic plants survive the application of the herbicide (background) in contrast to control plants which do not (foreground). (Figures from Meilan *et al.* (2000), kindly provided by Dr Rick Meilan.)

in trials with transgenic hybrid cottonwoods in the western USA (Figure 6). Due to high levels of natural insect pressure, the transgenic trees had lower damage and grew considerably faster than non-transgenic trees. The trees contained a synthetic *cry3a* gene that was driven by the cauliflower mosaic virus (CaMV) 35S promoter and flanked by matrix attachment regions (Meilan *et al.*, 2000).

Plants respond to attack from herbivores by activating gene complexes involved in defensive pathways. Characterization of the proteinase inhibitor system of the solanaceous plants (potato and tomato; Green and Ryan, 1972; Ryan, 1981, 1990) led to the identification and cloning of the proteinase inhibitor II (*pin2*) gene (Fox, 1986). The *pin2* gene is activated in response to wounding and its product inhibits the activity of trypsin and chymotrypsin in animal digestive systems. Klopfenstein *et al.* (1991) demonstrated the potential for using the potato *pin2* gene as an engineered defence mechanism in hybrid poplar (*P. alba* × *grandidentata*). Fusions between the *pin2* coding sequence and either the *NOS* promoter or the *CaMV 35S* promoter were engineered into hybrid poplar (Klopfenstein *et al.*, 1997). These transgenic poplar were assayed for resistance to willow leaf beetle (*Plagioderma versicolora*), a major defoliator of *Populus* plantations. Larvae fed on these transgenic poplars weighed less than those fed on control plants. These same larvae also showed a trend towards longer development times when fed leaves from the transgenic poplar. Greater resistance to *Chrysomela tremulae*, a leaf-feeding beetle, was achieved in hybrid poplar (*P. tremula* × *tremuloides*) by over-expressing the gene encoding oryzacystatin, *OCl*, a cysteine proteinase inhibitor (Leplé *et al.*, 1995). A mortality rate of 43.5% was observed when *C. tremulae* was reared on transgenic poplar over-expressing *OCl*, in comparison to the 4.5% mortality observed when the beetles were cultured on control plants. More recently, white poplar plants were engineered to express a novel *Arabidopsis thaliana* cysteine inhibitor (*AtCYS*) gene (Delledonne *et al.*, 2001). Tests showed that the expression of *AtCYS* in transgenic poplar conferred resistance to the chrysomelid beetle, *Chrysomela populi* (Delledonne *et al.*, 2001).

## Herbicide resistance

Herbicide resistant crops have been one of the major products of the first generation of agricultural biotechnology. They are intended to reduce weed control costs, increase control flexibility, facilitate the use of low-tillage (and thus reduced erosion) cropping systems, and enable broad-spectrum, environmentally benign herbicides to be more readily employed.

The first successful transformation of a woody species was reported in *Populus alba* × *grandidentata* using *Agrobacterium tumefaciens* (Fillatti *et al.*, 1987). Transgenic hybrid poplars with a reduced sensitivity to glyphosate, an extensively used broad-spectrum herbicide, were produced. Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is a key enzyme in the biosynthesis

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of aromatic amino acids (Cole, 1985). Reduced sensitivity to glyphosate was obtained by expressing a mutant form of the EPSP synthase (*aroA*). The same approach was employed in the production of glyphosate resistant European larch (*Larix decidua* Mill., Shin *et al.*, 1994). The resistance of hybrid poplars (*P. tremula* × *alba*) to glyphosate was later improved by directing the mutant EPSP synthase to the chloroplast, where it occurs naturally, using a chloroplast transit peptide (Donahue *et al.*, 1994). These transgenic poplars displayed greater resistance to glyphosate and a higher accumulation of chlorophyll. Using the same approach Brasileiro *et al.* (1992) produced a transgenic poplar resistant to the herbicide chlorsulphuron. Agrobacterium-mediated transformation was used to transform plants with a mutant acetolactate synthase (*crs1-1*) gene cloned from a chlorsulphuron resistant line of *Arabidopsis thaliana* (Haughn *et al.*, 1988). The transgenic poplars produced were resistant to high levels of herbicide application and also represent the first transgenic woody species produced using the gene's natural promoter.

De Block (1990) employed a different approach in the production of poplar clones resistant to the herbicide phosphinotricin (PPT). PPT inhibits glutamine synthase in the plant, which results in the accumulation of toxic levels of ammonium. Over-expressing the *bar* gene encoding the enzyme PAT, which inactivates PPT by acetylating it, conferred resistance in poplar. More recently, transgenic poplars have been produced that employ both methods of conferring resistance. Strauss *et al.* (1997) reported on the generation of transgenic poplars harbouring two different resistance genes – one that enabled the degradation of glyphosate, and a mutant EPSP synthase that had a much reduced sensitivity to the herbicide. During 2 years of field trials, selected lines of hybrid aspens and hybrid cottonwoods that contained these genes showed stable, high levels of tolerance, and an absence of growth penalty, to levels of herbicide application that were well above those used commercially (Meilan *et al.*, 2000) (Figure 6).

### The road ahead along forestry's fertile crescent

Recent evidence suggests that as few as five major loci can account for the most significant differences between domesticated maize and its undomesticated wild progenitor, teosinte (Doebley and Stec, 1993). Starting with its progenitor teosinte, maize has enjoyed a period of domestication that is likely to have extended over hundreds, if not thousands, of generations. This is in striking contrast with forest tree domestication, which has proceeded in earnest for only a few generations; lagging many generations behind most food

crop plants. As illustrated above, molecular genetics provides tools that may allow forest tree improvement to make up some of this lost ground. In the forestry community, the modern-day equivalents of the early teosinte breeders are molecular biologists; identifying and using genes to facilitate the domestication of forest trees.

Forestry's 'Fertile Crescent' is about to receive a huge infusion of intellectual capital. The entire poplar genome will be sequenced by the end of 2003, through the efforts of an international consortium, led by the US Department of Energy's Joint Genome Institute <<http://www.ornl.gov/ipgc/>>. If the experience with *Arabidopsis* is anything to go by, the availability of the entire genome sequence of poplar will usher forth a new era in our understanding of the basic biology of a tree species. Undoubtedly this new knowledge will enable powerful new strategies to be devised to hasten the domestication of poplar, an important tree species in its own right, as well as providing a test-bed for strategies aimed at the directed improvement of other tree species.

During the early stages of food crop domestication, breeders were fettered by the biological and environmental constraints imposed by the crop and their locale. While the domestication of forest tree species is still in its infancy, it proceeds with a baseline of scientific knowledge that far exceeds that available to early food crop breeders. This knowledge may allow forest tree breeders to circumvent the problems that were encountered by their early food crop-breeding counterparts. Nevertheless, tree improvement efforts proceed against a backdrop of scepticism by some sectors of society – including some sectors that are prepared to engage in criminal activities to put a stop to improvement efforts (Kaiser, 2001; Service, 2001). Present-day tree breeders working in forestry's 'Fertile Crescent' will have to be prepared to tackle not only the pragmatic issues associated with their efforts, but will also have to be prepared to deal with some contrasting ideological views.

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## Appendix: forest trees targeted for domestication

### Gymnosperms

Because of their broad geographical ranges, tolerance of infertile and droughty soils, and valued long fibres, gymnosperms are amongst the most widely harvested of all tree species. Gymnosperms have long been the stalwarts for providing wood for the production of everything from newsprint, matchsticks and grocery bags to lumber for construction and furniture manufacturing. While gymnosperms produce wood with desirable fibre characteristics, they are slow growing relative to the most highly productive angiosperms, and suffer from problems associated with wood chemistry (e.g. lignin content) and cell wall characteristics (e.g. microfibril angle). Many researchers are engaged in the improvement of gymnosperm species, most notably *Pinus taeda*, *Pinus radiata*, *Pinus pinaster*, *Pseudotsuga menziesii*, *Picea abies*, *Picea sitchensis* and *Picea nigra*. Most improvement efforts are aimed at the common goals of increasing volume growth, improving wood quality and enhancing herbivore resistance.

### Poplars

Trees in the genus *Populus* and their hybrids, known variably as poplars, aspens and cottonwoods, are fast-growing temperate trees. They have been used in plantation forestry in many parts of North America, Asia and Continental Europe. Valued largely for their fibres for the production of fine pulps and paper, poplars are being selected for increased volume growth and improved wood properties. In addition, there is great interest in enhancing disease resistance in poplars, particularly to rust pathogens. Poplars are amenable to both vegetative propagation and genetic engineering. The productivity of poplar plantations has been enhanced by vegetatively

propagating clones with desirable traits. There has been a considerable effort aimed at modifying herbivore resistance, herbicide tolerance, wood quality and reproductive development using genetic engineering (see main text). Poplar is the consensus 'model tree'. In keeping with this status, the poplar genome will be completely sequenced by the Joint Genome Institute by the end of 2003.

### Eucalypts

Trees in the genus *Eucalyptus* are amongst the most widely planted commercial tree species, particularly in tropical and subtropical climates in the Southern hemisphere. Eucalypts are valued for their rapid annual volume growth, and for fibre characteristics that are good for the production of pulp and paper and some kinds of lumber. As is the case for poplar, 'domestication' of eucalypts has involved extensive use of vegetative propagation of individual trees, including hybrids, with superior traits. Unlike poplar, many eucalypt species have been relatively recalcitrant to genetic engineering and have thus been the subject of much fewer transgenic studies.

### Hardwoods

Trees such as oaks (e.g. *Quercus robur* and *Quercus patrea*), walnut (*Juglans regia*) and mahogany (*Swietenia humilis*) are highly valued for their wood quality, which is generally used in the production of fine furniture and veneers. The domestication of these trees is in its infancy relative to those outlined above. Generally these trees are harvested from 'wild' forests and are not grown for wood production in plantations; however, in recent years there several efforts have been initiated to improve these species in a directed fashion.

### Biomass producers

Trees that accumulate large amounts of biomass on an annual basis are of great interest for the conversion of that biomass into energy. Trees such as willow and poplar have been the focus of tree improvement efforts aimed at producing more biomass for energy production. Willow and poplar both have a significant capacity for regrowth (coppice) after being cut back to the roots; therefore, biomass from these trees can be harvested in a manner analogous to forage crops like alfalfa.