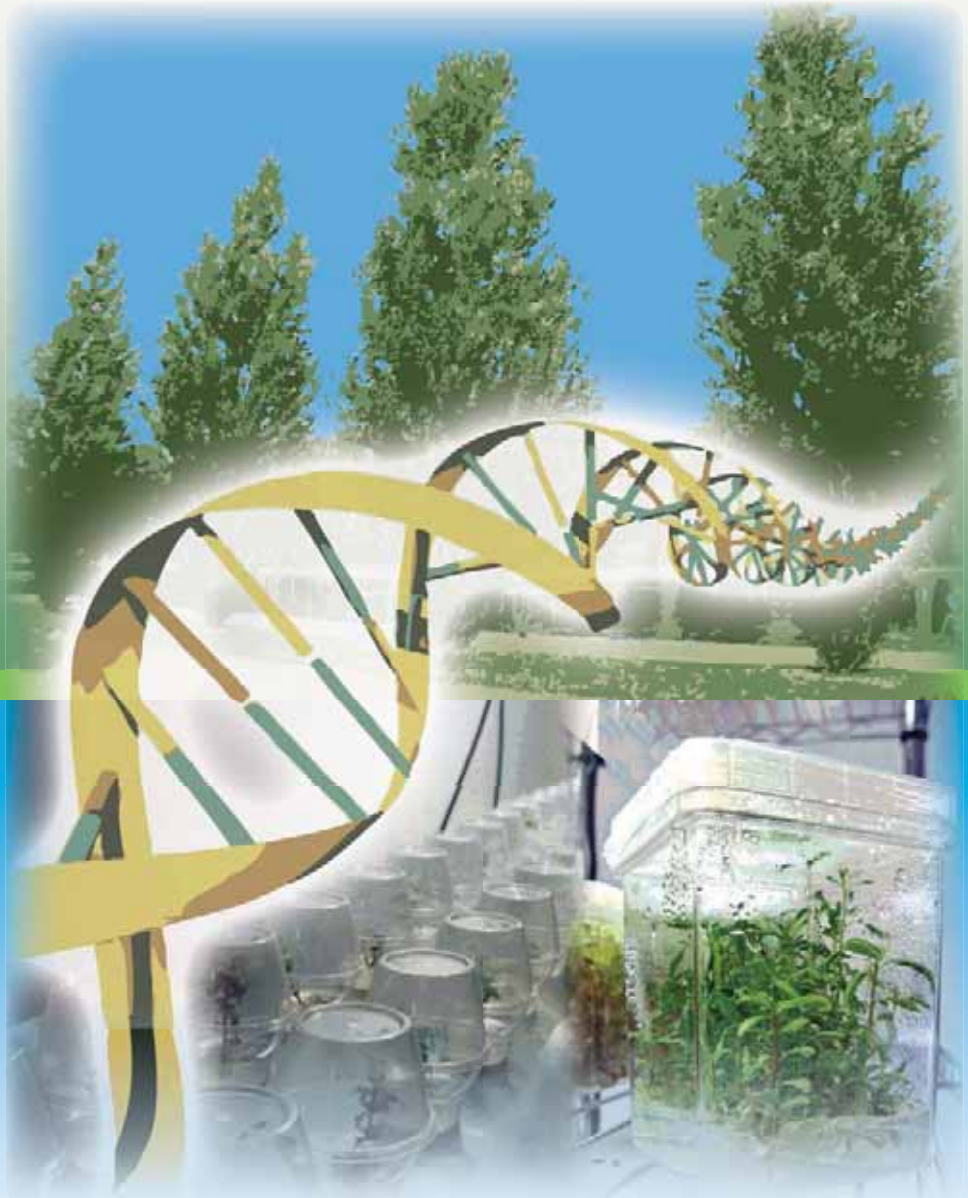
FORESTS
and
GENETICALLY MODIFIED TREES



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FORESTS
and
GENETICALLY MODIFIED TREES

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Contents

Foreword

The world's forests generate substantial economic benefits, and also provide countless ecosystem services and social, cultural and spiritual benefits on which it is more difficult to place an economic value. Aside from being a source of fibre, forests help protect air and water quality, mitigate climate change by storing vast quantities of carbon, and provide a home, temple and playground for many people.

Because of these intangible values and the long life span of trees, it is impossible to treat forests as a commodity within an agricultural model. The gap between forests and agricultural systems has become especially clear in the context of genetic engineering. The successful introduction of genetic engineering in agriculture, albeit for a limited number of traits and species, prompted forest scientists and managers to consider its use as a management and production tool in forestry. This subject has generated heated debates and violent reactions, which have often lacked the support of objective information. Furthermore, the existing scientific information has been contradictory, allowing for questions concerning its credibility. The competitive zeal of some biotechnology owners has added more fuel to the debate.

In the course of this debate, the term 'biotechnology' has often been wrongly used as synonymous with genetic engineering. In light of this confusion, the International Union of Forest Research Organizations (IUFRO) has formed a task force to address genetic engineering in forestry. Its mandate is to report and present factual information covering both the scientific and social dimensions of genetic engineering technology (also known as genetic transformation, gene technology or genetic modification). This publication, developed under the auspices of the IUFRO task force, has been created to present independent information gathered from the world's leading experts on the many facets of this subject. It is not intended to advocate any particular position towards genetic engineering or its application in forestry. Each chapter represents the views of its author(s), and not necessarily those of FAO or IUFRO.

The publication is divided into two parts. The first deals with the science of genetic engineering in forest trees: the position of genetic engineering in the biotechnology spectrum, how it is carried out, traits of interest, gene flow, genetic containment, integration of the technology in tree improvement programmes, and experience of its commercialization in China. The second part covers ethical, environmental, social, regulatory and trade aspects, and examines the technology's potential outside the realm of timber production.

We hope that the material presented here will assist readers in forming their own independent opinions on the place of genetic engineering in forestry.

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Acronyms

ABA	abscisic acid
AFLP	amplified fragment length polymorphism
APHIS	Animal and Plant Health Inspection Service, United States of America
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
Bt	*Bacillus thuringiensis*
CAD	cinnamoyl alcohol dehydrogenase
CAld5H	coniferaldehyde 5-hydroxylase
CCoAOMT	caffeoyl-coenyzme A O-methyltransferase
CCR	cinnamyl CoA reductase
cDNA	complementary DNA
CLB	cottonwood leaf beetle (*Chrysomela scripta*)
COMT	caffeic acid O-methyltransferase
DD-PCR	differential display PCR
DDRT-PCR	differential display reverse transcriptase PCR
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNM	dominant negative mutation
EC	European Communities
ECS	*see:* γ-ECS
EEC	European Economic Community
EIS	environmental impact statement
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvyl-3-phosphoshikimate synthase
EST	expressed sequence tag
EU	European Union
FDA	Food and Drug Administration of the USDA
G	guaiacyl [lignin]
GA	gibberellic acid
GIS	geographical information system
GMO	genetically modified organism
GR	glutathione reductase
GS	glutamine synthetase
GSH	glutathione
GSS	GSH synthetase
GST	glutathione S-transferase
GUS	β-glucuronidase [gene]

HGT	horizontal gene transfer
IAA	indole-3-acetic acid
IPR	intellectual property rights
MAB	marker-assisted breeding
MALDI-TOF	matrix-assisted laser desorption/ionization – time-of-flight mass spectrometer
MAR	matrix attachment region
MAS	marker-assisted selection
MAT	multiautonomous transformation system
mRNA	messenger RNA
NEPA	National Environmental Policy Act [of the United States of America]
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPT	phosphinothricin
qRT-PCR	quantitative reverse transcriptase-PCR
QTL	quantitative trait locus
QTN	quantitative trait nucleotide
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-PCR
S	syringal [lignin]
SNP	single nucleotide polymorphism
SSH	suppression subtractive hybridization
TACCF	The American Chestnut Cooperators Foundation
TACF	The American Chestnut Foundation
TCE	trichloroethylene
T-DNA	Transfer-DNA
USDA	United States Department of Agriculture
USEPA	Unied States Environmental Protection Agency
VIGS	virus-induced gene silencing
XET	xyloglucan transglycosylase
γ-EC	γ-glutamylcysteine
γ-ECS	γ-glutamylcysteine synthetase

Part 1

THE SCIENCE OF GENETIC MODIFICATION IN FOREST TREES

1. Genetic modification as a component of forest biotechnology

C. Walter and M. Menzies

While the term "biotechnology" refers to a broad spectrum of modern tools and the application of those tools, it is frequently equated with genetic engineering by the lay public. FAO noted in their 2004 report *The State of Food and Agriculture* that "biotechnology is more than genetic engineering" (FAO, 2004a). In fact, 81% of all biotechnology activities in forestry over the past ten years were not related to genetic modification (Wheeler, 2004).

There are many definitions of biotechnology and they differ in their scope. FAO (2001) defines the term biotechnology as "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use". This definition, although accurate for the specific purposes for which it was intended, may contribute to the confusion surrounding the term. A simpler definition might be "the application of biological knowledge to practical needs such as technologies for altering reproduction, or technologies for locating, identifying, comparing or otherwise manipulating genes".

In short, forest biotechnology is associated with a broad spectrum of modern methods applicable to agricultural and forest science, only some of which are related to genetic engineering. In forestry, the definition of biotechnology covers all aspects of tree breeding and plant cloning, DNA genotyping and gene manipulation, and gene transfer.

Forest biotechnologies can be classified in many ways (Yanchuk, 2001; Wheeler, 2004), but here they are grouped under five major, though undoubtedly overlapping, categories (Henderson and Walter, 2006; Trontin *et al.*, 2007; El-Kassaby, 2003, 2004):

- propagation;
- molecular markers;
- marker-assisted selection (MAS) and marker-assisted breeding (MAB);
- genomics, metabolomics and proteomics;
- genetic modification or genetic engineering.

This chapter provides a brief discussion of these technologies in the context of existing or proposed deployment in commercial forestry. However, this should be read only as an introduction, and the reader is referred to the vast literature available on those subjects.

PROPAGATION

Plant cloning has been used for centuries for tree breeding and propagation using grafts and cuttings. Chinese fir (*Cunninghamia lanceolata*) has been propagated by cuttings for clonal forestry in China for more than 800 years (Li and Ritchie, 1999) and Japanese cedar (*Cryptomeria japonica*) has been propagated clonally by cuttings in Japan for plantations since the beginning of the fifteenth century (Toda, 1974). Some tree species are easier than others to propagate by cuttings. Easy-to-root hardwood species, such as poplars (*Populus* spp.), willows (*Salix* spp.) and some eucalypt (*Eucalyptus*) species, and conifer species, such as spruces (*Larix* spp.), redwood (*Sequoia sempervirens*), and some pines (*Pinus* spp.), are widely planted as cuttings in family or clonal plantations (Ritchie, 1991; Ahuja and Libby, 1993; Assis, Fett-Neto and Alfenas, 2004; Menzies and Aimers-Halliday, 2004). In the future, the use of vegetatively propagated trees for intensively managed, high-yielding plantations is expected to increase in all regions of the world.

While the main use of propagation technologies has been for forest establishment of genetically-improved families or clones, there is also a conservation use for those species that are at risk, rare, endangered or of special cultural, economic or ecological value (Benson, 2003). Integrating traditional methods such as *in situ* conservation and seed storage with biotechnologies such as micropropagation and cryopreservation can provide successful solutions.

Micropropagation

Micropropagation refers to the *in vitro* vegetative multiplication of selected plant genotypes, using organogenesis and/or somatic embryogenesis. Approximately 34% of all biotechnology activities reported in forestry over the past ten years related to propagation (Chaix and Monteuuis, 2004; Wheeler, 2004). Micropropagation is used to multiply (bulk-up) desirable genotypes or phenotypes to create large numbers of genetically identical individuals of clones or varieties. These techniques are gaining increased attention by foresters and tree breeders because vegetative propagation offers a unique opportunity to bypass the genetic mixing associated with sexual reproduction.

Organogenesis

While macropropagation methods, such as cuttings, involve comparatively large pieces of tissue, micropropagation by organogenesis involves *in vitro* culture of very small plant parts, tissues or cells, particularly meristems from germinating embryos or juvenile plant apices. There are a number of stages in organogenesis, involving sterilization and shoot initiation, shoot elongation and multiplication, rooting and acclimatization. Sterilization is typically done with a diluted bleach solution, followed by initiation of shoots on an appropriate tissue culture medium. Shoots can develop from existing axillary meristems or from meristems of adventitious origin. Adventitious meristems can be stimulated from plant tissue, such as cotyledons or leaves, by exposure to a pulse of the plant hormone, cytokinin. Plants arising from shoots of adventitious origin may show undesirable

advanced maturation characteristics (Frampton and Isik, 1987). There have been many different media developed for organogenesis, depending on the species (McCown and Sellmer, 1987). Following shoot initiation, shoots are elongated on a medium without cytokinin. The addition of 0.5–1.0% activated charcoal may be beneficial. Once shoots have elongated sufficiently, they can be cut into nodal sections or topped to stimulate lateral side shoot or shoot clump development, which can then be separated and elongated. When sufficient multiplication has been achieved, the shoots can be stimulated to form roots by transferring them to a medium containing auxin. Rooting may be done *in vitro* or *ex vitro*, depending on the species. Venting of the culture container by using a hole in the container lid covered with a permeable membrane or cotton wool during the time in auxin medium may help acclimatization for transfer *ex vitro*. Similarly, the container lid may be left loosened or unwrapped to allow some gaseous exchange and exposure to ambient humidity. Once shoots are transferred *ex vitro* and have rooted, the humidity may be gradually reduced to ambient conditions in an acclimatization phase.

There are a number of methods available for maintaining or storing of clones in tissue culture by organogenesis, including repeated subculture (serial propagation), minimal growth media, cool storage and cryopreservation. Radiata pine clones have been maintained as shoots for more than ten years with repeated subculture every 6–8 weeks (Horgan, Skudder and Holden, 1997). However, long-term success at halting ageing is uncertain and the costs are high because of the requirement for regular transfers and a controlled environment. Using diluted nutrient concentrations in the media does reduce the need for regular subculturing, and radiata pine shoots have been maintained successfully for four years at 20–22 °C with annual subculturing (Horgan, Skudder and Holden, 1997). Successful cryopreservation of organogenic material has proved to be more difficult. Cotyledons from radiata pine zygotic embryos have been successfully frozen and thawed (Hargreaves *et al.*, 1999). Cryopreservation of axillary meristems is also being attempted (Hargreaves *et al.*, 1997) and results are now very promising (Hargreaves and Menzies, 2007). Organogenesis methods have been developed for a large number of forestry species for large-scale production, including hardwoods such as poplars, willows and eucalypts, and for conifers such as coast redwoods, radiata pine (*Pinus radiata*), loblolly pine (*Pinus taeda*) and Douglas fir (*Pseudotsuga menziesii*). More detailed protocols for various hardwoods and conifers can be found in Bonga and Durzan (1987a, b) and Bajaj (1986, 1989, 1991).

Embryogenesis

Another micropropagation technology that has been more recently developed and has promising applications for clonal forestry is somatic embryogenesis. Successful embryogenesis was first reported for sweetgum (*Liquidambar styraciflua*) in 1980 (Sommer and Brown, 1980) and for spruce (*Picea abies*) in the mid-1980s (Hakman and von Arnold, 1985; Chalupa 1985). Since then, somatic embryogenesis has been

investigated for many forestry species, including hardwoods such as poplars, willows and eucalypts, and conifers such as spruces, larch (*Larix* spp.), pines and Douglas fir. Embryogenesis differs from organogenesis in that somatic embryos are formed from embryogenically competent somatic cells *in vitro*, with both shoot and root axes, and these embryos will germinate, whereas with organogenesis shoots are developed, and these must be rooted as mini-cuttings.

As in organogenesis, there are a number of stages for embryogenesis, involving initiation of embryogenic tissue, multiplication, development and maturation, germination and acclimatization. Typically, embryogenic tissue is established from immature seeds, just after fertilization, using either embryos within intact megagametophytes or excised embryos. Tissue can be maintained or multiplied in a relatively undifferentiated state. However, by changing the medium, embryos can be stimulated to develop into bullet-stage embryos with suspensors. Further medium changes, including the addition of abscisic acid, increasing the osmotic potential, and controlled desiccation using water-vapour-permeable plastic film, stimulate the embryos to develop and mature into the cotyledonary stage. These embryos can be harvested and, after germination under sterile conditions, transferred to containers in a greenhouse. The somatic seedlings are transferred to larger containers or lined out in a nursery bed when they are large enough. More detailed protocols for various hardwoods and conifers can be found in Bajaj (1989, 1991), Jain, Gupta and Newton (1999, 2000) and Jain and Gupta (2005).

An important advantage of embryogenesis is the ability to maintain or store clones through cryopreservation. Reliable cryogenic storage of embryogenic tissue at –196 °C has been possible for many years (Cyr, 1999; Gupta, Timmis and Holmstrom, 2005). Typically, free water is removed by the use of a higher osmoticum medium, followed by the addition of a cryoprotectant, such as sorbitol and dimethylsulphoxide (DMSO). This avoids the formation of the ice crystals that cause cell disruption and death. Similarly, thawing is done rapidly to avoid ice crystal formation.

The efficiency of embryogenesis needs further improvement, but the technology has the potential to produce unlimited quantities of embryos of desirable genotypes at costs cheaper than current control-pollinated seed prices. These benefits will be achieved once genotype capture is improved, automation technology is designed and artificial seed is developed. Micropropagation, and in particular embryogenesis, is the gateway to genetic engineering (Henderson and Walter, 2006). While *Agrobacterium tumefaciens* transformation is most successful with hardwood species, using organogenic or embryogenic technologies, biolistic transformation can be used most successfully with embryogenic cultures of both softwoods and hardwoods. This means that the development of genetically modified trees is dependant on the availability of a reliable, reproducible propagation system (Campbell *et al.*, 2003).

Choosing the appropriate system

A range of propagation systems are available for clonal deployment and they each have advantages and disadvantages. Micropropagation systems have the advantages of high potential multiplication rates, potentially reliable cooled storage or cryopreservation, and amenability to genetic modification. However, major disadvantages are that the techniques may not work for a considerable proportion of genotypes, plant quality may be poor and costs are high. Nursery cuttings systems have lower multiplication rates and allow short-term clonal storage through stool-bed systems, but can reliably produce good quality plants at lower cost than current micropropagation systems. A hybrid system might be the best option. For example, organogenesis or embryogenesis could be used initially to capture and cryopreserve genotypes and to produce sufficient plants for clonal testing. Once clones had been selected for clonal production, sufficient individuals could be produced by micropropagation to be planted as stock plants for the production of cuttings, producing more robust and cheaper plants for outplanting (Menzies and Aimers-Halliday 1997). Also, if embryogenesis is producing low numbers of germinating somatic seedlings for some clones, the germinating plants can be transferred to an organogenesis multiplication system while still sterile to increase plant numbers before transfer *ex vitro*.

MOLECULAR MARKERS, MAS, QTL DETECTION AND FINGERPRINTING

The introduction of biochemical (e.g. terpenes and flavanoids) and Mendelian-inherited protein (allozymes) markers in the latter quarter of the past century drove a rapid increase in evolutionary biology studies in forestry. These markers also found valuable application in seed orchard management (Wheeler, Adams and Hamrick, 1993; El-Kassaby, 2000). In the past decade, the development of molecular markers based directly on DNA polymorphisms has largely replaced allozymes for most practical and scientific applications. This replacement was accelerated by the development of the polymerase chain reaction (PCR) technique. Molecular markers come in many forms, each with an array of benefits and drawbacks (Ritland and Ritland, 2000). The utility of these molecular markers and the analytical methods used differ according to the type of question asked and the nature of the markers (dominant vs co-dominant).

Molecular markers are routinely used for a number of research and development and practical applications in forestry, the most common of which is the estimation of genetic diversity in natural and artificial populations. According to Chaix and Monteuuis (2005), over 25% of all biotechnology activity reported in the past ten years related to marker application, predominately focused on measures of diversity. Other applications include the study of gene flow and mating systems, tracking clonal and seedling materials in breeding programmes, paternity studies, gene conservation, and construction of genetic linkage maps. Recently, a new approach to tree breeding that relies on molecular markers for full pedigree reconstruction following polycross mating was proposed (Lambeth *et al.*, 2001). This technology allows for making greater gains while reducing breeding and

testing costs. The use of markers for MAS and MAB will be discussed in the next section. In short, the application of molecular marker technology in forestry is extensive and likely to expand in the years ahead.

Marker-assisted selection and marker-assisted breeding

MAS and MAB refer to approaches to tree improvement that rely on the statistical association of molecular markers with desirable genetic variants. With the development of new and easily obtained molecular markers in the 1990s, the prospect for practising MAS/MAB was bright. Fifteen years of research around the globe has both tempered and rejuvenated this prospect.

Initially, MAS was attempted by creating genetic linkage maps using molecular markers in segregating populations (pedigrees or crosses), and placing quantitative trait loci (QTLs) that explained some portion of the variation in a trait of interest (e.g. wood density) on those maps. Markers are identified as being in close genetic linkage with the genes responsible for the trait of interest, and can be used to select for the desired alleles of those genes. In addition to MAS, potential applications for QTL maps include the genetic dissection of complex quantitative traits, and the provision of guidance for selection and prioritization of candidate genes (Wheeler *et al.*, 2005). QTL maps have been created for over two dozen forest tree species (Sewell and Neale, 2000). Though highly informative, QTL maps are difficult and costly to produce, and have utility limited largely to the pedigrees for which they were created. Use of this technology for MAS is modest, but finds strong advocates for selected applications in North America, Europe and New Zealand.

Currently, research on another approach to identifying QTLs using natural populations rather than pedigrees is receiving increasing attention in forestry and agriculture. This technology, called association genetics, proposes finding markers that tag the actual genetic variants that cause a phenotypic response (i.e. markers occurring within the gene of interest) (Neale and Savolainen, 2004). This approach holds great promise for MAS and MAB, and applications within forestry are possible within the next ten years.

Genomics

Genomics is a recent field, with many subdisciplines (Krutovskii and Neale, 2001). Over the past six years, substantial resources have been invested in the genomics sciences of humans, agronomic crops and forest trees. Genomics encompasses a wide range of activities, including gene discovery, gene space and genome sequencing, gene function determination, comparative studies among species, genera and families, physical mapping and the burgeoning field of bio-informatics. The ultimate goal of genomics is to identify every gene and its related function in an organism.

The completion of a whole-genome sequence for *Populus trichocarpa* (Tuskan *et al.*, 2006) has laid the foundation for reaching this goal for a model species. Efforts follow to replicate this deed in *Eucalyptus* sp. and *Pinus* sp., though

progress may be slower due to larger genome sizes, in particular for pines. Gene and expressed sequence tag (EST) (cDNA) libraries for conifers by far exceed one million entries; however, not all entries are readily available to the scientific community due to private ownership. The immediate applications of genomics include identification of candidate genes for association studies and targets for genetic modification studies. Also, comparative studies of genes from different trees have revealed the great similarity among taxa throughout the conifers, and raise hope that what is learned from one species will benefit many others.

Genomic sciences, like the other '-omics', namely metabolomics and proteomics, require substantial investment and are done on a very large scale, primarily by commercial entities with highly-trained laboratory staff, technology protected by intellectual property rights (IPR) and vast bio-informatics and associated statistical capacity. In general, genomics currently represents the most rapidly expanding area of biotechnological research; however, in forestry, most of the activities are concentrating on high throughput gene discovery and function elucidation. Characterization of genetic components of disease or pest resistance is a rapidly expanding field (Ellis *et al.*, 2001; Gartland, Kellison and Fenning, 2002). Other applications are expected to increase to complement traditional tree improvement through association genetics (Neale and Savolainen, 2004).

Proteomics

Proteomics is the large-scale study of the proteins expressed by an organism, particularly protein structure and function. The term 'proteomics' was coined to make an analogy with genomics, the study of the genes. The proteome of an organism is the set of proteins it produces during its life, and the genome of the organism is the set of genes it contains.

Proteomics is often considered the next step in the study of biological systems, after genomics. It is much more complicated than genomics, mostly because while an organism's genome is fairly constant, a proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment. Another major difficulty is the complexity of proteins relative to nucleic acids. For example, in the human body there are about 25 000 identified genes, but an estimated >500 000 proteins are derived from these genes. This increased complexity derives from mechanisms such as alternative splicing, protein modification (glycosylation, phosphorylation) and protein degradation.

Proteomics has attracted much interest because it yields information that is potentially more complex and informative in comparison with that gained from genomic studies. The level of transcription of a gene provides an approximate estimate of its level of expression into a protein. An mRNA produced in abundance may be degraded rapidly, modified or translated inefficiently. This could result in reduced amounts or types of protein being produced. In addition, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules.

Proteomic studies require significant analytical and biocomputing capability, including instrumentation such as electrophoresis, crystallography, infrared and mass spectroscopy, and matrix-assisted laser desorption/ionization – time-of-flight mass spectrometer (MALDI-TOF) equipment.

Proteomics can be of value to forestry in a number of ways. For example, a proteomic study with somatic embryogenesis in *Picea glauca* identified a number of differentially expressed proteins across different stages of embryogenesis (Lippert *et al.*, 2005). The knowledge gained from such experiments may help to better understand and manipulate the process of embryogenesis.

Metabolomics

Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" - specifically, the study of their small-molecule metabolite profiles. The metabolome represents the collection of all metabolites in a biological organism, which are the end products of its gene expression. Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell. One of the challenges of systems biology is to integrate proteomic, transcriptomic and metabolomic information to provide a more complete picture of living organisms. The typical technical approach to metabolomics is through mass spectroscopy.

Metabolomics can be an excellent tool for determining the phenotype caused by a genetic manipulation, such as gene deletion or insertion. Sometimes this can be a sufficient goal in itself, such as to detect any phenotypic changes in a genetically modified tree, and to compare this with the naturally occurring variation in a tree population. It can also be used to understand variation that is induced by various factors such as genetic or environmental factors. For example, a metabolomic study with field-planted Douglas fir found that environmental variation was greater than genetic variation (Robinson *et al.*, 2007).

GENETIC ENGINEERING

Biotechnological advancements in crop improvement through genetic engineering have attracted great attention from both the scientific and lay communities. This is as true for forestry as it is for agriculture. In fact, genetic modification is so embedded in the public conscientiousness that it is often considered synonymous with the term biotechnology. However, genetic engineering represents only one-fifth of the total biotechnology activities published in the past ten years (Walter and Killerby, 2004). Genetic modification is frequently seen as the most controversial use of biotechnology (Dale, 1999; Stewart, Richards and Halfhill, 2000; Thompson-Campbell, 2000; Dale, Clarke and Fontes, 2002; Conner, Glare and Nap, 2003; Burdon and Walter, 2004; Walter, 2004a, b; Walter and Fenning, 2004).

A major apprehension with genetic modification is the possible widespread gene transfer via escapes and hybridization and/or introgression with related native species. This concern is particularly felt in areas where inter-fertile species

are present in the vicinity of a plantation of genetically modified plants and when measures to prevent gene flow are not considered. Various approaches have been considered to ensure containment of genetically modified organisms (GMOs) through sterility (Brunner *et al.*, 2007).

Compared with the advances made in agricultural biotechnology, which can now be seen through looking back at more than ten years of successful commercial application, forest genetic engineering has lagged behind. This is mainly due to much fewer resources, longer rotation times of the crop and significant hurdles to overcome with regard to efficient tissue culture and propagation technologies. The more recent development of efficient plant tissue culture techniques has allowed forestry to emulate what has been achieved for agricultural and horticultural species. While there have been major advances with conventional tree breeding, there are some desirable traits that are not available in the tree species of choice. Possible traits of interest include herbicide and insect resistance, and modified lignin and cellulose content (Hu *et al.*, 1999; Bishop-Hurley *et al.*, 2001; Pilate *et al.*, 2002; Grace *et al.*, 2005). Also, more recently, research has focused on traits that are associated with the wood secondary cell wall and that have the potential to make transformational changes to wood-based products (Wagner *et al.*, 2007; Li *et al.*, 2003; Moeller *et al.*, 2005). Of increasing interest is the current trend towards a bio-based economy that derives resource materials from plant matter rather than petrochemicals.

GENETIC MODIFICATION TECHNOLOGIES

Two main technologies are available to transfer foreign DNA into plant cells, and then regenerate plants from these transformed cells. These technologies are the use of bacterium, typically *Agrobacterium tumefaciens* (Gelvin, 2003), or biolistics (gene gun) (Klein *et al.*, 1987). *A. tumefaciens* is a bacterium that causes crown gall disease in some, particularly dicotyledonous, plants. The bacterium characteristically infects a wound, and incorporates a segment of Transfer-DNA (T-DNA) (syn. Ti [Tumour inducing] DNA) into the host genome. This DNA codes for the production of plant hormones and its expression in the host plant cell leads to undifferentiated growth. The T-DNA resides on a bacterial plasmid that also carries other genes (virulence or *vir* genes), which are responsible for the transfer of the T-DNA into the plant cells The *A. tumefaciens* T-DNA can be replaced by any gene(s) of interest, which will then be transferred to plant cells during *A. tumefaciens* infection. Poplar was the first hardwood species to be transformed using this technology, with a herbicide resistance gene in 1987 (Fillatti *et al.*, 1987). Conifer species are difficult to transform using *A. tumefaciens*, although successful transformations of larch (*Larix decidua*) (Huang, Diner and Karnosky, 1991), pine (*Pinus radiata*) (Grant, Cooper and Dalr, 2004; Charity *et al.*, 2005) and spruce (*Picea* spp.) (Klimaszewska *et al.*, 2001; Le *et al.*, 2001) species has been reported (Henderson and Walter, 2006).

Biolistic techniques have now been developed to stably transform species that are difficult to transform using *A. tumefaciens* (Walter *et al.*, 1998, 1999; Find

et al., 2005; Henderson and Walter, 2006; Trontin *et al.*, 2007). For this technology, the DNA is coated onto small metal particles (tungsten or gold) and these are propelled by various methods fast enough to puncture target cells. Typically, a pulse of pressurized helium is used to inject the particles into the target cells. Provided that the cell is not irretrievably damaged, the DNA can be taken up by the cell and integrated into its genome. Any transformed cells need to be actively selected from non-transformed cells, so that chimeric cell lines are avoided. This can be achieved by including a selectable marker gene in the transferred DNA, such as for antibiotic resistance. Following the transformation event, the cells are cultured on a medium containing the antibiotic. Over time, only stably-transformed cells will survive this exposure to an antibiotic, and so transformed cell lines can be established and tested for the presence of the new DNA. The efficiency of transclone production using biolistic techniques is usually slightly higher than when *A. tumefaciens* is used as a vector for gene transfer. However, recent modifications to the biolistic process (Walter, unpublished) have increased the efficiency significantly, so that more than 200 transclones can be produced by one operator in a single day. Transgenic plants can be regenerated from these cell lines and evaluated in greenhouse and field tests.

The successful expression of genes that are of commercial interest has already been demonstrated in laboratory and field experiments. These include the modification of lignin and cellulose biosynthesis (Hu *et al.*, 1999; Pilate *et al.*, 2002), herbicide resistance (Bishop-Hurley *et al.*, 2001), and insect resistance (Grace *et al.*, 2005). Field tests of transgenic pine plants produced through biolistic techniques have also demonstrated the long-term stability of the introduced gene, up eight years of age (Walter, in preparation).

Genetic modification technology is still new to forestry. However, relatively numerous (124) introduced traits of transgenic trees have been under regulatory examination in the United States of America (McLean and Charest, 2000), and a commercial plantation of genetically-modified poplar trees has been reported in China (Su *et al.*, 2003). A new wave of transgenic trees with improved secondary cell wall characteristics (improved pulpability, increased cellulose content, better stability) will soon be available for field testing and subsequent commercial deployment in plantation forestry. In many cases, particularly where interfertile species are present, reproductive sterility will be required to prevent introgression of transgenes into native populations (Brunner *et al.*, 2007; Höfig *et al.*, 2006).

Forestry genetic modification activities are taking place in at least 35 countries, 16 of which host some form of experimental field trials (Wheeler, 2004). These field trials are generally small (12 to 2 850 plants in reported studies) and typically of short duration. In many countries, such trials must be destroyed before seed production occurs. In other countries, experimentation is restricted to laboratories or greenhouses. To date, only China (Wang, 2004) has reported the establishment of approved, commercial plantations of genetically modified trees. While the majority of activities on genetic modification are experimental and regulated

under very strict conditions, concerns about genetically modified trees are similar to those about agricultural crops.

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2. Biotechnology techniques

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Biotechnology can be divided into two broad areas: conventional breeding and molecular genetics. The former has been used for centuries to improve plant and animal species to satisfy human needs. Advances in molecular genetics have been rapidly adopted by the scientific community over the last two decades, and they complement tools already available to conventional breeders.

Molecular genetics can itself be subdivided into two distinct categories. In the first, which could be called 'non-controversial technologies', the plant genome is not altered. This category comprises molecular markers, which are used for DNA fingerprinting and MAS (e.g. QTL mapping and association genetics); sequence analysis (genomic DNA, cDNA libraries [ESTs], and bacterial artificial chromosome [BAC] clones), which aid in gene discovery; and *in vitro* propagation (e.g. somatic embryogenesis). The benefits of research using these technologies are increased genetic gain per generation through improved selection in conventional breeding programmes, faster deployment of genetically improved material to plantations, and a deeper understanding of the genes controlling commercially important traits.

The second major subdivision of molecular genetics, termed 'controversial technologies', includes recombinant DNA and gene-transfer techniques. These are the basis for genetic engineering, which is defined as the stable, usually heritable, modification of an organism's genetic makeup via asexual gene transfer, regardless of the origin and nature of the introduced gene. The product of this process is generally referred to as a genetically modified organism (GMO). Genetic engineering offers the opportunity to add new genes to existing, elite genotypes. Although much progress has been made, genetically engineered forest species are not likely to be deployed commercially in much of the world for several more years. One reason for this delay is our limited understanding of the key genes that contribute to the control of commercially important traits, such as wood properties, flowering control and pest resistance. Research in these areas will broaden our knowledge of the genetic and physiological mechanisms that govern tree growth and development. In addition, it will allow the assessment of risks associated with these controversial technologies–assessments that will be required if we are to produce genetically improved material for meeting the growing societal demands for high-quality wood and fibre (Farnum, Lucier and Meilan, 2007).

To make more rapid progress with tree biotechnology, certain innovations are needed, including improved regeneration protocols, alternative *in vitro* selection strategies, dependable excision mechanisms and reliable confinement strategies. One limitation is in our understanding of the roles played by genes controlling key aspects of tree development. Poplar is widely accepted as the model tree for

forest biology owing to its small genome, expanding molecular resources, fast growth, and the relative ease with which it can be clonally propagated *ex vitro* and transformed and regenerated *in vitro* (Bradshaw *et al.*, 2000; Wullschleger, Jansson and Taylor, 2002). The recently released *Populus trichocarpa* genome sequence (Tuskan *et al.*, 2006) and newly developed genomics approaches have already and will continue to expedite gene discovery. The knowledge gained through our work with poplar can then be applied to other tree species.

TECHNIQUES

Recombinant DNA

The application of a variety of techniques collectively referred to as 'recombinant DNA technology' permits the study of gene structure and function, gene transfer to various species, and the efficient expression of their products. Using microbiological methods, it is possible to combine genetic material from various organisms in novel ways. Through these techniques it has been possible to expand our knowledge concerning the way in which genes are regulated, eukaryotes synthesize proteins, and eukaryotic genomes are organized. With regard to genetic engineering, recombinant DNA techniques are essential for:

- identifying genes responsible for specific traits;
- isolating these genes;
- creating genetic constructs harbouring both these genes and flanking regulatory sequences needed for expression in the host organism (in our case, a tree);
- selecting transgenic cells (generally by using an antibiotic or herbicide resistance gene).

Once genetically modified plants have been produced, this technology also allows us to select the best individuals with preferred levels of integration and expression and to monitor, at the molecular level, whether transgene integration and expression are maintained from one growing season to the next, after sexual reproduction, and in various environments.

Transformation

The main steps required for the production of GMOs are:

- stably introducing a novel piece of DNA into the genome of a cell (i.e. transformation);
- isolating transgenic plant cells on a medium containing a selection agent (e.g. the antibiotic or herbicide against which the selectable marker gene imparts resistance);
- regenerating whole plants from the transformed cells through *in vitro* culture;
- screening various transgenic lines that result from independent transformation events on the basis of insert copy number and configuration, and expression.

To date, much of the research on genetic engineering of trees has concentrated on optimizing transformation. Three gene-transfer techniques are commonly

utilized here: protoplast transformation, biolistics and *Agrobacterium*-mediated transformation. Historically, angiosperms were transformed primarily through the use of *Agrobacterium tumefaciens*. Because of early difficulties encountered when transforming conifers with common *Agrobacterium* strains, gymnosperms were initially transformed using particle bombardment (Pena and Seguin, 2001). These problems have now largely been resolved, and several different species are being efficiently transformed via standard *Agrobacterium* strains (e.g. Pilate *et al.*, 1999; Tang, Newton and Weidner, 2007; Tereso *et al.*, 2006). However, except for larch (*Larix kaempferi* × *L. decidua*) (Levee *et al.*, 1997), much work remains to be done on the other steps leading to the production of genetically modified trees, particularly with regard to the regeneration of whole plants from transgenic cells. Plants are regenerated through one of two methods: direct organogenesis or somatic embryogenesis. The latter leads to the production of embryos from somatic tissues, whereas the former involves the generation of organs, such as shoots and roots, from various mature tissues or undifferentiated cell masses derived therefrom. No matter which approach is used, *in vitro* regeneration is often a genotype-dependent process.

Protoplast transformation

Protoplasts are derived by enzymatically digesting the walls of plant cells that are usually isolated from the leaf mesophyll, and are often grown in a liquid suspension culture. Frequently, protoplasts can be transformed either by direct DNA uptake, following polyethylene glycol pre-treatment, or by electroporation. Although many studies have resulted in successful transient expression of a transgene in cell-derived protoplasts (Bekkaoui, Tautorus and Dunstan, 1995), very few have described the regeneration of transgenic trees (e.g. Chupeau, Pautot and Chupeau, 1994). This is probably due to difficulties in regenerating whole plants from protoplasts.

Biolistics

Particle bombardment relies on the delivery of DNA-coated tungsten or gold microprojectiles, which are accelerated variously by ignited gunpowder, compressed gases (helium, nitrogen or carbon dioxide) or electrical discharge (Hansen and Wright, 1999). Although this technique was used to produce some of the first transgenic plants from recalcitrant coniferous or monocotyledonous species (Klein *et al.*, 1988; Ellis *et al.*, 1993), such transformation efficiency remains generally low, and usually results in a high number of transgene inserts in the genome. For these reasons, direct DNA transfer techniques have been avoided in favour of *Agrobacterium*-mediated protocols.

Agrobacterium-mediated transformation.

Agrobacterium tumefaciens is a soil-borne bacterium responsible for crown gall, a disease of dicotyledonous plants that causes chaotic cell proliferation at the infection site, ultimately leading to the development of a plant tumour. During

the complex infection process, bacterial DNA is stably incorporated into the plant genome. Today *A. tumefaciens* co-cultivation is the most widely used and preferred method for transforming many types of plants (reviewed by Gelvin, 2003).

A. tumefaciens harbours a large, tumour-inducing (Ti) plasmid, which encodes several products needed to transfer a piece of its DNA into the host-plant genome. This transferred sequence, called T-DNA, contains a region delimited by two borders, and carries genes that are responsible for tumour development and for the synthesis of opines (molecules that serve as a carbon and nitrogen source for the bacterium, and which result from an association between amino acids and sugars. The virulence genes (*Vir*), located outside the T-DNA region on the Ti plasmid, facilitate T-DNA transfer.

This naturally occurring mechanism for DNA transfer has been exploited by plant biotechnologists, who have demonstrated that the bacterium recognizes the DNA to be transferred to the plant cell genome by its unique borders. An *A. tumefaciens* strain is said to be disarmed when the genes within those T-DNA borders are removed. Another plasmid, a binary vector that contains the genes of interest between the border sequences, is then transformed into the disarmed strain of *A. tumefaciens*. The *Vir* genes located on the disarmed vector are able to act in trans.

The transfer of T-DNA into the host-plant genome takes place following the co-cultivation of explants (generally leaf disks, petioles, stem internodes or root segments) with the bacterium. The explants are then extensively washed to remove excess bacterium before being maintained on media containing bacteriostatins (e.g. cefotaxime or timentin) and the appropriate selection agent. Transgenic cells are multiplied then transferred to a series of media that have been optimized to contain the proper amounts of nutrients and plant growth regulators so that the various phases of plant regeneration are induced through either somatic embryogenesis or organogenesis.

The first genetically modified tree, a poplar, was produced 20 years ago (Fillatti *et al.*, 1987). Today, the number of forest tree species for which transformation and regeneration techniques have been optimized remains low; they include aspen, cottonwood, eucalyptus and walnut. Recently, transformation and regeneration protocols have been developed for several gymnosperms, mostly species within the genera *Pinus*, *Larix* and *Picea*. Within each of these species, only a few genotypes have been amenable to the recovery of transgenic plants. In general, for a wide range of genotypes, effective plant regeneration has been more difficult to achieve through organogenesis than through somatic embryogenesis.

Transgene type and its control

A gene comprises a coding sequence that is preceded by a promoter, which controls where, when and to what extent it will be expressed in a plant. This coding sequence might originate from a different species and therefore may not be present in the host plant. For example, Bt genes, which confer resistance to insects, are derived from a bacterium, *Bacillus thuringiensis*. Alternatively, the

transgene may already exist in the host plant (i.e. an endogene). For example, ferulate-5-hydroxylase (F5H) is an enzyme specific for the synthesis of syringyl lignins; homologues of this gene are found in angiosperm trees. In general, foreign genes are relatively easy to express in the host plant. Depending on the configuration of the genetic construct (e.g. the orientation of the coding sequence or the occurrence of an inverted repeat), expression of the introduced gene may be ectopic (e.g. expressed in a tissue or at a stage not ordinarily seen in the wild-type plant), elevated or down-regulated (e.g. RNA interference (RNAi)). Moreover, a promoter could be fused to a reporter gene, such as β-glucuronidase (GUS) (Jefferson, Burgess and Hirsch, 1986) or to the green fluorescence protein (GFP) gene from jellyfish (*Aequoria victoria*) (Haseloff *et al.*, 1997), which can be used to reveal the pattern of expression conferred by a given promoter.

IDENTIFYING CANDIDATE GENES

Mutation analysis

Several experimental approaches have been taken to isolate genes that either confer a commercially useful trait or control a key aspect of plant development. The first, mutation analysis, involves screening thousands and possibly millions of seedlings for rare mutations that might aid in identifying desirable genes. This is a random, hit-or-miss approach that is slow, labour-intensive and sporadic when applied to tree species. In addition, because trees have long generation times, mate by cross-pollination and are highly heterozygous, rare recessive mutations are difficult to detect. A directed programme of inbreeding could be employed to expose recessive mutations, but inbreeding can also result in trees with poor form and low vigour owing to their high genetic loads, confounding attempts to identify valuable alleles. Tree improvement through these conventional means could require many decades, even with rapid advances in the area of plant genetics and the ease with which biotechnological tools can be applied to certain tree species (e.g. poplar; Bradshaw and Strauss, 2001).

In silico cloning

A second method for identifying candidate genes involves utilizing information from other model plants, such as the herbaceous annual *Arabidopsis thaliana*, to identify tree orthologs. An example of this approach is the identification of the *NAC1* gene, a root-specific member of a family of transcriptional regulators in plants. A mutation in *NAC1* diminishes lateral root formation and perturbs expression of *AIR3* (Xie *et al.*, 2000), a downstream gene associated with the emergence of lateral roots (Neuteboom *et al.*, 1999a, b). Furthermore, transgenic complementation with a functional *NAC1* gene restores lateral root formation, and overexpression results in a proliferation of lateral roots. Thus, the *NAC1* gene product appears to be both necessary and sufficient for lateral root formation. In this case, both sequence and functional information are being tested for functionality via transgenesis (B. Goldfarb, personal communication, North Carolina State University).

Forward genomics

A third way to facilitate gene discovery relies on the use of direct, random mutagenesis. Gene and enhancer trapping are methods for insertion-based gene discovery that both reference genome sequence data and result in a dominant phenotype (Springer, 2000). In short, gene-trap vectors carry a reporter gene lacking a functional promoter, while enhancer-trap constructs contain a minimal promoter preceding a reporter gene. In each case, the reporter gene is expressed in a fashion that imitates the normal expression pattern of the native gene at the insertion site, as has been demonstrated for *Arabidopsis* gene- and enhancer-trap lines (e.g. Springer *et al.*, 1995; Gu *et al.*, 1998; Pruitt *et al.*, 2000). The genomic region flanking the insertion site is amplified using PCR and sequenced; alignment of the flanking sequence with the genome sequence allows immediate mapping of insertions (Sundaresan *et al.*, 1995). This technique has recently been applied to identify genes likely to be involved in vascularization (Groover *et al.*, 2004). A similar strategy, using a luciferase-based promoter-trap vector, has allowed the identification of tissue- or cell-specific promoters (Johansson *et al.*, 2003).

Another forward genomics approach, namely activation tagging, utilizes a strong enhancer element that is randomly inserted into the genome and can be effective some distance from a promoter (Weigel *et al.*, 2000). Elevated expression of the nearby native gene may result in an aberrant phenotype. Lines exhibiting an obvious difference (early flowering, modifications in crown form, adventitious root development, etc.) are then analysed for the causative gene. Overexpression of some native genes (e.g. those affecting wood quality) may not give rise to a visually apparent change. In such cases, high throughput analyses are needed for screening a population of transgenics. The feasibility of this approach has already been demonstrated in poplar (Busov *et al.*, 2003). The recent release of the annotated draft of the *Populus trichocarpa* genome (www.phytozome.net/poplar.php) is facilitating the isolation and characterization of loci underpinning mutations found in similar ways.

Microarrays

A fourth approach to identifying candidate genes utilizes differential gene expression. The development of microarray technology has provided biologists with a powerful tool for studying the effects of gene expression on development and environmental responses (Brown and Botstein, 1999; Rishi, Nelson and Goyal, 2002). Expression levels of entire suites of genes, of both known and unknown function, can be measured simultaneously rather than one or a few genes at a time. This approach has already been successful in many systems. For root formation, a screen of loblolly pine shoots given a rooting treatment (auxin pulse) yielded a putative membrane transport protein that was induced by auxin treatment in juvenile (rooting) but not in mature (non-rooting) stem bases (Busov *et al.*, 2004). This gene shows homology to a large multigene family in *Arabidopsis*, members of which are similar to what was first classified as a nodulin from alfalfa.

PCR-based techniques

The fifth molecular technique to identify candidate genes is based on PCR, and includes suppression subtractive hybridization (SSH), differential display PCR (DD-PCR), and cDNA-AFLP (amplified fragment length polymorphism).

SSH is a PCR-based technique that was developed for the generation of subtracted cDNA libraries, and combines normalization and subtraction in a single procedure. Diatchenko *et al.* (1996) demonstrated that SSH could result in the enrichment of rare sequences by over 1000-fold in one round of subtractive hybridization. This technique has been a powerful tool for many molecular genetic and positional cloning studies to identify developmental, tissue-specific and differentially expressed genes (Matsumoto, 2006). For example, using SSH, bract-specific genes have been successfully identified in the ornamental tree *Davidia involucrata* (Li *et al.*, 2002), and genes responsive to benzothiadiazole (BTH; used to induce systemic acquired resistance) in the tropical fruit tree papaya (Qiu *et al.*, 2004). Genes involved in flowering have also been isolated from carnation (*Dianthus caryophyllus*; Ok *et al.*, 2003) and black wattle (*Acacia mangium*; Wang, Cao and Hong, 2005) using this method.

DD-PCR is another widely used method for detecting altered gene expression between samples, often derived from the treated and untreated individuals from the same genotype or species. An amplification is done using a primer that hybridizes to the poly(A) tail and an arbitrary 5' primer. The first application of this technology was reported by Liang and Pardee (1992), and has since been used with a wide variety of organisms, including bacteria, plants, yeast, flies and higher animals, to expedite gene discovery. A Myb transcription factor HbMyb1 associated with a physiological syndrome known as tapping panel dryness has been identified and characterized from rubber trees using differential display reverse transcriptase PCR (DDRT-PCR) (Chen *et al.*, 2002). Transcriptional profiling of gene expression from leaves of apricot (*Prunus armeniaca*) was conducted by DDRT-PCR and up- or down-regulated genes in response to European stone fruit yellows phytoplasma infection were identified (Carginale *et al.*, 2004). A significant disadvantage of this technique is its high percentage of false-positives (Zegzouti *et al.*, 1997).

cDNA-AFLP was first used by Bachem *et al.* (1996) to analyse differential gene expression during potato tuber development and was subsequently modified by Breyne *et al.* (2003). It too is a PCR-based method, which starts with cDNA synthesis, using random hexamer primers and total or mRNA as a template. Following digestion with two different restriction enzymes, adapters are ligated before amplification via PCR. This method has proven to be an efficient tool for differential quantitative transcript profiling and a useful alternative to microarrays (Breyne *et al.*, 2003). cDNA-AFLP was used to identify transcripts that accumulated in mature embryos and in *in vitro*-cultured plantlets subjected to desiccation or abscisic acid (ABA) treatment in almond (*Prunus amygdalus*; Campalans, Pages and Messeguer, 2001). Using this approach a novel gene, designated *Mal-DDNA*, was cloned and confirmed to play an important role in lowering the acidity of apple fruit (Yao *et al.*, 2007).

RNA interface

Double-stranded RNA-mediated gene suppression, also known as RNA interference (RNAi), was first reported in *Caenorhabditis elegans* a decade ago (Fire *et al.*, 1998). It is currently the most widely used method to down-regulate gene expression. It can be used to knock out all copies of a given gene, thus providing insight into its functionality. However, it does not always result in complete inhibition of a gene's expression. Recent advances in targeted gene mutagenesis and replacement using the yeast *RAD54* gene (Shaked, Melamed-Bessudo and Levy, 2005) or zinc-finger nucleases (Lloyd *et al.*, 2005; Wright *et al.*, 2005) may eventually lead to efficient methods for engineering null alleles in trees.

IMPROVEMENTS NEEDED

Regeneration

Regeneration protocols are typically optimized for a single genotype by conducting complex, labour-intensive, complete-factorial experiments. A more universal protocol has not been developed because of a lack of fundamental understanding of how plant cells acquire the competence to regenerate *in vitro*. Using rapidly advancing genomics tools, it is now possible to unravel this mystery. The research community now has access to a chip on which sequence information for all poplar genes has been spotted. Using this microarray, it is possible to identify genes that interfere with or promote regeneration by evaluating expression levels for all genes in tissues that differ in their regeneration potential, before and after being induced to regenerate. In addition, gene expression profiling that is done on tissues gathered during the juvenility-to-maturity transition could help identify genes affecting regeneration, in a similar manner to the approach described by Brunner and Nilsson (2004) to identify genes involved in flowering control.

Selection systems

As described above, a selectable marker gene is linked to the gene of interest that is being inserted. Transformed cells can then be isolated on a medium containing the appropriate selection agent. While this method is convenient, it is often problematic. First, performing subsequent rounds of transformation may not be possible because only a limited number of selectable marker genes are available. Second, various selection agents can have dramatic and negative effects on regeneration. Finally, the presence of a selectable marker gene is usually an impediment to gaining public acceptance of genetically engineered plants.

Recently, alternative selection systems have been developed. These are based on a growth medium that lacks a substance needed for metabolic activity or proper development. A particularly attractive option exploits the inability of a cell to regenerate a whole plant without the addition of a phytohormone, or its derivative, to the culture medium at a precise step in the regeneration process. For example, most regeneration protocols rely on an exogenous supply of cytokinin to induce differentiation of adventitious shoots or embryos from transgenic calli.

The *GUS* gene, a common reporter, encodes an enzyme that cleaves glucuronide residues. The glucuronide derivative of benzyladenine is biologically inactive; if it is the sole cytokinin incorporated in the induction medium, regeneration will not occur. However, upon hydrolysis by β-glucuronidase, a biologically active cytokinin is liberated to induce regeneration (Okkels, Ward and Joersbo, 1997). This supplement must necessarily be transitory because cytokinin can inhibit subsequent steps in development.

Another positive selection strategy involves inserting a gene whose product imparts a metabolic advantage to the transformed cell. Mannose is a sugar that plants are unable to metabolize; cells starve when grown on a medium containing mannose as the sole carbon source. When taken up by the cells, this sugar is phosphorylated by a native hexokinase. However, plants lack a native phosphomannose isomerase gene, which encodes an enzyme that catalyses the conversion of mannose to a usable six-carbon sugar (Joersbo *et al.*, 1998). Similarly, xylose isomerase, another enzyme that plants lack, is able to convert xylose to a sugar that can be utilized (Haldrup, Petersen and Okkels, 1998). Regeneration protocols that exploit positive-selection strategies such as these can be up to ten fold more efficient than those that rely on more traditional, negative-selection strategies.

Excision systems

The ability to delete unwanted pieces of DNA reliably is a valuable tool for both basic and applied research. Excision systems can remove selectable marker genes, thereby alleviating public concern and allowing for easy re-transformation using vectors derived from a common backbone. Moreover, some alternative regeneration methods (e.g. MAT, discussed below) depend on excision for their success. Because transposons have proven too unreliable, alternative systems, such as *Cre/lox* (Russell, Hoopes and Odell, 1992), FLP/FRT (Lyznik, Rao and Hodges, 1996) and *R/RS* (Onouchi *et al.*, 1995), have been utilized. Excision vectors typically include a recombinase gene, usually under the control of an inducible promoter, and recognition sites that flank the DNA being targeted for removal. However, these systems have not proven to be reliable in certain plants. Thus, it is necessary to determine which is the most appropriate for use with various tree species. For each system, one must ascertain the efficacy of the recombinase and how cleanly it excises the target sequence. Moreover, it is imperative to have an inducible promoter that functions reliably in the plant being transformed.

Producing marker-free plants

The recently developed multiautonomous transformation system (MAT) allows for the production of transgenic plants lacking selectable marker genes from a variety of species (e.g. tobacco, aspen, rice, snapdragon) (Ebinuma *et al.*, 1997; Ebinuma and Komamine, 2001). These vectors harbour *Agrobacterium* genes (*ipt* or *rol*) that control sensitivity to or the biosynthesis of phytohormones. Cells transformed with these vectors regenerate into plants with either a 'shooty'

or 'hairy-root' phenotype. MAT vectors also contain a site-specific, inducible recombinase for excision of both the recombinase and the oncogenes. This alternative production system is attractive because it has the potential to increase both the yield and speed with which transgenic plants can be produced, and may eliminate the need for specific selection and regeneration conditions, making it possible to transform a wider array of genotypes. Such a system will also be useful for stacking genes in forest trees, as described by Halpin and Boerjan (2003).

Mitigating transgene spread

The Coordinated Framework of the United States Animal and Plant Health Inspection Service (APHIS) now gives consideration to transgenic woody perennials. It is likely that before such trees can be deployed commercially, a method to mitigate the risk of transgene spread in the environment will be required, particularly in the cases when the introduced gene will improve the fitness of the genetically engineered tree. Many researchers are investigating ways to modify floral development to satisfy this need. The two most common approaches are to engineer trees that are either reproductively sterile or have delayed flowering. The latter may be particularly useful for short-rotation intensive culture (SRIC), where trees are harvested before the onset of maturation. Nevertheless, the main techniques being employed to modify floral development are:

- cell ablation (floral-specific expression of a cytotoxin gene);
- RNAi (silencing native genes via short, interfering RNAs);
- dominant negative mutations (DNMs), which lead to the production of a dysfunctional version of a gene product, such as a transcription factor (reviewed by Meilan *et al.*, 2001).

Because of functional redundancy, suppression of more than one floral regulatory gene is likely to be needed to achieve complete sterility. Where redundancy is obvious, RNAi constructs can be designed to silence effectively several members of a multigene family (Waterhouse and Helliwell, 2003). It is also advisable to utilize multiple techniques (e.g. cell ablation, RNAi or DNM, alone or in combination) to alter the expression of genes in more than one family to increase the likelihood of developing a durable confinement strategy. Transgene expression has been found to be unstable under various conditions (Brandle *et al.*, 1995; Köhne *et al.*, 1998; Metz, Jacobsen and Stiekema, 1997; Neumann *et al.*, 1997; Scorza *et al.*, 2001). Matrix attachment regions (MARs) have been used to enhance and stabilize transgene expression (Han, Ma and Strauss, 1997; Allen, Spiker and Thompson, 2000); however, there is some question about their utility (Li *et al.*, 2008). Given the potential for instability, it will be imperative to conduct multiyear field studies, in a variety of environments, and extending past the onset of maturity, in order to ensure the reliability of a given confinement system.

Progress in this area has been hampered by the inherent, delayed maturation of trees. Even the five- to seven-year juvenile period for poplar is a serious impediment. There is a report of a *Populus alba* genotype (6K10) that can be

induced to flower precociously, but it is of limited practical use (Meilan *et al.*, 2004). Its induction regime is lengthy and complex, and specialized equipment is required. In addition, not every plant in a population responds to induction. Moreover, the efficiency with which the genotype can be transformed and regenerated is very low. Because both male and female sterility will be needed, poplar is dioecious and 6K10 is a female, confinement systems will need to be tested in another poplar genotype. Early-flowering genotypes are rare and many trees do not respond well to treatments that induce precocious flowering (Meilan, 1997). Thus, there is a need for alternative genotypes that can be reliably and efficiently induced to flower.

BIO-INFORMATICS TECHNOLOGY

Bio-informatics is an interdisciplinary approach that utilizes computational and statistical techniques to aid in solving biological problems at the molecular level. Initially, bio-informatic tools were merely used to store, retrieve and analyse nucleic acid and protein sequence information. The field is now evolving rapidly, and being employed in newly emerging disciplines such as comparative genomics, transcriptomics, functional genomics and structural genomics. Below we briefly discuss some of the basic bio-informatics applications that are commonly used today.

Sequence analysis

One of the fundamental goals of sequence analysis is to determine the similarity of unknown or 'query' sequences to those previously identified and stored in various databases. A commonly used algorithm known as BLAST (basic local alignment search tool) provides a way to rapidly search nucleotide and protein databases. Since BLAST performs both local and global alignments, regions of similarity embedded in other, seemingly unrelated, proteins can be detected. Sequence similarity can provide important clues concerning the function of uncharacterized genes and the proteins they encode.

Other sequence-analysis tools are available to aid in determining the biological function and structure of genes and proteins, or to cluster them into related families based on their sequence information. Some software packages need to be purchased, others are available at no cost. The European Molecular Biology Open Software Suite (EMBOSS) is free, open-source software that can be downloaded from http://emboss.sourceforge.net/. It integrates many bio-informatics tools for sequence analysis into a single environment and can be used to analyse DNA and protein sequence in a variety of formats. Within EMBOSS there are hundreds of applications covering areas such as sequence alignment, rapid database searching for sequence patterns (e.g. to identify islands or repeats), protein motif identification (domain analysis), codon usage analysis for small genomes, and rapid identification of sequence patterns in large sequence sets. In addition, because extensive libraries are provided with this package, it is possible for users to develop and release software of their own. An example of another integrated

bio-informatics software can be found at http://ca.expasy.org/tools. As with EMBOSS, this package is helpful for characterizing and predicting the function of biomolecules of interest. Other commonly used sequence analysis applications include ClustalW and IMAGE.

Structure prediction

There are also software packages that can predict protein structure based on its sequence information or that of the gene encoded by it. Understanding protein structure is the key to revealing its function. Currently there are many programs for performing primary, secondary and tertiary structural analyses. ProtParam is a tool that computes physical or chemical parameters for a protein, such as molecular weight, amino acid and atomic composition, isoelectric point, extinction coefficient, estimated half-life, stability index and aliphatic index, based on user-entered sequence information. RasMol is an excellent graphics tool for visualizing macromolecular structure in order to help elucidate function. Other structure-prediction programs include Dowser, FastDNAml, LOOPP, MapMaker/QTL and PAML.

THE -OMICS

The 'omics' suffix is used to describe disciplines in which researchers analyse biological interactions on a genome-wide scale. The associated prefix indicates the object of study in each field. Examples include genomics, transcriptomics, metabolomics and proteomics. These encompass the study of the genetic make-up, the complete set of mRNA produced, the collection of metabolites, and protein function and interaction, respectively, in organisms, tissues or cells. The main focus of -omics is on gathering information at a given level and using computer-based tools to identify relationships in order to understand heterogeneous, biological networks, often with the ultimate goal of manipulating regulatory mechanisms. Omics require a multidisciplinary approach, bringing scientists together from a variety of fields to interpret the data collected.

APPLICATIONS

Rapidly emerging biotechnological tools can be used to help us better understand how biological systems function. The resulting discoveries allow us to introduce novel or alter existing traits that are useful to humans. Chapter 4 by McDonnell *et al.* in this volume provides a description of some commercially important and environmentally beneficial traits that have been incorporated into trees.

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3. Genetic containment of forest plantations

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> "It is essential that new molecular gene-containment strategies... be developed and introduced."
>
> **Editorial, *Nature Biotechnology*, 20: 527 (2002)**

CONTEXT FOR GENE CONTAINMENT APPROACHES

In an ideal world, industrial forest plantations would operate in harmony with, and in isolation from, natural ecosystems. Plantations would occur within a landscape designed to maintain biodiversity and minimize ecological impacts of plantations on external ecosystems, and economic goals would be the primary consideration within plantations. However, the reality is that plantations have multiple ecological connections with other managed and wild ecosystems and operate in a social milieu where their actual and perceived impacts may or may not be tolerated. Regulations, laws, and marketplace mechanisms such as certification systems set limits on the kinds of activities that may occur within plantations and on the impacts that these activities may have outside of plantations. All of these mechanisms strongly constrain research and commercial application of genetically engineered trees (reviews in Strauss and Bradshaw, 2004). Genetically engineered, genetically modified or transgenic organisms, as used in this review paper, are defined as those that have been modified using recombinant DNA and asexual gene transfer methods – regardless of the source of the DNA employed.

Forest certification systems represent a growing mechanism for expression of social preferences in the marketplace (Cashore, Auld and Newsom, 2003). One major forestry certification system aimed at environmental and social compliance, that of the Forest Stewardship Council, bans all forms of genetically engineered trees on certified lands. This rule is absolute; it applies regardless of the level of containment, whether the genes are from the same or different species, whether the goal is purely scientific research vs application, or whether the primary aim is the solution of substantial environmental problems rather than economic benefits (Strauss *et al.*, 2001a, b). Such a broad ban, which covers even contained research with environmental goals, is difficult to justify on scientific grounds, especially given the long-standing scientific consensus that "product not process" should dominate risk assessment for genetically engineered organisms (Snow *et al.*, 2005). It shows that social considerations can overwhelm technical innovations.

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Thus, containment systems may be required even for genes where no significant biological impact, or even a positive environmental effect, is expected to occur. By allowing effective isolation of trees produced in different ways on the landscape, containment systems should provide a mechanism whereby different social values can more easily co-exist.

However, genetic mechanisms for isolation have never before been required even when highly bred or exotic species have been used in agriculture or forestry; their novelty, therefore, creates new forms of social controversy. Although genetic containment systems have long been called for by ecologists and other scientists to reduce a number of undesired effects of genetically engineered crops (NRC, 2004; Snow *et al.*, 2005), there has been strong pressure on companies and governments against use of any forms of 'Terminator-like' containment technology (ETC, 2006). For example, a law against the use of such technology in Brazil (Law 11,105/05, banning "...the commercialization of any form of Gene Use Restriction Technology (GURTs)") delayed approval of a field trial of a reduced-lignin, putatively sterile eucalypt (ISAAA, 2006). In agriculture, these concerns primarily are about control of intellectual property and the forced repurchase of seed by farmers. But in the forestry area, there has also been activism against containment technology because of a lack of confidence that it will be fully effective, concerns about loss of biodiversity associated with modification or loss of floral tissues (Cummins and Ho, 2005), and legal uncertainties and liability risks from the dispersal of patented genes. These biological concerns occur despite the intention to use such technology mainly in plantations that, due to breeding, high planting density and short life spans, already produce few flowers and seeds compared with long-lived and open-grown trees. The powerful inverse association between forest stand density and degree of tree reproduction is widely known (Daniel, Helms and Baker, 1979). There is also an abundance of means to avoid and mitigate such effects at gene to landscape levels (Johnson and Kirby, 2004; Strauss and Brunner, 2004). Government regulations against the dispersal of genes from research trials also pose very substantial barriers to field research to study the efficiency of containment mechanisms (Strauss *et al.*, 2004; Valenzuela and Strauss, 2005). Thus, genetic containment technology is, itself, difficult and highly controversial, requiring special social conditions even to carry out research.

From a biological viewpoint, however, there are good reasons to employ containment technologies to control some forms of highly domesticated, exotic or genetically engineered organisms. Once genes or organisms move beyond plantation boundaries, the risks to external ecosystems are virtually impossible to control, and as with other biological introductions of mobile organisms, may be irreversible. Novel organisms of all kinds may impair the health of some wild ecosystems or create management problems for human-dominated ecosystems (James *et al.*, 1998). If we could confidently segregate intensely domesticated trees by control of reproduction, it would avoid the need for much of the complex, imprecise and costly ecological research that would otherwise be required to try to understand and predict impacts of spread. The costs and obstacles to conducting

commercially relevant environmental research with genetically engineered trees are great and occur for a number of reasons:

- laboratory cost of genetically engineered tree production, including production and study of many kinds of gene constructs and gene transfer events;
- ecological complexity in space and time and high stochastic variance in gene flow and related ecological processes, requiring many sites, environmental conditions, long time frames and large spatial scales;
- cost of needed patents, licenses, publication agreements, and transactions for access to genes intended for commercial use (required if results are to be directly relevant to regulatory decisions);
- cost of record keeping and compliance with regulations, which can be very demanding and legally risky for complex programmes that span many years and sites;
- uncertainty over what data regulators will require due to vagueness in regulatory standards and political volatility creating substantial changes in regulations or their interpretations over time;
- risk of spread into the environment during research, including costly steps to prevent any spread (e.g. premature termination of trials, bagging all flowers in test plantings, use of non-commercial but sterile genotypes, or use of geographically distant planting environments);
- disincentives to undertaking costly and risky research, as a result of possible marketplace rejection and separation costs; other significant disincentives result from primary ownership of the genes and gene transfer methods generally being out of the hands of the tree breeders and producers that bear most of the risks and costs of field testing.

These very formidable obstacles, many of which have substantial similarities in many other crop species, have forced companies and governments to ask whether these obstacles do more harm than good by blocking economically and environmentally beneficial technologies. It has also prompted calls for regulations that would place genetically engineered organisms into risk categories that call for dramatically different levels of research and containment depending on the novelty and risk of the new traits (Bradford *et al.*, 2005). For example, it has been suggested that 'genomics guided transgenes (GGTs)', where the expression of native or functionally homologous genes are altered in a manner analogous to conventional breeding, and 'domestication transgenes' that encode traits highly likely to reduce fitness in the wild, should be put into a low risk category or exempted from regulation entirely (Strauss, 2003). In contrast, new types of genetically engineered plants that are more likely to produce ecologically novel traits, or produce hazardous forms of pharmaceutical or industrial compounds, would be regulated with increased stringency. The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA), which regulates all field research in the United States of America, is currently undergoing a major review, with one goal being the creation of risk categories. The obstacles to

field research have also called for increased emphasis on ecogenetic models, where the spread and impacts of transgenes with different properties, and under different environmental and social conditions, can be studied over decades as they spread within the containment of a computer (reviewed below).

The sense for a mandate to use containment technologies was also inspired by the creation of genetically engineering-based male and female sterility mechanisms during the early 1990s (Mariani *et al.*, 1990, 1992), when the possibilities of plant biotechnology seemed limitless, public acceptance was not an issue, and regulatory hurdles appeared modest (reviewed below). It was also stimulated by the suggestion of 'mitigation' genes that can both increase value in managed environments and reduce competitive ability in the wild (Gressel, 1999). If gene spread creates irreversible risks and social discomfort, and technology exists to greatly reduce these risks, is it not the ethical responsibility of scientists and companies to act to minimize these risks? The incorporation of biosafety features into genetically engineered organisms during their design has been promoted as key elements of good stewardship (Doering, 2004).

Unfortunately, as discussed above and in genetic detail below, applying containment technology to trees is an extremely costly and difficult endeavour. Caution is therefore warranted in assuming that containment systems – even the use of genes with a neutral or negative effect on fitness – present good stewardship. If genetic containment were incomplete, genes that provide a significant and evolutionarily highly stable selective advantage (should such transgenes be feasible to create and deploy), could eventually spread widely. Even neutral or deleterious genes can persist and even become fixed in wild populations in situations where transgenes numerically swamp native genes (Haygood, Ives and Andow, 2003). Obtaining licences to the set of patents that cover all of the elements of the best containment technology can also be very costly or impossible. At the same time, it is also likely that the spread of fitness-improving transgenes could, in some cases, provide ecological benefits. A gene for resistance against a serious exotic pest of trees such as the chestnut blight or Asian longhorn beetle might provide large ecological benefits by maintaining or restoring healthy ecological dominants and their dependent communities. Genes for general pest or abiotic stress resistance, including against native herbivores or pathogens, might also provide net ecological benefits by increasing the vigour of a native organism like poplar, which provides habitat for myriad dependent organisms (Whitham *et al.*, 2006), even if some introduced herbivores or plant species were disadvantaged as a consequence. It is therefore essential that containment technology is not indiscriminately required by regulations or used when its net benefits are questionable.

The goal of the remainder of this paper is to review the state of sterility technology that might be useful for sexual containment of trees used in clonal forestry and ornamental horticulture. We previously reviewed the many options for sex-specific sterility and inducible sterility/fertility (Strauss *et al.*, 1995) that might be used to enable continued seed propagation. Here, we focus on complete sterility under some form of vegetative propagation. Only after a simple method for strong

and bisexual sterility is shown to be effective and socially accepted is it likely that more sophisticated methods for fertility control will be developed and deployed.

TECHNICAL APPROACHES AND THEIR ADVANTAGES AND DISADVANTAGES

Below, we discuss the main approaches to engineering containment relevant to forest trees. In addition, via electronic searches, we have scanned the recent (2000 to time of writing) scientific and patent (United States Patent and Trademark Office (US PTO]) literature and presented representative examples of developments. Tables 3-1 and 3-2 summarize the kinds of approaches being taken, nearly all of which are relevant to one kind of tree species or another.

TABLE 3-1
Selected literature on genetic engineering of sterility published from 2000 onwards

Phenotype	Mechanism	Promoter	Active gene	Plant species	Reference
Delayed flowering					
Late flowering	Overexpression of *FLM*	35S CaMV	*Flowering Locus M*	*Arabidopsis*	Scortecci, Michaels and Amasino, 2001
	AGL20/shoot apical meristem	35S CaMV	*AGAMOUS LIKE 20*	*Arabidopsis*	Borner *et al.*, 2000
Cell ablation					
Male sterility	Altered pollen development	Endosperm specific promoter, *AGP2*	Fission yeast *cdc25*	Wheat	Chrimes *et al.*, 2005
	Pollen sterility	Rice tapetum promoter (*TAP*)	Barnase/rice tapetum gene *rts*	Creeping bentgrass	Luo *et al.*, 2005
	Alteration in tapetal cells	*Tapetum A9* promoter	Chimeric gene in transgenic plant	*Arabidopsis*	Guerineau *et al.*, 2003
	Abnormal pollen	*BcA9*	*DTx-A*	*Brassica*	Lee *et al.*, 2003
	Tapetal dysfunction	*TA29* promoter	*RIP*	Tobacco	Cho *et al.*, 2001
	Reduced pollen viability	Pollen specific promoter G9	Chimeric genes *G9 uidA* and *G9-RNase*	Tobacco	Bernd-Souza *et al.*, 2000
Male and female sterility	Floral organ ablation with otherwise normal growth	*PopulusPTD*	*DTA*	Tobacco, poplar, *Arabidopsis*	Skinner *et al.*, 2003
Recoverable block of function (RBF)	Inducible fertility	Sulfhydryl endopeptidase, heat-shock promoter	*Barnase* (the blocking construct) and *barstar* (recovering construct)	Tobacco	Kuvshinov *et al.*, 2001
Gene suppression					
Male sterility	Distorted pollen morphology	Various	*AtMYB32 AtMYB4*	*Arabidopsis*	Preston *et al.*, 2004
	Temperature sensitive male sterility due to silencing choline biosynthesis	*S*-adeno syl-L-methionine	Phosphoethanolamine *N*-methyltransferase (PEAMT)	*Arabidopsis*	Mou *et al.*, 2002
	Mitochondrial dysfunction	Tapetum specific promoter	Antisense pyruvate dehydrogenase E1 α subunit	Sugar beet	Yui *et al.*, 2003
	Abnormal pollen	*Nin88* promoter	Antisense *Nin88*	Tobacco	Goetz *et al.*, 2001
	Abnormal pollen	Glutenin subunit gene promoter	Antisense sucrose non-fermenting-1-related (SnRKl) protein kinase	Barley	Zhang *et al.*, 2001
Restoration of fertility	Glucanase gene suppression	*pA9*	Sense and antisense PR glucanase	Tobacco	Hird *et al.*, 2000

TABLE 3-2
Selected patents on genetic engineering of sterility published from 2000 onwards

Phenotype	Mechanism	Promoter	Active gene/Protein	Species	Reference
Time of flowering					
Altered floral development	Expression of floral meristem identity protein	Modified native promoter	*CAULIFLOWER* (*CAL*), *APETELA 1* (*API*), *LEAFY* (*LFY*)	Angiosperm or gymnosperm	Yanofsky, 2000
Cytotoxin ablation					
Suicide gene to ablate gamete	Any of several cytotoxic genes expressed in gametes	Male- or female-specific promoter expressed in gamete	Various "suicide" genes (*barnase, tasselseed2, diphtheria toxin A*)	Rice	Dellaporta and Moreno, 2004
Female sterility	Enhance fruit development or induce sterility	*DefH9* promoter	DNases, RNases, proteases, glucanases, lipases, toxins, etc.	Many	Spena *et al.*, 2002
Gene suppression					
Male sterility	Calcium/calmodulin-dependent protein kinase (CCaMK) expression	Developmental stage-specific anther promoter	Antisense RNA	Tobacco	Poovaiah, Patil and Takezawa, 2002
Reversible male sterility	Biosynthesis of amino acids inhibited in male reproductive organs, reversible by application of those amino acids	Male organ-specific promoter	Antisense RNA	*Arabidopsis*, tobacco	Dirks *et al.*, 2001
Male sterility	Supression of A*TH1* gene to control flowering time	35S CaMV	Antisense A*TH1*	*Arabidopsis*	Smeekens, Weisbeek and Proveniers, 2005
Delayed flowering time	Loss of function of *SIN1* by RNAi	35S CaMV	Short integuments 1 protein	Unspecified	Ray and Golden, 2004
	RNAi construct	Constitutive, inducible, or tissue-specific promoter	Sequence similar to transgene or endogenous gene	Unspecified	Waterhouse and Wang, 2002
Floral promoters					
Male sterility	Anther development-specific genes and promoters	Tapetum, pollen	Antisense RNA or any gene that compromises pollen viability	*Brassica, Arabidopsis*, tobacco	Knox, Singh and Xu, 2004
Female sterility	Regulatory region of corn silk/pistil genes	*C3* promoter	Silk-specific gene, *C3*	Maize	Ouellet *et al.*, 2003
Restoration of fertility to cytoplasmic male sterile plants	Wild-type *atp6*	*AP3* promoter	Wild-type *atp6* gene fused to a mitochondrial transit peptide	*Brassica*	Brown, 2002
Conditional male sterility	Upon application of acetylated toxin	Stamen-selective promoters	Deacetylase	Wheat	Quandt, Bartsch and Knittel, 2002
Male and female sterility	Poplar floral homeotic genes and promoters	Native promoters	*PTLF, PTD, PTAG-1, PTAG-2*	Poplar	Strauss *et al.*, 2002
Male sterility	Recessive mutant causes sterility	*Ms41-A* promoter	*Ms41-A*	*Arabidopsis*, maize,	Baudot *et al.*, 2001
Male sterility	Absence of a functional callase enzyme	*MsMOS* promoter	*msMOS*	Soy	Davis, 2000

TABLE 3-2 (CONTINUED)

Phenotype	Mechanism	Promoter	Active gene/Protein	Species	Reference
Protein interference					
Reversible male sterility	Dominant negative genes under anther-promoter reversed by expression of a repressor	Anther-specific promoter and *lexA* operator	Any cytotoxic methylase or growth-inhibiting gene	Maize	Cigan and Albertsen, 2002
Cytoplasmic male sterility	ATP synthesis in mitochondria inhibited	Ubiquitin promoter	Unedited *Nad 9* gene	Rice, wheat, maize, soybean	Patell *et al.*, 2003
Male sterility	Biotin-binding polypeptide ablates male gamete tissue, fertility can be restored	Promoter regulated by the *LexA* operon expressed in anther	Botin-binding polypeptide and inhibitory proteins	*Arabidopsis* and tobacco	Albertsen and Huffman, 2002
Male sterility	Repressor protein under male promoter repressed by antisense RNA	Male flower specific promoter	Repressor protein	Multiple	Bridges *et al.*, 2001
Male sterility	Protein that disturbs metabolism, development and gene for reversibility	Stamen-specific promoter	A sterility RNA. protein or polypeptide	*Brassica,* maize, rice	Michiels, Botterman and Cornelissen, 2000
Mitigation					
Male sterile and dwarf	Unknown	Native promoter	*dfl1* gene	Safflower	Weisker, 1995
Dwarf plants	*GA* insensitive	Native promoter	Mutant of *GA1*	*Arabidopsis*	Harberd *et al.*, 2004
Dwarf plants	Rht mutant dominant allele causes *GA*–insensivity	Native promoter	Mutant of *Rht* (D8)	Rice	Harberd, Richards and Peng, 2004

There are five major approaches to containment. One approach, mitigation (e.g. Al-Ahmad, Galili and Gressel, 2004), is a directed form of plant domestication such that the fitness benefits of transgenes are effectively cancelled by tight linkage to a gene that is beneficial within farms or plantations, but deleterious elsewhere. It has the advantage of being applicable to vegetative and sexual dispersal, which is useful for species like poplars that can spread vegetatively. Mitigation genes could also be combined with sterility genes to provide a second layer of containment. Genes that reduce the rate of height growth in forest trees, especially for shade-intolerant species like poplars (Daniel, Helms and Baker, 1979), are expected to provide a very powerful competitive disadvantage in competition with wild trees (Strauss *et al.*, 2004). Only two patents for dwarfism genes are shown under mitigation in Table 3-3 (Harberd, Richards and Peng, 2004; Harberd *et al.*, 2004), though there are a number of such genes now reported in both the scientific and patent literature. It is unclear, however, if such genes could be used and still maintain or improve yield and adaptability in plantation grown trees, but such studies are underway (e.g. Strauss *et al.*, 2004; Busov *et al.*, 2006).

The other forms of containment affect sexual reproduction, which is overwhelmingly the most important means for large-scale propagule spread in most tree species. There are basically four genetic engineering approaches: ablation, where floral tissues are effectively destroyed or made non-functional

TABLE 3-3
Summary of studies on stability of transgene expression in plants

Taxa	Gene	Number of events (unstable)[1]	Environment	Propagation	Generations or years	Associated factors	Non-associated factors	Reference
Chrysanth-emum	*35S-gus*	17(0)	Greenhouse	Vegetative	1 generation			Pavingerová *et al.*, 1994
Citrus	*35S-uidA, NOS-nptII*	70 (0)	Screenhouse	Vegetative	4–5 years	Copy number	T-DNA rearrangements	Cervera *et al.*, 2000
Poplar	*FMV-cp4, FMV-gox*	40 (1)	Field	Vegetative	4 years			Meilan *et al.*, 2002
Poplar	*35S-rolC*	6–22 (2–6)	*In vitro,* greenhouse, field	Vegetative	5–6 years	T-DNA repeat formation, flanking AT-rich sequence		Kumar and Fladung, 2001
Poplar	*35S-uidA, EuCAD-uidA*	44 (0)	*In vitro,* greenhouse, field	Vegetative	6 years		Copy number, extra vector sequence	Hawkins *et al.*, 2003
Poplar	*35S-ASCAD 35S-ASCOMT*	4	Field	Vegetative	4 years			Pilate *et al.*, 2002
Potato	*Gus, nptII*	2	*In vitro,* greenhouse	Vegetative	2 years			Borkowska *et al.*, 1995
Potato	*Nptll, gus, ocs, rolA, and C*	4	Greenhouse	Vegetative	3 generations			Ottaviani, Hanisch ten Cate and Doting, 1992
Sugar cane	*Ubi-bar*	1	Greenhouse	Vegetative	3 generations		Contained five copies	Gallo-Meagher and Irvine, 1996
Sugar cane	*Pat*	1	Field	Vegetative	3 generations		Contained nine copies	Leibbrandt and Snyman, 2003
Tall fescue	*Actinl-gus*	2	Growth room	Vegetative	5 generations			Bettany *et al.*, 1998
Arabidopsis	*NOS-nptII*	7	*In vitro*	Sexual	4 generations	Promoter methylation		Kilby, Leyser and Furner, 1992
Arabidopsis	*35S-hpt*	28 (14)	*In vitro*	Sexual	1 generations	Copy number		Scheid, Paszkowski and Potrykus, 1991
Arabidopsis	*NOS-nptII*	111 (62)	*In vitro,* growth chamber	Sexual	3 generations	Construct configuration, temperature	Copy number	Meza *et al.*, 2001
Arabidopsis	*Fpl-dsFAD2*	1	Greenhouse	Sexual	4 generations			Stoutjesdijk *et al.*, 2002
Petunia	*35S-A1*	1	Field	Sexual	1 year	Promoter methylation, temperature, endogenous factors		Meyer *et al.*, 1992
Rice	*35S-bar, 35S-gusA*	12 (0–2)		Sexual	3 generations	Presence of truncated transgene sequences	Copy number, position effect	Kohli *et al.*, 1999
Rice	*Ltp2-gus*	3	Greenhouse	Sexual	5 generations	Partial rearranged transgene		Morina, Olsen and Shimamoto, 1999
Tobacco	*NOS-nptII*	2	*In vitro*	Sexual	3 generations			Müller *et al.*, 1987
Tobacco	*NOS-nptII*	18 (5×10^{-5} ~5.9×10^{-4})[2]	*In vitro*	Sexual	1 generation	Environmental stress	MAR	Conner *et al.*, 1998
Tobacco	*35s-hpt, 35s-cat*	4	*In vitro*	Sexual	8 generations	T-DNA flanking sequences, position effect, extra vector sequence		Iglesias *et al.*, 1997

[1] Unstable events given in parentheses only where data on ten or more independent events reported.
[2] Frequency of kanamycin-sensitive seedlings derived from each event.

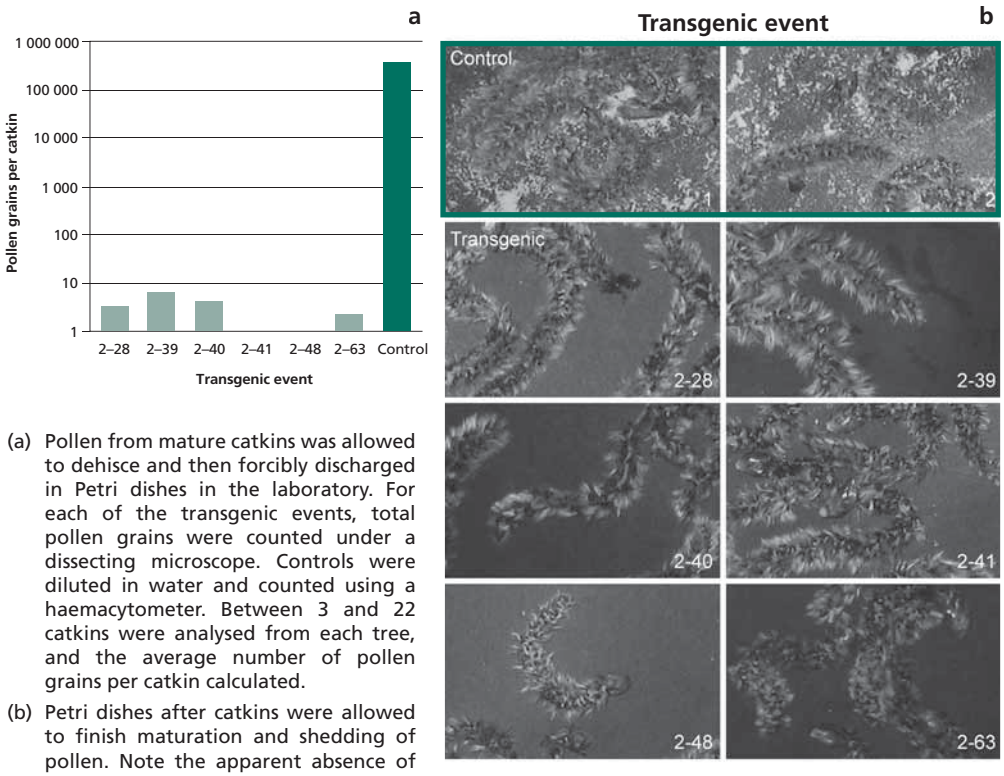
by a cytotoxin; excision, where some or all functional transgenes are removed from gametes before their release; gene suppression, where the activity of one or more genes essential for reproduction are impaired at the DNA, RNA or protein levels; and repression, where the onset of flowering is postponed by modifying the expression of genes that promote vegetative growth or repress the transition to reproductive growth.

Ablation approaches

Genetic ablation methods employ promoters active in specific cells to control the expression of a deleterious gene, usually encoding a cytotoxin (e.g. Burgess *et al.*, 2002). However, many kinds of deleterious genes may be employed, as demonstrated by the patent applications of Dellaporta and Moreno (2004) and Spena *et al.* (2002), which cite in addition to the widely used RNases and protein synthesis inhibitors (Table 3-1), DNases, proteases, glucanases and lipases. Höfig *et al.* (2006) recently reported that targeted expression of stilbene synthase, which interferes with pollen function, gave a high rate of male sterility. For engineering reproductive sterility, a floral predominant promoter has been used to control the expression of a cytotoxin such as the ribonuclease barnase (Mariani *et al.*, 1990). Ideally, cytotoxin expression will be confined to floral cells; however, it appears that many floral promoters are not expressed exclusively in floral tissues (e.g. Brunner *et al.*, 2000; Rottmann *et al.*, 2000), and even low levels of unintended cytotoxin expression may impair tree growth (Skinner *et al.*, 2000). Thus, great care is needed in selection of promoters and cytotoxins. Skinner *et al.*, (2003) showed how the promoter of the poplar floral homeotic gene PTD, used to drive the cytotoxin DTA, gave rise to high levels of sterility in tobacco and *Arabidopsis* and did not impair vegetative growth in a greenhouse trial. The tapetal specific promoter TA29 from tobacco, when fused to barnase, caused very high levels of male sterility in field-grown poplars (Figures 3-1 and 3-2). However, Wei *et al.* (2007), studying poplar, and Lemmetyinen, Keinonen and Sopanen (2004) and Lánnenpáá *et al.* (2005), studying birch, found that many transgenic events with floral homeotic promoter::barnase fusions showed abnormal growth or morphology in the greenhouse. In an attempt to avoid deleterious effects on growth seen with the poplar *LEAFY* (*PTLF*) promoter driving barnase, barstar, a specific inhibitor of barnase, was co-expressed in transgenic poplars using various promoters, and it was found that gene insertion events with low ratios of barstar to barnase activity had abnormal growth and morphology (Figure 3-3), and that even among plants with normal growth and morphology in the greenhouse, those events with barnase grew slower in the field than events with only barstar or that lacked both genes (Wei *et al.*, 2007). We found that we were unable to regenerate any transgenic poplars containing an intact *pAPETALA1::DTA* transgene, a likely result of leaky expression (root and leaf) seen with this promoter in transgenic poplars with *pAPETALA1::GUS* fusion genes (data not shown). Thus, ablation-based systems need to be carefully engineered in trees via judicious choice of promoters, cytotoxins and vectors, and then carefully field tested.

FIGURE 3-1

Pollen production from catkins of a non-transgenic control and several transgenic trees that originated from different gene transfer events, after ten years growth in the field in Oregon, United States of America



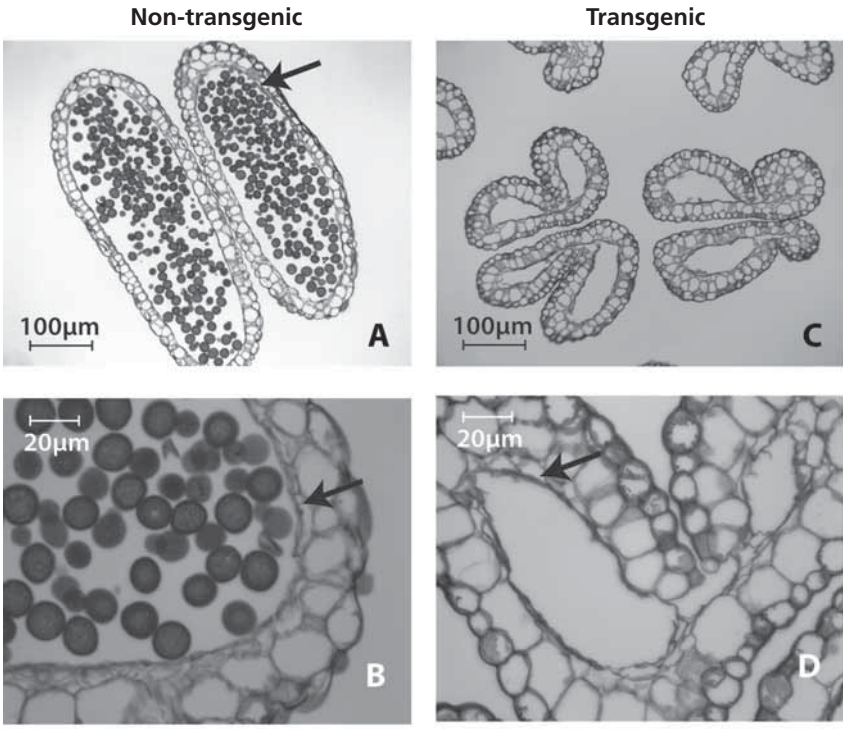
(a) Pollen from mature catkins was allowed to dehisce and then forcibly discharged in Petri dishes in the laboratory. For each of the transgenic events, total pollen grains were counted under a dissecting microscope. Controls were diluted in water and counted using a haemacytometer. Between 3 and 22 catkins were analysed from each tree, and the average number of pollen grains per catkin calculated.

(b) Petri dishes after catkins were allowed to finish maturation and shedding of pollen. Note the apparent absence of pollen from the six different transgenic events sampled compared with the non-transgenic control samples.

Gene excision approaches

There have been considerable efforts to develop more precise means for manipulation of transgenes and their genomic locations via the use of site-specific recombinase systems such as *cre/lox* from bacteriophage P1 (reviewed in Gilbertson, 2003). Although the primary goals have been the removal of selectable marker genes and the targeting of transgenes to defined locations, a more recent application has been to use them to selectively remove transgenes before the release of seeds and pollen. By flanking transgenes with recombinase recognition sites and placing the recombinase under the control of a floral predominant promoter, it appears that very high levels of transgene excision can be obtained. Mlynárová, Conner and Nap (2006) used the microspore-predominant NTM19 promoter to control expression of an intron-containing *cre* gene to successfully excise GUS encoding transgenes from tobacco pollen at a rate above 99.98%. No

FIGURE 3-2
Transverse sections of nearly mature anthers from a transgenic, putatively male-sterile field-grown poplar and a non-transgenic control poplar of the same age

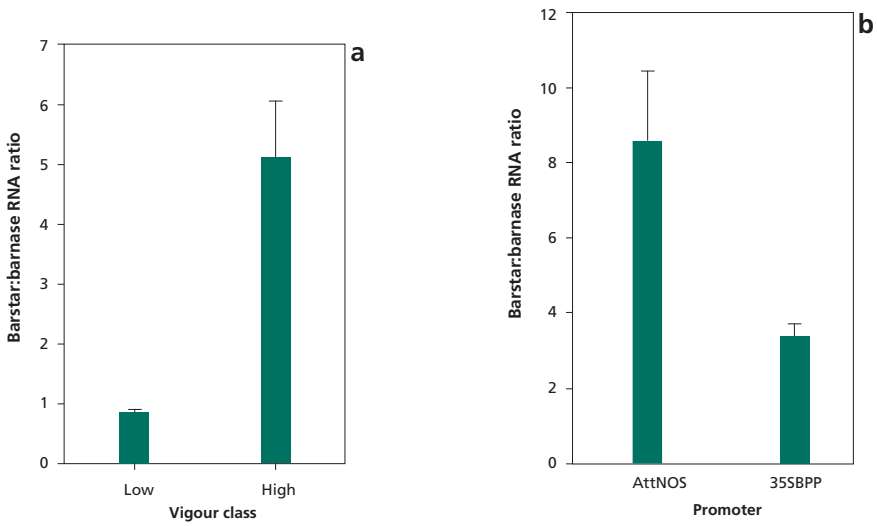


Slides in top row were taken at ×100 magnification; those below were taken at ×400 magnification. Samples were fixed, dehydrated, embedded in glycol GMA methacrylate plastic, sectioned and mounted on slides. Sections were stained in 0.5% Toluidine Blue O in citrate buffer. Arrows point to tapetal layer (absent or disorganized in transgenics).

excision activity was detected other than in target tissues. Li and Pei (2006 and personal communication) used the promoter of the bisexually expressed PAB5 gene (Belostotsky and Meagher, 1996) to drive either or both the *cre* or FLP recombinase genes, targeting loxP-FRT fusion recognition sites. Based on GUS activity examined in more than 25 000 T_1 progeny per transgenic event, they reported a 100% rate of transgene removal from both male and female gametes of tobacco in 18 of 45 events studied. Although this is a promising system for transgene containment in vegetatively propagated plants, its effectiveness in the long term under field conditions is unknown, and predicting and verifying that gametes will lack transgenes in large trees when they begin flowering will be difficult. It is also distinct from the other approaches in that it does not impair fertility, and thus would provide containment of only the excised transgenes – not of exotic or highly domesticated organisms. However, reproductive transgene excision could be used in combination with a sterility transgene to provide a more robust containment system.

FIGURE 3-3

Ratio of barstar:barnase RNA from shoot tips of greenhouse-grown trees with barnase driven by the poplar LEAFY (PTLF) gene promoter, and barstar driven by one of three promoters



(a) Transgenic events with the highest ratios had the greatest vegetative growth, and those with the lowest ratios tended to be stunted or have abnormal physiology.

(b) The NOS promoter directed twice the level of barstar expression compared to the 35S basal promoter and the basal promoter with an omega enhancer element (mean shown). All data are expressed relative to barnase expression from a pPTLF::barnase gene.

Gene suppression approaches

The activity of genes essential for fertility can be suppressed by transcriptional gene suppression, posttranscriptional gene suppression, blocking the activity of the encoded protein, or by directed mutation or deletion. As shown in Tables 3.2 and 3.3, there have been a great variety of genes and approaches in various plant species that have been successfully used to impart sterility and/or restore fertility. This includes targeting of signal transduction proteins (Zhang *et al.*, 2001; Poovaiah, Patil and Takezawa, 2002), amino acid metabolism (Dirks *et al.*, 2001), choline biosynthesis (Mou *et al.*, 2002), transcription factors (Preston *et al.*, 2004; Smeekens, Weisbeek and Proveniers, 2005), methylases or methyltransferases (Cigan and Albertsen, 2002; Luo *et al.*, 2005) and mitochondrial genes (Patell *et al.*, 2003; Yui *et al.*, 2003).

RNA interference and related methods

Double-stranded RNA (dsRNA) can induce a variety of sequence-specific gene suppression processes in plants, animals and fungi (reviewed in Baulcombe, 2004; Matzke and Birchler, 2005). RNA-mediated gene suppression, also called RNA interference (RNAi), is now widely exploited to reduce the expression of specific genes (reviewed in Watson *et al.*, 2005). Virus-induced gene silencing (VIGS)

vectors are one option for inducing sequence-specific suppression and have great potential for functional genomics (Burch-Smith *et al.*, 2004 and discussed below), but are not suited to stable introduction of a biosafety trait.

Stable transformation of transgenes containing an inverted repeat or hairpin sequence corresponding to a transcribed region of the target gene has been effective in a variety of plants, and post-transcriptional suppression has been shown to be stably inherited over several generations (Chuang and Meyerowitz, 2000; Wesley *et al.*, 2001). However, stability through rounds of vegetative propagation and across multiple years in field environments has not been extensively studied (discussed below). Inverted-repeat transgenes of promoter regions can induce methylation and transcriptional gene suppression of endogenous plant promoters, and this approach was used to engineer male sterility in maize (Cigan, Unger-Wallace and Haug-Collet, 2005). Nonetheless, there have been relatively few studies, and thus its utility as a gene suppression approach is uncertain. Moreover, it appears that promoters vary in their sensitivity to different types of cytosine methylation, depending on their sequence composition (Matzke *et al.*, 2004).

Multiple genes can be silenced by using a conserved region or by joining sequence segments of multiple genes together to create a compound RNAi transgene (reviewed in Watson *et al.*, 2005). This capability is especially important for sterility systems where a redundant approach is desirable to produce a highly robust and reliable biosafety trait. Because of genetic redundancy in the regulation of flowering and many taxon-specific gene duplications and losses (Irish and Litt, 2005), the extent and configuration of redundancy required for robust and effective RNAi suppression will vary between species.

A population of transgenic events carrying the same RNAi transgene typically exhibit highly diverse levels of suppression. Although RNAi transgenics that phenocopy null mutations in floral regulatory and other genes have been obtained, strong suppression can be infrequent (Chuang and Meyerowitz, 2000; Stoutjesdijk *et al.*, 2002). In addition, the level of endogene suppression appears to be target-specific (Kerschen *et al.*, 2004). The endogenous expression level of the target gene appears to influence the effectiveness of RNA-mediated silencing, but does not appear to be the only gene-specific determinant of RNAi effectiveness (Han, H. Griffiths and D. Grierson, 2004; Kerschen *et al.*, 2004; Wagner *et al.*, 2005).

Possible additional determinants include spatiotemporal expression, RNA turnover and sequence composition. Single-copy RNAi transgenics are preferable because multicopy events appear more variable with respect to level of suppression and stability, perhaps because multicopy transgenes are more susceptible to transcriptional gene suppression (Kerschen *et al.*, 2004). For practical application, successful transformation events (i.e. those exhibiting strong suppression) must be identifiable via molecular tests when trees are still juvenile. This potentially limits the utility of this approach because many target genes are specifically or predominantly expressed in floral tissues. We have produced transgenic poplars carrying RNAi transgenes targeting various genes regulating floral onset and floral organ development. Using vegetative tissue from poplar transgenics still in tissue

culture or the greenhouse, we have been able to identify events exhibiting strong target endogene suppression using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; Figure 3-4), suggesting that RNAi transgenic trees with greatly reduced fertility can be selected at an early, non-flowering stage.

Pleiotropic effects of RNAi methods can be significant. Non-target effects of dsRNAs are well-known in animal systems (Jackson and Linsley, 2004). However, this does not appear to be a common problem in plants for well-targeted dsRNAs, perhaps because both siRNAs and miRNAs require high levels of complementarity with their target (Watson *et al.*, 2005; Schwab *et al.*, 2005). Transitive suppression, whereby suppression spreads from the initiator sequence to an adjacent region, could potentially cause pleiotropic effects in plants. However, several plant studies have shown that transitive suppression occurred when the target was a transgene, but did not occur when an endogene was the target (Vaistij, Jones and Baulcombe, 2002; Petersen and Albrechtsen, 2005; Miki, Itoh and Shimamoto, 2005). Why transitive silencing appears to commonly occur with transgenes, but not endogenes, is unknown. However, to date, a few studies have looked for transitive silencing with endogene targets.

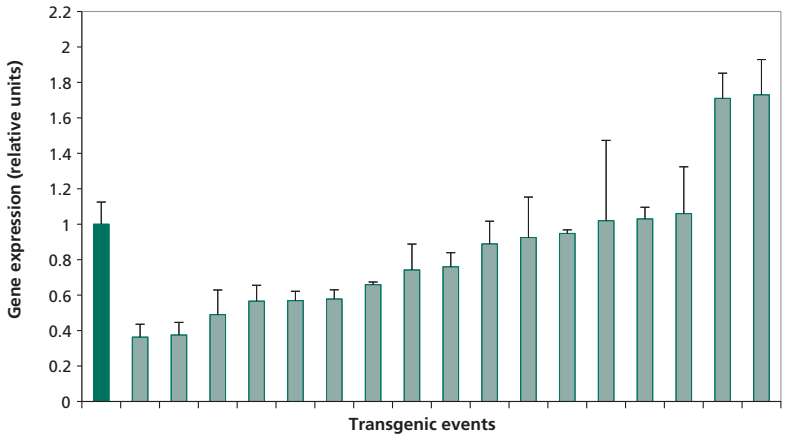
DOMINANT NEGATIVE PROTEINS

Alternative approaches to repressing floral genes include introduction of dominant negative mutant forms of the target endogene and artificial transcription factors. Several studies have identified dominant negative mutant forms of plant signal transduction proteins and transcription factors, including MADS box genes regulating floral development (e.g. Jeon *et al.*, 2000; Dievart *et al.*, 2003; Ferrario *et al.*, 2004). Most dominant negative forms appear to exploit the modular nature of these proteins and that they often form multiprotein complexes. For example, a dominant negative protein might be able to interact with other proteins, but the protein complex cannot bind DNA. Based on studies of rice and mammalian MADS-box genes, we used site-specific mutagenesis to alter amino acids predicted to be necessary for dimerization and/or DNA binding in *AG* and *APE-TALA1(AP1)*. Constitutive expression induced strong loss-of-function phenotypes at a frequency of approximately 30% in primary *Arabidopsis* transformants, and these transgenes are now being evaluated in poplar and sweetgum (data not shown).

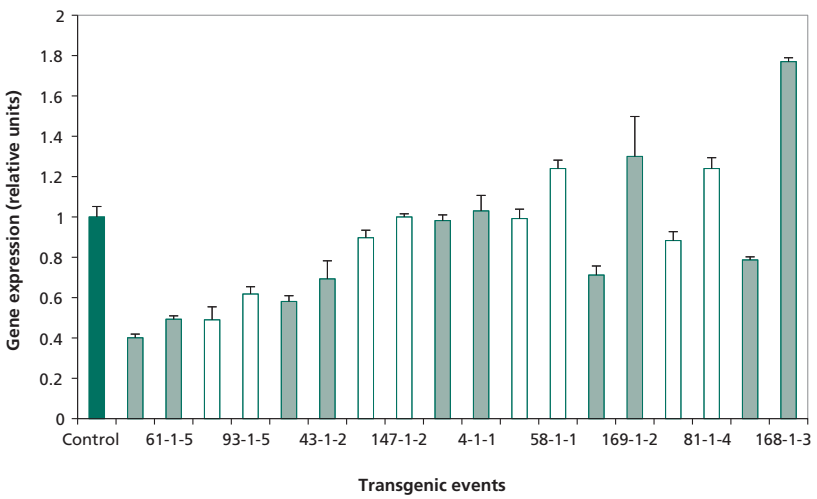
Another option for dominant repression of transcription factor activity is the introduction of chimeric transgenes that are translational fusions of the selected transcription factor coding region and a repression domain such as the ERF amphiphilic repressor (EAR) motif (Hiratsu *et al.*, 2003). Expression of EAR chimeras has proven to be useful for producing phenocopies of double knockouts in *Arabidopsis* and thus, can overcome the problem of genetic redundancy among gene duplicates. Recently, Mitsuda *et al.* (2006) used this chimeric repressor approach with *AP3*, *AG*, *LEAFY*, and a floral expressed *MYB* gene, and reported very high levels of sterility in *Arabidopsis* and/or rice. Recent studies have also shown that synthetic zinc-finger domains fused to a transcriptional activation or repression domain are highly effective for manipulating the expression of specific

FIGURE 3-4

Range of RNAi gene suppression (top) and repeatability among biological replicates (bottom) for floral genes expressed in vegetative tissues



Relative expression level of native PTLF gene in selected poplar PTLF-RNAi transgenic trees and non-transgenic controls of poplar clone 353-53 (*Populus tremula* × *tremuloides*). Expression was determined by qRT-PCR analysis of native transcripts in vegetative shoots (a ubiquitin gene served as an internal control). Each datum represents a pool of total RNA from four to five ramets per transgenic event; error bars are standard deviations over three PCR technical replicates.



Relative expression level of native *Poplar SOC1(PSOC1)* gene in pairs of biological replicates (RNA extraction from different ramets) of selected *PSOC1*-RNAi transgenic trees and non-transgenic controls. qRT-PCR methods as in top graph. Data are means of independent qRT-PCR runs for two different ramets for single transgenic events; error bars are standard deviations over the average of two PCR technical replicates (r^2=0.41). Pairs (shading) show biological replicates per event.

genes (reviewed in Segal, Stege and Barbas, 2003). By combining pre-defined zinc-finger modules appropriately, three- or six-finger domains can be created that specifically bind to a selected 12 to 18 bp DNA sequence. For example, a transgene containing a human repression domain, fused to a zinc-finger module designed to bind to a site in the *AP3* promoter, was able to repress endogenous *AP3* expression and induce a loss-of-function phenotype (Guan *et al.*, 2002).

It remains to be determined how these different methods of gene suppression compare with respect to frequency of transformants exhibiting strong repression or loss-of-function phenotypes, and stability over multiple years, in the field. It is also important to investigate whether pleiotropic effects are more common with certain methods. As discussed above, deleterious side-effects are not always evident under controlled conditions, but may appear as a cumulative effect of tree development, especially in the field. Although most studies have used strong constitutive promoters, tissue-specific promoters have been successfully used for RNAi and other repression methods. Promoters directing more restricted expression could reduce the occurrence of pleiotropic effects. However, they might be less effective at inducing strong, stable sterility.

Targeted gene mutagenesis and replacement

The long-sought-after goal of routinely creating precise deletions, insertions or mutations with plant genes has been elusive, largely due to the propensity for random rather than homologous DNA recombination in plants. However, recent studies have demonstrated new strategies that achieve substantial improvements in the rate of targeted mutagenesis and gene replacement. By constitutively expressing the yeast *RAD54* gene, a member of the *SWI2/SNF2* chromatin remodelling gene family, Shaked, Melamed-Bessudo and Levy (2005) achieved gene targeting frequencies of 3 to 17% in *Arabidopsis*. Another approach employs the zinc-finger modules discussed above for targeted gene repression. In this case, the zinc-finger domain is fused to a nuclease to introduce double-strand breaks at specific genomic sites. In one study, zinc-finger nucleases (ZFN) were expressed in *Arabidopsis* to create breaks that were subsequently repaired by non-homologous end joining, resulting in site-specific insertion/deletion mutations at frequencies of 2–20% (Lloyd *et al.*, 2005). Using a ZFN to facilitate gene replacement via homologous recombination, Wright *et al.* (2005) achieved 10% gene targeting efficiency. Both *ZFN* and donor genes had been introduced into tobacco protoplasts via electroporation. In four of seven tobacco plants that were homozygous for the target reporter gene, the desired gene replacement occurred on both chromosomes; such a capability is critical for induction of sterility as loss of function effects are expected to be recessive, and breeding for homozygosity in trees is generally not feasible.

Genetic redundancy further complicates introducing sterility via gene targeting (e.g. both alleles of two or more genes might need to be replaced or mutated). However, replacement of only one allele of one gene with a dominant suppression transgene might be more effective in achieving reliable sterility than random

integration of the sterility transgene because it would reduce wild-type gene dosage and may avoid position effects that can occur with random transgene integration. A key factor limiting the use of gene targeting is ease and efficiency of transformation in the species or genotype of interest. The feasibility of gene targeting is dependent of the combined frequencies of transformation and gene targeting and ease of transformation, regeneration and selection. *In planta* transformation is routine for *Arabidopsis* and that allows production and screening of a large number of transgenics with little effort; no similar system exists for trees.

One caveat to gene mutation or deletion is that recent studies suggest the possibility that there might be cases where it is not permanent. *Arabidopsis* hothead (*hth*) mutants can inherit allele-specific DNA sequences at multiple loci that were not present in the genomes of their parents, but were present in an earlier ancestor (Lolle *et al.*, 2005). Under certain environmental conditions, varieties of flax exhibit highly specific DNA changes at multiple loci from parents to progeny, including a large insertion that is found in natural populations, but is not present in the genome of the progenitor (Chen, Schneeberger and Cullis, 2005). To explain the non-Mendelian inheritance of *hth* mutants, Lolle *et al.* (2005) proposed that a cache of stable RNA serves as the template for extra-genomic DNA sequence reversion; however, others have posited alternative explanations (e.g. Comai and Cartwright, 2005). It is unclear whether this type of reversion could occur somatically in trees (e.g. during vegetative propagation or under certain stressful conditions). Rates of transgene instability under vegetative growth appear to be considerably lower than under sexual reproduction (discussed below).

Repressors of flowering

The activities of some strong repressors of the transition to flowering are directly correlated with their expression level (reviewed in Boss *et al.*, 2004). Thus, constitutive expression or overexpression of a floral repressor in appropriate tissues may be effective at long-term postponement of flowering. Because of the multiple pathways promoting flowering, this approach might delay, rather than prevent, the transition to flowering, but if flowering were delayed until long after harvest age, it still could be an effective biosafety approach. In addition, a floral repressor transgene could be combined with a different sterility transgene, such as one suppressing genes necessary for reproductive organ development, to provide redundancy. Overexpression of a floral repressor might be more likely to induce pleiotropic effects that, as discussed above, might not be apparent until trees are field-tested. Maintaining trees in a purely vegetative phase throughout their rotation cycle, whether by overexpression of a floral repressor, suppression of a floral promoter, or both, is highly desirable because this would completely prevent resource allocation to reproductive structures. However, depending on the tree taxon and environment, development of sterile reproductive structures might not be desirable if, for example, the plantation provides important habitat for birds or beneficial insects that feed on flower parts.

REPRODUCTIVE GENE MOLECULAR BIOLOGY AND GENOMICS IN TREES

Analysis of floral gene homologs

Most published studies of genes controlling flowering in trees have described the isolation and gene expression patterns of homologs of genes known to control various stages of flowering in *Arabidopsis* (e.g. Southerton *et al.*, 1998; Sheppard *et al.*, 2000; Cseke, Zheng and Podila, 2003). Results from heterologous overexpression in *Arabidopsis* and tobacco have also been reported, and these studies have usually shown a phenotype similar to that induced by overexpression of the *Arabidopsis* homolog (e.g. Kyozuka *et al.*, 1997; Rutledge *et al.*, 1998; Elo *et al.*, 2001). Functional gene studies of flowering in trees are rare because of the lack of sufficiently efficient transformation systems to produce multiple-event transgenic populations for large numbers of target genes. In addition, the multiple-year non-flowering phase of trees requires long and costly time spans and large areas for field research. *LFY* and *AP1* and tree orthologs of *FT*, which accelerate flowering when overexpressed in *Arabidopsis*, have been shown to induce early flowering in poplar and/or citrus, potentially bypassing the long time delays to flowering (Weigel and Nilsson, 1995; Rottmann *et al.*, 2000; Pena *et al.*, 2001; Endo *et al.*, 2005; Böhlenius *et al.*, 2006; Hsu *et al.*, 2006). In some cases, however, the inflorescences have been abnormal or gametes inviable (Rottmann *et al.*, 2000; Hsu *et al.*, 2006); induction of at least some *FT* homologs may bypass this problem (Böhlenius *et al.*, 2006).

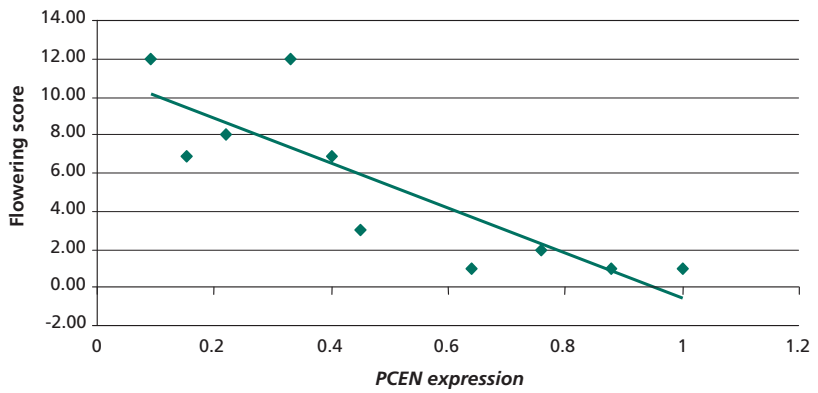
Both overexpression and antisense constructs of the silver birch genes, BpMADS1 and BpMADS6, homologs of *SEPALLATA3* and *AG*, were transformed into an early flowering birch genotype (Lemmetyinen *et al.*, 2004). Although mutant phenotypes were somewhat inconsistent or rare, suppression of BpMADS1 appeared to cause some inflorescences to partially revert to vegetative shoots, and in two BpMADS6 transgenics, some male inflorescences lacked stamens, suggesting functions similar to their *Arabidopsis* counterparts. In *PTLF* antisense poplar transgenics that flowered after several years in the field, some male transgenic events produced mutant flowers with homeotic conversion similar to *lfy* mutants (data not shown). Phenotypes were consistent between catkins from a single transgenic event, but catkins typically displayed a basal to tip gradient with flowers at the tip having a more severe mutant phenotype; thus, basal flowers often produced stamens that were wild-type in appearance. However, in the transgenic event with the most severe mutant phenotype, few flowers with stamens were observed. RNAi transgenes have been reported to be more efficient at inducing suppression than antisense constructs (Wesley *et al.*, 2001), suggesting that RNAi versions of *PTLF* now entering field trials (data not shown) might give a higher rate of sterility both within and between events.

Encouraging results were found with RNAi studies of *PCENL1*, a poplar homolog of the *Arabidopsis* floral repressor, *TERMINALFLOWER 1*. Transgenic events that showed strong reduction in target endogene expression as determined by qRT-PCR initiated flowering earlier than wild-type in the field (Mohamed, 2006); the extent of precocious flowering was significantly correlated with the

level of endogene suppression (Figure 3-5). These studies suggest that RNAi suppression of orthologs of *Arabidopsis* genes that promote flowering, and do not appear to have any role in vegetative development, can be an effective method for introducing biosafety traits. They also suggest that transgenic events will need to be carefully screened to select lines exhibiting strong suppression. Where vegetative tissue expression is detectable, it should be possible to screen for desirable events during seedling growth, saving years of study and reducing the costs and issues of screening large numbers of field-grown trees.

The extent of overlap in genes and pathways regulating reproductive development in angiosperms and gymnosperms is poorly known. Most studies have focused on MADS-box genes. For example, studies have identified *Picea*, *Ginkgo*, *Gnetum* and *Cycas* genes belonging to the *AG* subfamily (Rutledge *et al.*, 1998; Shindo *et al.*, 1999; Jager *et al.*, 2003; Zhang *et al.*, 2004). The expression patterns of the gymnosperm *AG* homologs and phenotypes induced by heterologous ectopic expression or complementation of an *Arabidopsis ag* mutant support a conserved function in controlling reproductive organ development. Conifer homologs of the MADS-box B-class floral organ identity genes, the flowering time gene, *SOC1*, and *LEAFY* have also been identified (Tandre *et al.*, 1995; Sundstrom *et al.*, 1999; Mellerowicz *et al.*, 1998; Mouradov *et al.*, 1998). The Norway spruce gene *DAL10* belongs to a MADS-box subgroup that is possibly gymnosperm-specific

FIGURE 3-5
Association of expression level of native *PCENL1* transcripts and flowering of field-grown *PCENL1* RNAi transgenic trees of poplar clone 717-1B4 (*P. tremula* × *alba*)



Expression was measured by qRT-PCR as described in Figure 3-4. Pools of RNA from two ramets per event were used for each assay. Final flower score was estimated as the number of flowering ramets per event × mean number of flowers for each event, rated using a scoring system for each tree (mean for an event) of 0 = no flowers, 1 =1 to 11 flowers, 2 = 11 to 30 flowers, and 3 = >30 flowers. Only those transgenic events that showed evidence of gene suppression (estimated expression below that of non-transgenic control) were included ($r^2 = 0.71$, $P < 0.01$).

and is specifically expressed in pollen and seed cones (Carlsbecker *et al.*, 2003). Another spruce MADS-box gene, *DAL1*, belongs to the *AGL6* subfamily and its expression correlates with maturation to the adult or flowering phase (Carlsbecker *et al.*, 2004).

Forward-looking genomics approaches

Although comparative studies indicate that similar genes and pathways control reproductive development in angiosperms and to an extent in gymnosperms, taxon-specific gene duplications and losses, and subsequent subfunctionalization and neofunctionalization, make predictions of gene function based solely on orthology or expression patterns problematic (Irish and Litt, 2005). The poplar genome sequence and an increasing number of large expressed sequence tags (EST) datasets for various tree taxa greatly facilitates identification of tree homologs to various *Arabidopsis* genes regulating flowering and their lineage-specific gene duplications and losses (Brunner and Nilsson, 2004). Moreover, the Floral Genome Project (www.floralgenome.org) (Albert *et al.*, 2005) and other projects (e.g. Brenner *et al.*, 2005) have developed extensive floral EST datasets from diverse plants including phylogenetically important eudicots, non-grass monocots, basal angiosperms and gymnosperms. Although many of the floral EST sets are not from trees, comparative floral genomics studies are still informative because tree taxa occur in almost all eudicot orders (Groover, 2005). These extensive sequence resources are beginning to reveal patterns of conservation and divergence of families of floral regulatory genes (e.g. Zahn *et al.*, 2006).

Genomic platforms for analysing gene networks controlling flowering in trees will enable selection of genes and design of sterility strategies with greater precision and effectiveness. Global expression analyses of *Arabidopsis* development, responses to floral induction stimuli and spatial patterns in flowers of *Arabidopsis* mutants, have revealed tissue-predominant expression patterns and components of gene networks controlling floral initiation and floral organ development (Schmid *et al.*, 2003, 2005; Wellmer *et al.*, 2004). Bio-informatic analyses of co-expressed genes, chromatin immunoprecipitation studies and comparison of regulatory regions of orthologous genes can identify cis-regulatory elements associated with a particular response or process (e.g. Li, Zhong and Wong, 2005; Kreiman, 2004; Rombauts *et al.*, 2003). Yeast two-hybrid screens were used to develop a comprehensive interaction map of all *Arabidopsis* MADS domain proteins (de Folter *et al.*, 2005). Combined with global expression analysis, protein interaction studies would be especially useful for selecting genes and sterility methods unlikely to have pleiotropic effects. Similar strategies are beginning to be applied to poplar, and a new United States of America National Science Foundation Plant Genome Project is studying the transition to flowering in poplar. This includes use of overexpression and RNAi poplar transgenics for transcriptome analyses.

Comprehensive study of gene expression is more difficult in trees than annuals due to complex developmental phase changes and increasing size and tissue complexity across years. We have observed that some genes showing floral-

predominant expression in poplar show levels of vegetative expression that vary in intensity across an annual cycle of growth and dormancy (data not shown). Furthermore, trees are exposed to very variable abiotic and biotic conditions over many years that can markedly affect gene expression. For example, galling insects appear to induce ectopic organ developmental programmes that are similar to reproductive development; *LEAFY*, *API* and C-class MADS-box genes directing carpel development, but not B-class genes, are expressed during development of galls on grape vine leaves (J.C. Shultz, personal communication). This is especially problematic for ablation sterility systems where selection criteria for appropriate promoters are most stringent.

In addition to not having complete genome sequences, studies in most tree taxa are generally limited by lack of efficient transformation systems. Development of VIGS vectors for trees could be particularly valuable for studying genes controlling flowering. A VIGS vector has recently been developed for poplar (Naylor *et al.*, 2005), but unfortunately a poplar genotype that reliably flowers in the greenhouse in the absence of *FT* overexpression is not currently available. Some other tree species, such as eucalypts and apple, can be reliably induced to flower via use of plant hormones and cultural treatments.

As tree genomics tools and knowledge of candidate genes for flowering advance, it should be possible to clone genes that control onset of flowering using high-resolution quantitative trait locus (QTL) or association genetics approaches. This approach potentially allows discovery of mechanisms of reproductive development that are unique to trees, rather than relying on studies of herbaceous annual model plants for target gene identification. Liebhard *et al.* (2003) reported QTLs for juvenile phase in apple. Missiaggia, Piacezzi and Grattapaglia (2005) identified a QTL for very early flowering in eucalypts. For these studies, it will be essential to have large populations ready that include segregants with rare precocious flowering. To prevent flowering, these genes could then be suppressed or mutated, as discussed above.

STABILITY OF TRANSGENE EXPRESSION

It is well known that newly produced transgenic plants often exhibit instability in expression of transgenes, related endogenes and their encoded traits. It is also widely known that the level of instability varies widely among constructs, species and gene transfer methods. However, after field screening, gene insertion events with strong and stable expression are generally identified, and these are the ones focused upon during research and commercial development. The ability to identify highly stable transgenic events has been firmly established by the hundreds of millions of hectares of genetically engineered crops that have been grown by farmers, which contain a variety of genetic constructs in a variety of genotypes and species. These include commercialized trees (papaya, poplar), with traits induced via RNAi (papaya, tomato, squash) and with conventional transgene expression.

Questions remain, however, about the long-term stability of specific traits in vegetatively propagated crops, including containment traits and to what extent

stable expression can be identified and delivered in an efficient manner in breeding programmes with transgenics. It is also unclear how strong and stable a sterility phenotype must be to confer an adequate level of containment. A high level of stability of a leaf-expressed gene for herbicide resistance, imparted by genes derived from other species, does not guarantee that a native gene designed to suppress a floral meristem identity gene via RNAi will be sufficiently reliable for stringent, long-term containment goals. Because of the importance of stability of gene expression for genetic containment in trees, we review both what has been learned from studies in other vegetatively propagated crops, and then in the following section consider how a modelling approach can help to identify how much trait instability (i.e. reversion to fertility) might be biologically acceptable.

Due to the long life cycles of forest trees and the complex environments they experience, stability of expression of genetically engineered-introduced traits in trees has received considerable debate (Fladung, 1999; Hoenicka and Fladung, 2006a). In addition, possible genome instability due to effects of the gene transfer process and interaction with plant genome sequences adds to scientific uncertainties about long-term performance of primary transformants in the field. In an AFLP study with four *Agrobacterium*-transformed aspen transgenic lines carrying a *rolC* gene, 886 out of 889 (99.9%) of the amplified bands were common between the control and transgenics, suggesting very limited genetic engineering-associated genomic change compared with extensive wild AFLP polymorphism in poplar and most other tree species (reviewed in Hoenicka and Fladung, 2006b). In agronomic crops, it also appears that genomic variation imparted by transformation is modest compared to the extensive genomic variation present in traditionally bred and wild plants (Bradford *et al.*, 2005).

A number of factors have been implicated in transgene silencing, including insert number, chromosomal environment (position effect), T-DNA structure, environmental stress and endogenous factors (Table 3-3). Unfortunately, most of these factors do not seem to be consistent predictors of long-term stability. For example, there appears to be little association between insert number and instability, even though single-copy transgenes are widely assumed to be important for obtaining stable gene expression. Where transgene structure was studied, however, instability was often associated with transgene repeat structure, truncation, or other re-arrangements at or near transgene insertion sites (Table 3-3).

Transgene stability under vegetative propagation has been studied in poplar, citrus, tall fescue, sugar cane, chrysanthemum and potato. Transgene expression appears far less stable over sexually propagated generations than over vegetatively propagated generations (Table 3-3). Unfortunately, most studies have used a small number of transgenic events (<20), and are thus of limited relevance to commercial transformation and breeding programmes, which often screen many dozens or hundreds of events. Moreover, many of the published studies on stability of transgene expression have focused on unstable events observed in preliminary screens, and are thus biased with respect to the levels of instability expected in commercial programmes.

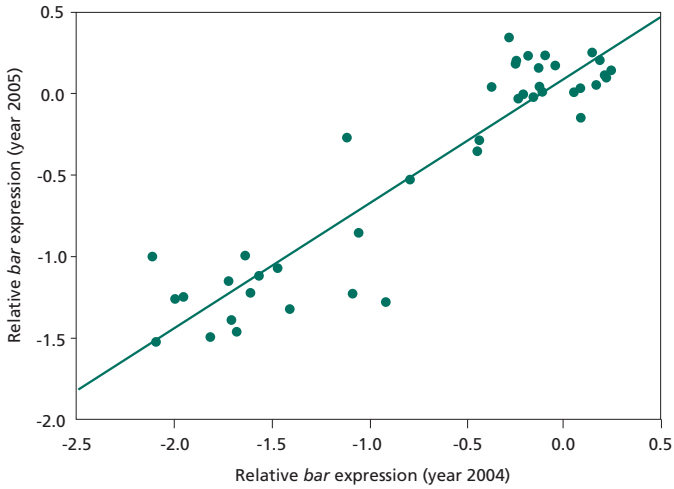
In a study similar to what a tree breeding programme might address, Meilan *et al.* (2002) reported high stability of herbicide resistance genes in 40 independent poplar transgenic events over four years in the field. Hawkins *et al.* (2003) reported stable expression of a GUS reporter gene in 44 independent poplar transgenic events over a period of six years under *in vitro*, greenhouse and field conditions. Histological GUS analysis in 70 transgenic events showed similar patterns of GUS expression over a period of four to five years in citrus (Cervera *et al.*, 2000). In contrast, in a study of 22 transgenic events carrying the morphological marker gene, *rolC*, phenotypic alteration or reversion was observed for up to one-third of the events during vegetative growth in either *in vitro*, greenhouse or field conditions (Kumar and Fladung, 2001). In biolistically transformed pine, Wagner *et al.* (2005) reported that the level of silencing of a cinnamyl alcohol dehydrogenase (CAD) gene during embryogenic propagation was associated with expression level.

Variation in stability of transgene expression among studies can result from uncontrolled differences in experimental protocols, as demonstrated by James *et al.* (2004). Because native and introduced genes show stochastic (Raser and O'Shea, 2004) and developmental variation in expression, it is important to pick a suitable control gene. For example, the strong and deleterious effects of variable expression of the *rolC* gene discussed above might be similar to the normal variation that occurs with many endogenes and transgenes, but its gene product is so powerful and toxic that its effect on development is amplified. In contrast, no such consequence, nor possibly any phenotypic effect at all, would be expected for similar levels of variation in a transgene encoding insect or herbicide resistance.

We have performed three stability studies using different transgene constructs (unpublished data). In one study, the *BAR* herbicide resistance gene was transferred into two poplar clones, and 32 transgenic events produced. The expression of the *BAR* gene was monitored on 384 plants over a period of eight years of repeated coppicing in the field. No instability or loss of the initial resistance phenotype was observed based on visualized herbicide damage and protein enzyme-linked immunosorbent assays (ELISA). In another study, the reporter genes *GFP* and *BAR* were assembled in the same binary vector, and transferred into two poplar clones. The expression levels were measured on 2 256 transgenic poplar trees generated from 404 independent transgenic events over three years in the greenhouse and the field. The expression of both genes was highly stable over three years, with no cases of gene silencing observed. However, the physical loss of transgene sequences was observed in three of the 80 transgenic events after they were regenerated via a second round of organogenesis in tissue culture.

In a third study, we examined the stability of RNAi silencing of a resident *BAR* gene in transgenic poplars that had been re-transformed with inverted repeats (IR) of either a section of the coding sequence or the promoter sequence of the *BAR* gene. RNAi silencing efficiency and stability were studied in 56 RNAi transgenic events over two years in the field. The results suggested that dsRNA of the *BAR* coding sequence was highly efficient in suppressing *BAR* expression; 80% of

FIGURE 3-6
Correlation of RNAi suppression in shoots of field-grown trees between year 2004 and year 2005



Expression of the targeted bar transgene for 42 gene insertion events were quantified with real-time RT-PCR (ubiquitin gene used as internal control) and then expressed relative to that of the parent transgenic genotypes and log transformed ($r^2 = 0.47$, $P < 0.001$)

the events showed more than 90% gene suppression. However, dsRNA of the *BAR* promoter sequence was much less efficient; only 6% of the events showed more than 90% suppression. Most importantly for gene containment, the degree of RNAi suppression appeared to be stable for both constructs over two years (Figure 3-6). These studies, plus the reporter gene studies described above, suggest that instability of gene expression may only rarely be a problem in vegetatively propagated trees, though longer-term studies are desirable.

STERILITY AS A QUANTITATIVE TRAIT: HOW MUCH DO WE NEED?

Complete prevention of sexual reproduction with 100% certainty is a daunting technical and social challenge. The long time frames and large numbers of potential reproductive meristems in transgenic tree plantations provide many opportunities for reversion to fertility, such that rare events become probable. Furthermore, transgenic approaches to sterility will incur added economic and regulatory costs and social resistance (discussed above). It is therefore critical to define if sterility is needed at all for biological or social reasons, and if so, what level and form is required. However, there does not seem to have been any serious field studies, in any crop, sufficient to estimate the operational effectiveness of containment genes (Ellstrand, 2003). Until many such studies are published, it would be unwise to assume that genes can be fully and safely contained in the near future. Conventional approaches to fertility reduction, including the use of hybrids or aneuploid germplasm (Bradshaw and Stettler, 1993), also generally do not provide complete containment. However, they could provide an option for deployment of some

transgenes in breeding programmes that use ploidy-modified trees. However, such genotypes are rare in most forest tree breeding programmes. Poplar and some other tree species are capable of dispersal and establishment of vegetative propagules, thereby potentially bypassing most containment measures based on sexual sterility. Though local spread from plantings can usually be managed, some degree of long-distance vegetative spread can occur through adventitious rooting from broken or abscised branches (Rood *et al.*, 2003). If transgene containment is an important goal, it is important to explore the consequences of all of the different modes and levels of reproduction under realistic ecological scenarios. This is best addressed in the context of a risk assessment and is facilitated by the use of ecological modelling.

Risk assessment includes hazard identification, exposure assessment, consequence assessment, risk characterization and delineation of mitigation options (Hill, 2005). Risk from transgene dispersal is sometimes treated as synonymous with the exposure portion of the process, and demonstrations of potential distributions of transgenic propagules are treated as examples of the inherent risks of forest biotechnology (e.g. Williams, 2005). However, the mere presence of transgenic propagules does not automatically constitute a negative endpoint (Stewart, Halfhill and Warwick, 2003). Production and dispersal of transgenic seed and pollen constitute the first steps in a network of processes contributing to introgression of transgenes to wild populations. Even with the extensive dispersal distances expected for forest trees (Nathan *et al.*, 2002), realized transgene introgression could still be extremely low due to sexual incompatibility with wild trees, lack of availability of safe sites for establishment, negative fitness effects of transgenes or domestication genes in a wild setting, and extensive dilution from non-transgenic planted and wild stands (Pilson and Prendeville, 2004; Hails and Morley, 2005). As discussed above, transgene dispersal could also have large net ecological benefits.

Trees create special challenges for generating the data necessary for assessing potential introgression. Very large temporal and spatial scales must be considered for movement of tree pollen and seeds (Nathan *et al.*, 2002; Smouse and Sork, 2004). Furthermore, long-distance gene flow is a disproportionately important determinant of rates of spread of introduced organisms or genes (Higgins and Richardson, 1999), and this process is subject to stochastic influences that make accurate measurement extremely challenging, if not impossible (Clark *et al.*, 2003). This difficulty is magnified when one considers the network of interacting, highly variable factors that determine establishment and spread in wild systems (Parker and Kareiva, 1996; Pilson and Prendeville, 2004). Therefore, realistic, replicated experiments cannot be performed at appropriate scales and time frames for predicting introgression of transgenes (Parker and Kareiva, 1996). However, data from non-transgenic populations can be used in simulations to provide useful estimates of what is likely to occur under various deployment situations and environments (Dunning *et al.*, 1995; Pilson and Prendeville, 2004).

Simulation approaches have been used successfully to investigate factors affecting the spread of transgenic insect-resistant oilseed rape varieties (Kelly *et al.*, 2005)

and to investigate factors affecting fitness of transgenic fish with enhanced growth (Howard, DeWoody and Muir, 2004). However, many of these kinds of studies have not taken into account realistic spatial distributions of transgenic organisms on the landscape relative to wild and managed habitats. The spatial dimensions of gene flow are an essential component of introgression because habitat availability and competition from wild relatives are likely to be two of the primary factors inhibiting spread of partially fertile transgenic trees, and these will be determined by management regimes and locations of wild populations on the landscape.

Many different types of models have been used for simulating dispersal and gene flow across a landscape (Nathan *et al.*, 2003). One approach is to devise mechanistic models of pollen and seed dispersal based on the physical properties of the propagules and the environment (Katul *et al.*, 2005; Nathan *et al.*, 2002; Clark *et al.*, 2003). Such models have a distinct advantage in that they are easily parameterized for a large number of species because flight characteristics of pollen and seeds are readily measured, detailed microclimatic data can be obtained for many sites, and the physics of dispersal by abiotic agents are fairly well characterized. Disadvantages include the large number of parameters that require estimation (particularly if realized gene flow is to be modelled) and the high computational requirements that limit the extent of the area and time frame that can be modelled (Nathan *et al.*, 2002).

An alternative approach is to model gene flow phenomenologically based on field observations of dispersal and demographic processes. A common method is to use reaction-diffusion models to depict the movement of an 'invasion front' using a diffusion approximation and logistic growth models (Fisher, 1937; Shigesada and Kawasaki, 1997). Alternatively, probability density functions of propagule movement and/or reproductive success can be used to determine the probability of dispersal between points on a lattice of habitat cells (Higgins and Cain, 2002; Lavorel, Smith and Reid, 1999). This approach has the advantage of being easily parameterized from historical data (e.g. a chronosequence of air photos or survey data) and readily integrated with geographical information systems (GIS). A major disadvantage is the difficulty of measuring contemporaneous realized gene flow on appropriate space and time scales to parameterize the models.

As an example of the latter approach, we developed a spatially explicit model of gene flow from hybrid poplar plantations based on observations of realized gene flow in wild populations (DiFazio, 2002; Slavov, DiFazio and Strauss, 2004). The model, called Simulation of Transgene Effects in a Variable Environment (STEVE), was applied to a landscape grid in northwest Oregon (23 km × 37 km, 100 m^2 cells) containing information about elevation, habitat type and poplar populations. The simulation has an annual time step, with modules to simulate creation and conversion of poplar patches, growth, reproduction, dispersal and competition within poplar cohorts. The primary objective of this model was to produce a framework for virtual experiments that could accommodate the diverse silvicultural, agronomic and ecological settings in which transgenic trees might be released, and to incorporate many different types of transgenic traits.

The findings of the STEVE model most germane to discussions of reproductive sterility come from simulations with different levels of innate fertility of transgenics and with various probabilities of reversion to fertility. Relative pollen production was calculated for each genotype within each sexually mature cohort of trees in each poplar cell. Representation of pollen and seed was entirely relative because the most important quantity is the ratio of transgenic to conventional genotypes in the propagule pools. Therefore, pollen production was directly proportional to the basal area of each genotype in a particular location on the landscape.

Relative fertility varied annually based on a user-defined standard deviation determined from annual field observations of flowering in plantations. In addition, transgenics with reduced fertility could have their fertility partially restored according to a user-defined probability. Vegetative propagule production was also stochastic and proportional to basal area. Pollen was dispersed within the immediate vicinity of male trees and across the landscape according to empirically determined dispersal kernels (Slavov, DiFazio and Strauss, 2004), and transgenic and conventional seed production was determined by the proportion of pollen of each genotype dispersed to female trees, modified by relative fertility factors.

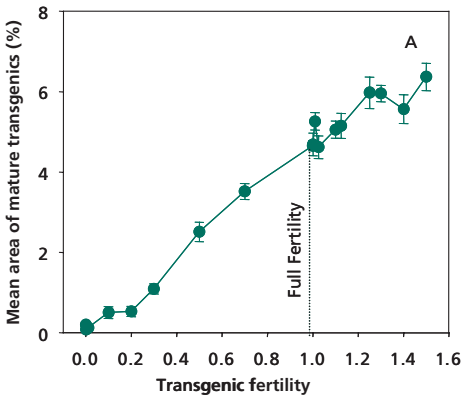
As expected, fertility of transgenic trees had a strong effect on rate of gene flow from transgenic plantations. With highly reduced fertility, gene flow was at some of the lowest levels observed for all scenarios tested: between 0.1 and 0.2%, compared with approximately 5% for fully fertile transgenic plantations. In addition, transgene flow rates were not distinguishable within the range of 0 to 1% of wild fertility, indicating that complete sterility was not required to attain maximum gene containment (Figure 3-7a). Thus, the reductions in fertility of approximately 10^5 that we have observed in the field (Figure 3-1) would appear to be far in excess of the level needed for effective mitigation in this scenario. (In practice, only the pollenless events might be chosen for commercial purposes.) The low level of gene flow that we observed for fully sterile plantations was due to movement of vegetative propagules in the vicinity of plantations. However, transgenic gene flow remained very low under a wide range of rates of vegetative establishment (Figure 3-7b), and gene flow rates were insensitive to changes in rates of vegetative establishment and shapes of vegetative dispersal curves (data not shown). Sexual fertility was therefore much more important than vegetative establishment in controlling gene flow in this system. Nearly 50% of the gene flow with low-fertility transgenics (fertility <0.1) was due to sexual reproduction, as demonstrated by simulations with vegetative establishment eliminated (Figure 3-7b).

Other investigations have also identified fertility as a major factor limiting plant spread. For example, a reduction of fertility of as little as 75% was projected to limit the spread of scotch broom (*Cytisus scoparius* L.), based on insect-protection assays and simulations (Rees and Paynter, 1997). Density of pines spreading from plantations in South Africa was sensitive to fecundity and age of reproductive maturity in spatially explicit simulations (Higgins, Richardson and Cowling, 1996). Spread of feral oilseed rape was hypothesized to be limited by seed input

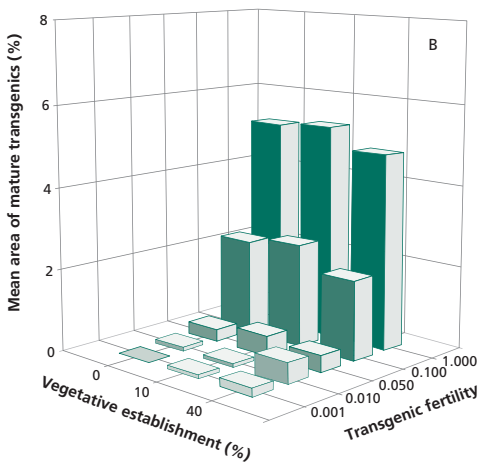
FIGURE 3-7

Simulated effects of transgenic fertility on transgene flow based on the STEVE model

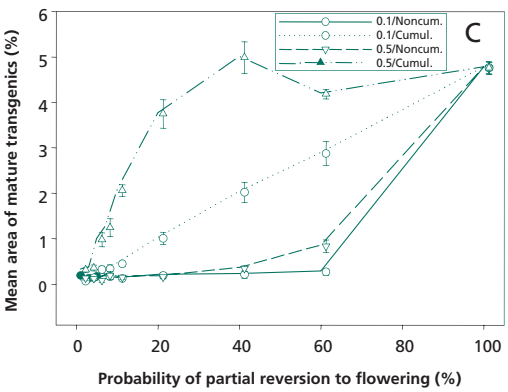
Simulations were conducted over a 50-year time period, and gene flow was indexed by the proportion of 100 m² *Populus cohorts* greater than ten years of age that contained at least one transgenic tree outside of plantations (Mean Area of Mature Transgenics). Responses were averaged over the final 25 years of the simulation to simplify presentation of results (responses stabilized by age 25 for the simulations shown).



(a) Effects of fertility of transgenic trees relative to non-transgenics.



(b) Interaction between vegetative establishment and fertility. Vegetative establishment is the proportion of established individuals in a new cohort that are derived from vegetative propagules. Variation in vegetative establishment had little overall effect on transgene flow, although a minor effect is apparent at low levels of fertility.



(c) Effects of unstable sterility on transgene flow. Probability of sterility breakdown is the probability of a reversion to fertility (x-axis), which is then restored with a fertility level of 0.1 or 0.5, sampled from a normal distribution with a standard deviation of 0.05 or 0.25, respectively. Reversion was permanent and cumulative (Cumul.) for each tree through time, or fertility was transient and reset to the original value each succeeding year of the simulation (Noncum.). Low values of instability had little effect on gene flow; a cumulative reversion rate of about 20%, with 50% fertility restoration, would be required for gene flow levels to approach those of fully fertile transgenic trees.

Source: DiFazio, 2002.

based on patterns of establishment along shipping (i.e. dispersal) routes (Crawley and Brown, 1995), and simulation modelling implicated seed viability as a major factor limiting spread of transgenic oilseed rape (Kelly *et al.*, 2005). Therefore, the effectiveness of partial sterility in attenuating gene flow is not surprising, but the model is useful in demonstrating the importance of different modes of reproduction (vegetative vs various degrees of sexual reproduction).

The model was also useful for exploring implications of unstable sterility. We simulated this by allowing some restoration of fertility for trees that began the simulations with highly reduced fertility (fertility level of 0.01 compared to wild-type trees) (Figure 3-7c). These simulations had three important parameters: the probability of reversion to fertility (sampled from a normal distribution), the level of fertility restoration for each reversion event (10 or 50%, sampled from a normal distribution), and the duration of the restoration (cumulative or permanent restoration vs non-cumulative or transient restoration, with reversion to the original fertility level each year). With a permanent restoration level of 50% per reversion event, a 20% probability of reversion was required for gene flow levels to approach those of fully fertile trees. With a permanent restoration level of 10%, gene flow was considerably less than full fertility, and this was true even with reversion rates as high as 60%. Gene flow with reversion rates up to 3% were nearly indistinguishable from that of trees with stable sterility. If reversion was not cumulative (i.e. fertility was reset to 0.01 each year for each tree), gene flow was still greatly reduced compared to wild trees and was marginally greater than for trees with stable sterility. These results were manifested across a broad range of probabilities of reversion. Reversion rates that we have observed under vegetative propagation for transgenic *Populus* (reported above) appear to be considerably below the rates required for significant effects on modelled transgene flow. In addition, such high rates of reversion would likely be detected with moderate pre-commercial screening and post-release monitoring efforts. The simulations discussed above dealt with sterility in relation to spread of neutral transgenes. Transgenes that enhanced the competitiveness of trees in wild settings caused greatly enhanced gene flow for fully fertile transgenic trees, but a tightly linked sterility gene was very effective at attenuating spread, even in the face of a strong selective advantage and incomplete sterility (DiFazio, 2002).

CONCLUSIONS

"In theory, there is no difference between theory and practice. In practice, there is."

Andrew S. Tannenbaum, TIGR (The Institute for Genomic Research)

There are many genes of interest for commercial purposes that are likely to present very low risks, either because they are very similar to native genes, because they will reduce fitness or be neutral in the wild, or because their benefits outweigh their detriments. At the same time, there may be crops, such as forms of bio-industrial crops that encode novel and potentially ecotoxic compounds, for which very strong biological containment would be clearly warranted. Nonetheless,

the loudest social resistance seems to focus not on the products, traits and their benefits vs risks, but on perception of 'contamination' by GMOs generally. Indeed, because of the long-known propensity for long distance movement of pollen and/or seed from most tree species, if complete containment is the social goal, there is unlikely to be any place for genetically engineered trees in forestry plantation or horticulture – at least not for many decades. The technologies and simulations presented assume that some level of transgene dispersal could be socially and biologically acceptable – much like dispersal of new or modified genes and chromosomes introduced by breeding continues to have high social acceptance.

It has often been said that plant sterility should be an easy trait to engineer; after all, there are dozens of ways to damage a motor so it does not work. Unfortunately, motors do not have the redundancy and resilience of biological systems that have evolved to reproduce 'at all costs', nor do vandalism-leaning auto mechanics face the large biological and social obstacles that researchers and companies do when trying to conduct field-relevant research with genetically engineered trees. To arrive at efficient, reliable, effective sterility systems, we make the following suggestions:

1. *Functional genomics in trees*. Much more basic functional genomics is required in model taxa that represent the major forestry species. In this research, the main candidate genes based on studies in *Arabidopsis* and other model plant species, combined with newly discovered genes from trees identified in QTL, EST or microarray studies of trees, would be repressed or over-expressed and their functions identified in the field or the greenhouse, hopefully under conditions of accelerated flowering. This should allow the most important genes and promoters to be identified, thus, informing efforts to combine genes in redundant, reliable systems. It is hoped that inducible systems that make use of the *FT* gene might provide the much needed acceleration in production of normal flowers (Böhlenius *et al.*, 2006).
2. *Transformation technology improvements*. Gene transfer, gene targeting and highly specific recombinase technology needs to be greatly improved if mutagenesis of floral genes, and efficient addition or removal of sterility genes in many genotypes, is to become feasible. This requires much basic research on innovative transformation, excision, and homologous recombination methods – first in model plant species; but then, considerable work will be required to transfer these systems to trees.
3. *Regulatory and intellectual property constraints.* Candidate sterility cassettes based on the results of suggestions 1 and 2 need to be designed to meet regulatory standards and have freedom to operate with respect to intellectual property. They must then be tested in a diversity of commercially relevant environments and genotypes for stability and pleiotropic effects. These should be combined with predictive assays where possible to enable their effectiveness and pleiotropy to be forecast from a young age. The current 'anti-commons' (Boettiger and Bennett, 2006), where the licences for each genetic and construct

element, and basic transformation technology, are owned by parties different from those bearing the costs and risks of this long-term research, appear to provide large disincentives to moving forward. High regulatory and licensing costs and market stigmas impede the 'adaptive management' approaches so common in forestry (where research and commercial development go hand-in-hand, a result of the high costs and long time frames for forestry research).

4. *Transparency*. Containment research, due to its cost, long time frame and high level of scrutiny from society, should ideally be conducted by non-commercial third parties. A similar model is applied for all environmental research by Weyerhaeuser Company because of the need for independent validation of results for social acceptance (P. Farnum, personal communication). It is doubtful that company-based research, where only selected results are presented to the public, will be trusted, yet this model continues to be followed by some biotechnology companies. Ironically, the "eco"-vandalism that is still common in Europe, and continues to be a concern in North America, limits the extent to which the details of field and laboratory research can be safely disclosed. It appears that both vandalism risks to companies and Forest Stewardship Council exclusion of genetically engineered trees from field trials – both motivated by ecological concerns over appropriate uses of forest biotechnology – are delaying, rather than promoting, the development of ecologically sound genetic engineering technologies.

Because of the rapid rate of growth of genetic information and technological innovations, we believe that highly efficient containment systems can be developed and their reliability established. Without such systems, which will require testing over many years, it appears that many kinds of transgenes may never obtain regulatory or social approval in many countries, greatly limiting the benefits that transgenic biotechnologies are likely to be capable of providing.

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4. Engineering trees with target traits

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Despite their unparalleled importance both ecologically and economically, very little is known about the molecular mechanisms that underpin the development, growth and health of forest trees. However, the past decade has yielded remarkable progress in elucidating the biochemical and genetic mechanisms controlling the growth and survival of annual plants. An understanding of these processes will inform efforts aimed at ensuring the long-term maintenance and sustainability of global forests. Much of this progress has been made through the application of what is collectively known as functional genomics.

Functional genomics entails the analysis of an organism's genetic material (the genome), and relates this to its form and function. Genomic analysis of a tree or model plant may identify gene(s), or in some cases spontaneous or induced mutations, which offer opportunities for directed modification of the corresponding trait(s). Using new forward genomics techniques (e.g. activation tagging and gene/enhancer traps), it is now possible to induce useful 'mutations'. The resulting phenotype may reflect the underlying gene's function and may point to further, desirable alterations. Newly available, high-throughput screening techniques may also identify useful, but cryptic, mutations that are not normally manifested as an obvious phenotype (Davis *et al.*, 2006; Ehlting *et al.*, 2005; Kelley *et al.*, 2004; Kent *et al.*, 2006; Labbe *et al.*, 2005; Tsuchikawa, 2007; Tuskan *et al.*, 1999; Wiklund *et al.*, 2005). Ultimately, functionality must be demonstrated by either silencing or overexpressing the putative gene.

Along with the great progress with the model plant *Arabidopsis thaliana*, recent publication of the first genomic sequence for a tree (*Populus trichocarpa*; Tuskan *et al.*, 2006), is revolutionizing our understanding of tree biology and permitting the exploitation of several powerful biotechnological techniques that can aid in the domestication of other tree species. The next decade should prove extremely exciting for tree researchers as they exploit the genomic sequence, and combine postgenomic technologies (e.g. cell biology, bio-informatics, transcriptome and proteome analyses, and metabolic profiling) to unravel many of the mysteries surrounding genotype-phenotype relationships in trees. Consideration of costs and benefits, including unintended effects, will influence the ultimate choice of a target trait. This is especially true when comparing directed target trait modification to alternative approaches (e.g. conventional breeding or cultural treatments).

BIOTIC STRESS

Damage to forest trees caused by both native and introduced pests is of global importance. These biotic stresses significantly affect forest growth and productivity, with substantial economic consequences. For example, in China in 1989, damage to hybrid poplar plantations by common defoliators such as the poplar lopper (*Apochemia cinerarua*) and the gypsy moth (*Lymantria dispar*) resulted in substantial (40%) stand loss (Hu *et al.*, 2001). Similarly, coniferous trees such as loblolly pine (*Pinus taeda*) are often damaged by the insect pests *Dendrolimus punctatus* and *Crypyothelea formosicola* (Tang and Tian, 2003), while white spruce is often negatively affected by defoliating insects such as the spruce budworm (*Choristoneura fumiferana*; Lachance *et al.*, 2007). In addition to defoliating insects, there are fungal, bacterial and viral pathogens that can affect forest health and productivity (Table 4-1). The following sections highlight the results of genetic modifications aimed at improving tree defences against damaging pests.

TABLE 4-1
Summary of genetic modifications in trees, targeting resistance to various pathogens

Gene	Modification	Effects	Reference
Bt *Cry3Aa*	Expressed in *E. coli*	Using *E. coli* as a preliminary system to test the effectiveness of a variety of Bt toxin, the protein caused high mortality in long-horned beetle larvae	Chen *et al.*, 2005
Bt *Cry1A*	Expressed in *Picea glauca* embryogenic callus	Low toxicity to spruce budworm when fed embryonic callus tissue	Ellis *et al.*, 1993
Synthetic Bt *Cry3Aa*	Expressed in hybrid poplar, *P. tremula* × *tremuloides*	Transgenic leaves toxic when fed to *Chrysomela tremulae* beetle	Genissel *et al.*, 2003
Bt *Cry1Ac*	Expressed in *Pinus radiata*, ubiquitin promoter (pr)	Some lines had higher resistance to painted apple moth; mature needles were more toxic compared with young needles	Grace *et al.*, 2005
Bt *Cry1Aa*	Expressed in *P. alba* × *grandidentata*	Gypsy moth fed more on mature leaves among transgenics	Kleiner *et al.*, 2003
Bt *Cry1Ab*	Expressed in *P. glauca*, ubiquitin pr.	Embryogenic, young somatic tissue and needles from 5 year field trial trees were toxic to spruce budworm	Lachance *et al.*, 2007
Synthetic *Cry1Ac*	Expressed in *Pinus taeda*, 35S pr.	64 to 75% mortality of *D. punctatus* Walker and *C. formosicola* Staud larvae after 7 days of feeding	Tang and Tian, 2003
Various forms of Bt toxin	Expressed in *P. nigra*, 2x35S pr.	Low expression of toxin, but high mortality of various pests and reduced damage to leaves on transgenic plants	Wang *et al.*, 1996
Bt toxin	Expressed in *P. alba* × *grandidentata* and *P. nigra* × *trichocarpa*, 35S pr.	Leaf feeding assay: high mortality to forest tent caterpillar and gypsy moth	McCown *et al.*, 1991
Polyphenol oxidase (PPO)	Overexpressed in *P. tremula* × *alba*	Increased levels of PPO in leaves, but no effect on feeding caterpillars	Barbehenn *et al.*, 2007
Kunitz trypsin proteinase inhibitor	Expressed in *P. nigra*	Feeding assays: no change to *Lymantria dispar* and *Clostera anastomosis* larval mortality or pupal weight	Confalonieri *et al.*, 1998
Cysteine proteinase inhibitor (*Atcys*)	Overexpressed in *P. alba*	Higher mortality to *Chrysomela populi* beetle larvae when fed transgenic leaves	Delledonne *et al.*, 2001

Note: Bt = *Bacillus thuringiensis*

Transgenic trees expressing Bt toxins

Insect pests are a major problem for poplar plantation managers. The two main classes of poplar pests are chrysomelid beetles and lepidopteran caterpillars, both of which are susceptible to microbial pesticides derived from different strains of *Bacillus thuringiensis* (Bt). This bacterium synthesizes polypeptides that are activated within the gut of certain insects, causing lesions and eventually insect death (Knowles and Dow, 1993). The insecticidal proteins, collectively referred to as Bt toxins, have been used safely as microbial pesticides in numerous crops (Carozzi and Koziel, 1997) for many years, both exogenously and endogenously. These toxins are relatively selective insecticides that have very few non-target effects (James, 1997). Several Bt strains have been identified, each affecting a select group of insects that are usually closely related phylogenetically (Thompson, Schnepf and Feitelson, 1995). Genetically modifying trees to produce forms of Bt toxin offers an appealing alternative for establishing plantations that are resistant to damage from a broad range of pests (Table 4-1).

Trees expressing Bt transgenes may be preferable to use of spray applications for several reasons. First, vegetation, soil and water surrounding the crop are not exposed to spray drift. Susceptible, non-target, insects in areas adjacent to the transgenic crop would not be exposed, reducing the potential for development of Bt resistance. Second, spray applications quickly degrade, persisting on leaves for, at most, a few days (Thompson, Schnepf and Feitelson, 1995; James, Croft and Strauss, 1999). Genetically engineered trees, however, can produce the toxin continuously, thereby avoiding sensitivities to application timing and the costs associated with repeated applications. Finally, because transgenic trees produce the toxin within plant tissues, it is possible to affect insects residing in the plant, such as wood borers and leaf folders. For some of these pests, no insecticides are currently available that target the life stage(s) most responsible for damage.

McCown *et al.* (1991) were the first to report on poplars that were stably transformed with a Bt toxin gene. One transgenic line in particular showed high levels of resistance to two pests: the forest tent caterpillar and the gypsy moth, as observed through leaf feeding experiments. Ellis *et al.* (1993) then described the first stable transformation of a conifer, white spruce (*Picea glauca*), with the *Cry1A* gene (a gene coding for a form of Bt toxin). Spruce budworms fed a diet of the transgenic embryonic tissues showed few signs of toxicity. The lack of toxicity was attributed to low transgene expression in embryogenic tissues. More recently, transgenic Monterey pine (*Pinus radiata*) expressing a Bt toxin gene showed variable resistance to damage from painted apple moth larvae (*Teia anartoides*), depending on the age (maturity) of needles (Grace *et al.*, 2005). These studies emphasize the importance of transgene expression levels and tissue specificity.

One example of successful engineering of poplar trees to combat an insect pest is found in the cottonwood leaf beetle (CLB, *Chrysomela scripta* Fabricius), the primary insect pest in poplar plantations. The CLB is a multivoltine insect that has a wide distribution, which can culminate in outbreaks causing severe defoliation, particularly in young plantations (Hart *et al.*, 1996). James, Croft and Strauss

(1999) have demonstrated that a Cry3A Bt toxin is highly effective against the CLB. In this study, a binary vector containing a *Cry3A* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter was used to produce 51 insect-resistant lines in four genotypes of *Populus* sp. The transgenic trees were field-tested in eastern Washington State, United States of America. This trial relied on insect pressure from the surrounding, commercial stands to evaluate insect resistance. Trees were evaluated for damage, basal diameter and height at various stages during the growing season. Virtually all of the Bt transgenics showed very low feeding damage, whereas the non-transgenic lines sustained significantly higher levels of defoliation. Moreover, in most cases, the mean growth for transgenic lines was greater than that for the non-transgenic controls within each clone (Meilan *et al.*, 2000).

Others have explored the impacts of transgenic expression of synthetic *Cry1A* genes. Leaves of transgenic *Populus tremula* × *P. tremuloides* expressing a synthetic form of Bt toxin (*Cry3Aa*) proved to be highly effective in resisting damage by the phytophagous beetle *Chrysomela tremulae* (Genissel *et al.*, 2003). The synthetic Bt gene was modified to possess dicotyledonous codon usage and no AT-rich regions, and beetles feeding on high- and low-expressing *Cry3Aa*-leaves died within days. Synthetic *Cry1A*c expressed in loblolly pine under the control of the 35S promoter resulted in a nearly eight-fold increase in toxicity to various insect larvae (e.g. *Dendrolimus punctatus* and *Crypyothelea formosicola*), in laboratory feeding experiments. Although verification of resistance in both short- and long-term field trials is needed, stable expression of synthetic Bt toxin appears to be an effective method to reduce damage and, hence, lost growth or mortality caused by various insect pests.

The potential for insects to develop resistance to genetically engineered crops is a major concern (DiCosty and Whalon, 1997; James, 1997; Roush and Shelton, 1997). Before insect-resistant transgenics can be commercialized, a resistance management plan must be developed. Many management strategies have been proposed based on prior experiences with pesticide resistance (e.g. Luttrell and Caprio, 1996; Roush, 1997; Gould, 1998; McGaughey, Gould and Gelernter, 1998). Combining resistance genes (pyramiding or stacking) is one way of reducing the risk of insects becoming resistant to Bt gene products (Nwanze *et al.*, 1995; Maredia and Mihm, 1997; Roush 1997). This approach has proven to be an effective strategy for resistance management with many insects, including the cotton bollworm (*Helicoverpa armigera*; Zhao *et al.*, 1997). However, in the United States of America, for example, in order to obtain approval from the United States Environmental Protection Agency (USEPA) to deploy trees containing a gene encoding a Bt toxin, additional studies of beetle dispersal will be needed.

Non-Bt modifications

Despite the relief offered by Bt toxins, both native and synthetic, against damage caused by insect pests, other research has targeted herbivore resistance using different compounds. For example, Confalonieri *et al.* (1998) generated *P. nigra*

expressing a soybean trypsin proteinase inhibitor (Kunitz proteinase inhibitor, *KTi3*). Although the transgenic Kunitz protein inhibited digestive proteinases of the polyphagous moths *Lymantria dispar* and *Clostera anastomosis in vitro*, leaf feeding bio-assays showed no increase of larval mortality as a result of the transgenic expression. Perhaps increased expression would improve resistance, or a different proteinase inhibitor would be more detrimental to the digestion processes of larvae. In contrast, greater success was achieved in white poplar (*P. alba*) expressing an *Arabidopsis* cysteine proteinase inhibitor (*Atcys*), which resulted in up to 100% mortality of chrysomelid beetle (*Chrysomela populi*) larvae after only 16 days of feeding on transgenic leaf tissue (Delledonne *et al.*, 2001). Interestingly, expression of the scorpion neurotoxin, AaIT, in hybrid poplars appears to impart resistance against the gypsy moth (*Lymantria diaper*; Wu *et al.*, 2000).

Fungal pathogens

Fungal infections can be equally damaging to forest trees. Genetic modifications using a variety of genes from several plants have been evaluated to improve fungal pathogen resistance, and have met with varying success. Expression of the bacterio-opsin gene in tobacco suggested that defence mechanisms would be elicited by its expression and pathogen resistance therefore increased (Mittler, Shulaev and Lam, 1995). However, the expression of a synthetic bacterio-opsin gene in hybrid poplars did not elicit a significant increase in defence-response against a variety of fungal pathogens, such as leaf rust, leaf and shoot blight, and stem canker (Mohamed *et al.*, 2001). Similarly, white poplar expressing grapevine stilbene synthase (StSy), which has been implicated in the production of resveratrol compounds, did not significantly affect the efficacy of resistance against a rust disease (*Melampsora pulcherrima*; Giorcelli *et al.*, 2004). Although these transgenic trees offered an interesting opportunity to produce pharmacologically valuable compounds, they were not a viable option to reduce loss due to rust (Giorcelli *et al.*, 2004). In contrast, transgenic poplars expressing a rabbit defensin gene (*NP-1*; Zhao *et al.*, 1999) or a chitinase (*CH5B*) appear to have increased resistance to a broad spectrum of fungal pathogens (Meng *et al.*, 2004). Hybrid poplars expressing a wheat germin-like oxalate oxidase gene, directed at metabolizing the oxalic acid produced by fungal pathogens, showed signs of delayed infection by *Davidiella populorum* (syn. *Septoria musiva*) (Liang *et al.*, 2001). Liang *et al.* (2002) have also investigated the ability of transgenic poplars expressing antimicrobial peptides to alter resistance against *Davidiella populorum*. Two-year-old transgenic trees expressing a combination of antimicrobial peptides did, indeed, show greater resistance in leaf disc assays, and initial field trials showed less frequent *Davidiella* cankers on transgenic trees (unpublished, but reported in Powell *et al.*, 2006). It is evident that increasing resistance against fungal pathogens requires the use of a variety, and perhaps a combination, of transgenic products.

Bacterial pathogens

Reports of genetic modifications resulting in increased resistance to bacterial pathogens are less common. Although bacterial damage occurs less frequently, serious infections of *Xanthomonas* spp. have been reported (Haworth and Spiers, 1988; De Kam, 1984). Transgenic poplar expressing the antimicrobial peptide, D4E1, showed mixed resistance against *Agrobacterium* and *Xanthomonas* infection (Mentag *et al.*, 2003). In particular, the transgenic line displaying the highest transgene transcript abundance showed a significant increase in resistance, as defined by reduced tumour formation after *Agrobacterium* inoculation or the development of smaller cankers following *Xanthomonas* infection. However, D4E1-transformants did not show improved resistance against fungal pathogens. It should be noted that resistance against one strain of *Agrobacterium*, C58, was not improved; therefore, D4E1 appears to have limited specificity (Mentag *et al.*, 2003).

Field trials

The value of transgenic trees will only be realized after the completion of many extensive field trials. In the area of pest and pathogen resistance, a number of field trials have been reported, and some trials have yielded contrasting findings. In one case, resistance appeared to be lower in the field than in laboratory tests, and resistance levels can vary depending on the tissue tested. For example, a three-year field trial of transgenic birch expressing sugar beet chitinase revealed that although plants showed greater resistance in greenhouse trials, in the field the birch were equally, if not more, susceptible to fungal diseases such as leaf spot, caused by *Pyrenopeziza betulicola* (Pasonen *et al.*, 2004). In contrast, Hu *et al.* (2001) conducted a three-year trial of Bt-transgenic *P. nigra* and showed a decrease in damage from defoliators: 10% leaf damage compared to 80 to 90% damage on control plants. This study had other significant implications, as it demonstrated that there was a concurrent decrease in the abundance of insect pupae in the soil on transgenic plots, and that non-transgenic, wild-type trees were more protected when grown near or amongst transgenics. In conifers, although Cry1Ab levels in needles from field-grown trees were lower than levels in embryonic tissues or somatic seedlings, Bt-transgenic spruce had improved resistance against spruce budworm (Lachance *et al.*, 2007). Mortality of larvae feeding on tissues from field-tested plants ranged from 44 to 100% for transgenic trees, compared with approximately 37% for controls. Five-year-old trees also appeared phenotypically normal. This study illustrates the inherent variation of genetic modifications, and emphasizes the need for long-term trials. Efforts to understand the broad changes caused by genetic modifications were also evaluated by Davis *et al.* (2006), who looked at the effects of a Bt transgene on wood properties in hybrid poplars, and found no significant difference in chemical composition.

Considerations

As with chemical sprays, it is extremely important to consider the potential for pests to become resistant to the transgene product. Although laboratory

experiments suggested that feeding on transgenic Bt tissues enhanced the development of Bt-resistant pests, field tests report no increase in Bt-resistant pests either on or near Bt crops (Tabashnik *et al.*, 2003). Natural variation present in the field may prevent the rapid evolution of resistant pests. Results from Hu *et al.* (2001) demonstrate the value of planting stands mixed with both non-transgenic and transgenic trees. Genetic modification in other non-forest trees may be relevant when assessing the long-term affects of field-grown, genetically modified trees. One such example is the production of virus-resistant papaya, which was genetically modified to resist infection by the papaya ring-spot virus (Lius *et al.*, 1997). The genetically modified papaya trees have been grown commercially since the mid-1990s, and continue to be of benefit in Hawaii (NASS, 2005). However, the movement of transgenes from engineered trees to other organisms is still of concern (Fuchs and Gonsalves, 2007).

New research has highlighted the production of antibodies within transgenic plant cells, which could aid in combating infection from various pathogens (Powell *et al.*, 2006). For example, the expression of a recombinant antibody in citrus trees may reduce the infectious nature of pathogen proteins and thereby reduce disease progression. Such new technologies will surely change the direction of some research programmes, and continued efforts to understand the genes involved in plant defence mechanisms will lead to new avenues to improve resistance (e.g. Ralph *et al.*, 2006). Although commercial deployment of transgenic trees is several years away, using genetic modifications to increase tree survival, reduce the impact of chemical sprays to the environment and increase economic value of forests has significant potential.

NUTRITION

Forest nutrition is a key factor affecting tree growth. Nitrogen and sulphur are two elements that are essential for normal growth and development, and are often in short supply. With afforestation and reforestation efforts increasingly occurring on marginal agricultural land, tree nutrition is a key target area for genetic improvements.

Nitrogen

Nitrogen availability is a common limiting factor in forest tree growth (Suárez *et al.*, 2002). Development depends not only on the inorganic nitrogen available in the soil, but also on recycling within the plant, particularly in situations with limited nitrogen. Glutamine synthetase (GS) plays a significant role in both nitrogen uptake and recycling, as it catalyses the incorporation of ammonium into glutamine, the precursor to glutamate. Glutamine is also the precursor for all other plant N-containing compounds (Miflin and Lea, 1980). In an attempt to alter tree growth, GS has been an important target for genetic engineering (Fu *et al.*, 2003).

There are two iso-enzymes of GS, one localized in the cytosol (GS1) and the other in the chloroplast (GS2; Gallardo *et al.*, 1999). In angiosperms, GS2 is thought to function in the assimilation of ammonium from nitrate and respiration,

while GS1 has been suggested to be involved in glutamine generation for transport within the plant (Lam *et al.*, 1996). In conifers, GS2 has not been identified; GS1 is expressed in the photosynthetic cells and has been proposed to be involved in the primary assimilation in roots and re-assimilation from other metabolic processes (Jing *et al.*, 2004). GS1 has been found to co-localize with QTLs associated with yield (Hirel *et al.*, 2001; Obara *et al.*, 2001).

Suárez *et al.* (2002) overexpressed pine GS1 in poplar, and the plants were shown to form GS protein that is different from the native version, and elevated levels of chlorophyll (Gallardo *et al.*, 1999). Transgenic plants grew significantly faster than non-transformed controls. The transgenic plants also show increased early vegetative growth, increased leaf area, and a greater number of internodes (Fu *et al.*, 2003). GS activity was increased by 66%, chlorophyll by 33% and protein content by 21%. The results suggest that GS activity in young leaves is an effective marker for vegetative development (Fu *et al.*, 2003), even under low-nitrogen conditions. GS activity was strongly correlated with height growth, more so than chlorophyll or protein content. It is possible that the GS expression could affect additional pathways involved in vegetative growth other than through enhanced nitrogen, as was seen in tobacco, where changes in photosynthetic and photorespiratory capacities resulted in improved growth (Fuentes *et al.*, 2001).

These same poplars trees were field-tested for three years. The plants were again shown to be taller than the corresponding controls and had increased protein, total GS activity/protein and ferredoxin-dependent glutamate synthase (Fd-GOGAT), but showed no change in the Rubisco large subunit or in water content (Jing *et al.*, 2004). No significant differences were seen in polysaccharide or lignin content in the stems, but stem diameter and bark protein content suggest that nitrogen reserves accumulated to a greater extent in the stems of transgenics (Jing *et al.*, 2004). This increased growth and nitrogen cycling in poplars with ectopic pine cytosolic GS expression may be of particular importance given that marginal lands are being increasingly reclaimed for tree plantations, a trend that is likely to increase with the demand for bio-energy.

Sulphur

Sulphur is an essential element found mostly in its reduced form as the amino acids cysteine and methionine. In plants, cysteine is used either in the synthesis of proteins, or can be further metabolized to methionine, glutathione (GSH) and phytochelatins. GSH plays several crucial roles in plants, including acting as an antioxidant, protecting against reactive oxygen species (Foyer *et al.*, 1995); as a substrate for glutathione S-transferases, enabling detoxification of xenobiotics (Marrs, 1996); as a precursor in the synthesis of phytochelatins, which participate in the detoxification of heavy metals (Cobbett, 2000); in the regulation of gene expression (Wingate, Lawton and Lamb, 1988); and in the storage and transport of reduced sulphur (Herschbach, Jouanin and Rennenberg, 1998).

GSH is synthesized in two steps. Initially, γ-glutamylcysteine synthetase (γ-ECS) catalyses the fusion of cysteine to glutamate, producing γ-glutamylcysteine,

which then reacts with glycine in the synthesis of GSH via GSH synthetase (GSS; Herschbach and Kopriva, 2002). There are three factors that control the rate of GSH synthesis in leaves: the availability of cysteine (Strohm *et al.*, 1995), feedback inhibition of γ-ECS by GSH (Schneider and Bergmann, 1995) and the availability of the enzyme γ-ECS (Farago and Brunold, 1994). Glycine may also be a limiting factor during prolonged darkness, as its synthesis is affected by photorespiration (Noctor *et al.*, 1999).

Given the biological significance of sulphur in plant development, it has been a key target for genetic engineering in trees, particularly GSH formation. Numerous strategies have been used in designing transgenics over the years. Originally, γ-ECS and GSS were up-regulated in the cytosol (Foyer *et al.*, 1995; Strohm *et al.*, 1995; Noctor *et al.*, 1996; Arisi *et al.*, 1997). Overexpression of GSS did not yield an increase in GSH, despite the supplemental supply of cysteine. In subsequent feeding studies, an exogenous supply of γ-EC overcame this limitation, leading to increases in GSH, suggesting that γ-ECS is the rate-limiting step (Strohm *et al.*, 1995). Overexpression of γ-ECS resulted in an increase in GSH without affecting the redox state, as well as a significant increase in γ-EC (Strohm *et al.*, 1995; Noctor *et al.*, 1996). An exogenous supply of cysteine resulted in further increases in GSH. Similar results were observed in transgenic tobacco (Creissen *et al.*, 1996); however, in tobacco there was increased sensitivity to oxidative stress, and necrotic lesions formed.

Additional studies revealed an increase in GSH in the phloem of poplars overexpressing γ-ECS, confirming its role as a major transport form of sulphur (Herschbach, Jouanin and Rennenberg, 1998). Furthermore, overexpression of γ-ECS in the chloroplast, using the 35S promoter along with the pea rbcS chloroplast transit peptide or rbcS itself, resulted in increased concentrations of foliar valine, leucine, isoleucine, tyrosine and lysine. These findings suggest that there is an additional indirect effect on nitrogen metabolism (Noctor *et al.*, 1998).

Similarly, the overexpression of glutathione reductase (GR) in the chloroplasts of poplar led to increased GSH pools (Foyer *et al.*, 1995). Although there were no quantifiable changes in photosynthetic rates under normal growing conditions, when photo-inhibition was induced by subjecting the transgenics to low temperatures and high light levels, the plants proved to be much less sensitive to stress compared with the corresponding controls. The improved stress tolerance was attributed to a number of factors, including: increased cellular recycling of GSH and ascorbate pools, helping to combat the production of harmful free radicals; and elevated GSH levels that may aid in stabilizing enzymes that require reduced thiol groups for activity, and could also exert indirect actions on protein synthesis and gene expression (Foyer *et al.*, 1995).

The significance of these findings extends beyond the bounds of general plant nutrition, as sulphur is a key component of compounds involved in stress and herbicide resistance. The implications of alteration of sulphur nutrition are discussed further below.

Herbicide resistance

Generally, there are three main breeding and improvement objectives, including improved growth rates, improved wood characteristics, and improved resistances to herbicides, pests and disease (Chupeau, Pautot and Chupeau, 1994). Improvements in herbicide resistance would allow for lower total herbicide use as well as application of more environmentally benign active ingredients, not to mention more flexibility with regard to the timing of application.

Glyphosate

Fillatti *et al.* (1987a, b) reported the first successful insertion of a herbicide-tolerance gene in trees, generating transgenic poplar (*P. alba* × *P. grandidentata*) expressing the *Salmonella typhimurium aroA* gene. A mutation in this gene resulted in the formation of a 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS) that is resistant to inhibition by glyphosate, the active ingredient in Roundup® herbicide (Comai, Sen and Stalker, 1983). Ectopic expression of *aroA* under the mannopine synthase promoter yielded surprisingly low levels of herbicide tolerance (Riemenschneider *et al.*, 1988), which was thought to be the result of low cytosolic expression of the gene. When the same poplar was transformed with *aroA* under the control of the 35S promoter and a chloroplast transit peptide, the resulting plants showed higher levels of glyphosate tolerance (Riemenschneider and Haissig, 1991; Donahue *et al.*, 1994).

Although use of the 35S promoter led to higher expression levels than mannopine synthase, and the transit peptide directed transgene product to the chloroplast, performance of these *aroA*-containing lines still fell short of expectations (Karnosky *et al.*, 1997). Following greenhouse spray tests, chlorophyll content in all transgenic lines was inversely correlated to glyphosate concentration, height growth was arrested following herbicide treatment, and only one line retained live leaves six weeks after treatment (Donahue *et al.*, 1994). It was clear from this work that the production of a herbicide-tolerant poplar line would not be as straightforward as originally thought.

More recently, Meilan *et al.* (2002a) tested a construct containing a glyphosate-tolerance gene, *CP4*, for its ability to impart glyphosate tolerance in poplar. *CP4* is an alternative form of EPSPS that originates from *Agrobacterium tumefaciens* strain CP4, and glyphosate has a low affinity for this form of the enzyme. Using an *Agrobacterium*-mediated transformation protocol (Leple *et al.*, 1992; Han *et al.*, 2000), Meilan *et al.* (2000) generated transgenic plants in 12 genotypes of hybrid poplar. The resultant transgenic plants were field-tested for two years on the west and east sides of the Cascade Mountains in Oregon, United States of America. Growth of the lines expressing *CP4* was significantly better than controls and lines expressing both the *CP4* and *GOX*, another gene implicated in herbicide tolerance. In addition, the lines expressing *CP4* only exhibited less damage in response to glyphosate treatment. This was the first report of transgenic poplars exhibiting high levels of glyphosate tolerance when grown under field conditions

(Meilan *et al.*, 2002b). Herbicide resistance has remained stable for over eight years in trees grown under field conditions (Li *et al.*, 2008).

Chlorsulphuron

Chlorsulphuron [chlorsulfuron] is a sulphonylurea herbicide that acts on acetolactate synthase and blocks the biosynthesis of valine and isoleucine (Ray, 1984). A mutant acetolactate synthase gene (*crs1-1*) from *Arabidopsis*, which confers resistance to chlorsulphuron, has been expressed in hybrid poplar (*Populus tremula* × *P. alba*) under the control of both the native and 2×35S promoter. Both promoters led to transgenic lines that were completely resistant to higher-than-normal field application rates of chlorsulphuron in greenhouse tests. Control poplar trees died within two to three weeks of treatment, whereas transgenic lines survived. Following slightly delayed growth and root development, the transgenic plants returned to normal growth following the treatment (Brasileiro *et al.*, 1992).

Chloroacetanilide

Acetochlor and metochlor are active ingredients in chloroacetanilide herbicides, which are detoxified by GSH-dependent reactions (Gullner, Komives and Rennenberg, 2001). GSH and the glutathione S-transferase (GST) family play crucial roles in the degradation of several herbicides. GSTs are able to catalyse conjugation reactions between a number of xenobiotics and GSH. Herbicide:GSH conjugates are less toxic and more water soluble than the herbicide molecules alone (Edwards, Dixon and Walbot, 2000). When poplars expressing γ-ECS in chloroplast or cytosol were exposed to acetochlor and metochlor dispersed in soil (Gullner, Komives and Rennenberg, 2001), the growth and biomass of all lines was markedly reduced, but the reduction was less dramatic in the transgenic lines relative to the non-transformed trees, and the growth rate of cytosol expressers was less affected than the chloroplast expressers. Foliar γ-EC and GSH levels increased in all lines, but more so in the transgenics poplar (Gullner, Komives and Rennenberg, 2001).

Glufosinate

Glufosinate (phosphinothricin, PPT) is the active ingredient in herbicides known as Basta® and Buster®, and is a structural analogue of glutamate. It inhibits glutamine synthetase, causing ammonium to accumulate, which at elevated concentrations is lethal (Bishop-Hurley *et al.*, 2001). This interaction causes irreversible inactivation of GS, which also blocks photorespiration, resulting in the depletion of leaf amino acid pools (Pascual *et al.*, 2008). Plants respond to PPT by developing necrosis, usually initiating at or near the apical meristem and spreading throughout the plant (Pascual *et al.*, 2008).

The *BAR* gene encodes for phosphinothricin acetyltransferase (PAT), which inactivates glufosinate by acetylating its free ammonium group (Thompson *et al.*,

TABLE 4-2
Summary of genetic modifications in trees, targeting resistance to herbicides

Gene	Modification	Effects	Reference
aroA (EPSP gene)	Overexpression in *Populus alba* × *P. grandidentata*	First record of insertion and expression of a foreign gene of agronomic importance in woody plants; slight resistance to glyphosate	Fillatti *et al.*, 1987
bar (PAT gene)	Overexpressed in *P. alba* × *tremula* and *P. trichocarpa* × *deltoides*	Transgenics did not accumulate NH_4^+ when treated with Basta®	De Block, 1990
crs 1-1	Overexpressed in *P. tremula* × *P. alba*	Resistance to chlorsulphuron in greenhouse tests	Brasileiro *et al.*, 1992
aroA	Overexpressed in *P. alba* × *P. grandidentata*	Conferred resistance to glyphosate	Donahue *et al.*, 1994
als, pat	Overexpressed in *P. tremula* × *P. alba*	Conferred herbicide resistance to calli	Chupeau, Pautot and Chupeau, 1994
aroA	Overexpressed in *Larix decidua*	Conferred resistance to glyphosate at moderate treatment levels	Shin *et al.*, 1994
bar	Overexpressed in *Eucalyptus camaldulensis*	Conferred resistance to herbicide at over twice the normal field application rate	Harcourt *et al.*, 2000
bar	Overexpressed in *Pinus radiata* and *Picea abies*	Conferred resistance to glufosinate in both species in greenhouse testing	Bishop-Hurley *et al.*, 2001
γ-ECS	Overexpression in *Populus tremula* × *P. alba*	Conferred resistance to acetochlor and metolachlor present in soil. Plants with cytosolic expression were more resistant than those with chloroplastic expression	Gullner, Komives and Rennenberg, 2001
GOX, *CP4*	Overexpressed in various poplar hybrids, including *P. trichocarpa* × *P. deltoides*	Genes shown to confer resistance up to 66% at low glyphosate levels, with 25% of lines showing increased growth following herbicide treatment. Lack of damage was attributed to *CP4*, as GOX was suspected to cause undesirable side effects. Twelve lines expressing only *cp4* had similar herbicide tolerance, but grew better and had less damage in response to treatment	Meilan *et al.*, 2002a Meilan *et al.*, 2002b
γ-ECS	Overexpressed in *P. tremula* × *P. alba*	No change in tolerance to paraquat	Bittsanszky *et al.*, 2006
GS	Overexpressed in *P. tremula* × *P. alba*	Conferred resistance to PPT	Pascual *et al.*, 2008
bar	Overexpressed in *P. alba*	Conferred resistance to Basta® at normal field dosage; poplar still tolerant at twice the normal field dosage	Confalonieri *et al.*, 2000

1987). The effectiveness of *BAR* in conferring herbicide resistance has been shown in many tree species. Poplar explants transformed with *BAR* were able to survive and grow on glufosinate-containing medium at the callus phase, proving it to be an effective selectable marker gene (Chupeau, Pautot and Chupeau, 1994). Similar experiments have been carried out in other species (see Table 4-2).

Poplar overexpressing the pine GS gene showed increased resistance to PPT. Resistance was measured at 5, 25 and 100 μM PPT. At 5 μM, there was limited effect on all plants, while at 25 μM, 75% of control plants died and 50–100% of the transgenic plants in each line remained viable. At 100 μM all of the wild-type trees were dead within eight days, whereas 20–45% of the transgenics survived (Pascual *et al.*, 2008).

Abiotic stress

Environmental stresses can significantly affect productivity. Low temperatures and high salt concentrations during the growing season can damage seedlings, leading

to impaired growth or even death (Blennow and Lindkvist, 2000). Because certain plants and bacteria are able to survive these harsh conditions, genomic tools have been used to identify target genes within the protective pathways and impart stress tolerance through genetic modification (Cushman and Bohnert, 2000).

Increased resistance to many types of stress has already been achieved for several plant species. Expression of anti-freeze or ice nucleation genes has been shown to improve freeze tolerance in tobacco and maize protoplasts (Baertlein *et al.*, 1992; Murata *et al.*, 1992; Georges, Saleem and Cutler, 1990). Two antifreeze genes, glucose-6-phosphate dehydrogenase (PsG6PDH) and anti-freeze protein (PsAFP), have been introduced into poplar and freeze-resistance tests are currently underway (Lin and Zhang, 2004).

Control mechanisms for abiotic stresses are based on the activation or regulation of specific stress-related genes, which can be involved in controlling transcription, cell signalling, protecting membranes and proteins, or scavenging free-radicals, or a combination (Wang, Vinocur and Altman, 2003). Several different abiotic stresses often activate similar signalling pathways and cellular responses (Knight and Knight, 2001; Zhu, 2002) and therefore result in similar plant phenotypes. As such, transcription factors hold particular interest because of their potential to increase tolerance to multiple stresses via the overexpression of a single transcription factor. An ERF/AP2-type transcription factor has been of particular interest because when overexpressed in *Arabidopsis*, it caused the simultaneous up-regulation of pathogen- and cold-response (*COR*) genes (Yi *et al.*, 2004). Similarly, the overexpression of the dehydration response element 1A (DREB1A) transcription factor in *Arabidopsis* caused increased tolerance to multiple abiotic stresses by the increased *COR* expression (Kasuga *et al.*, 1999; Jaglo-Ottosen *et al.*, 1998). Gains in the tolerance of abiotic stress have also been seen in forest trees.

Ozone stress

Ozone is formed by photochemical reactions between nitrogen oxides, hydrocarbons and carbon monoxide, and is highly phytotoxic (Lelieveld and Crutzen, 1990). At elevated concentrations it elicits changes in plant biochemical and physiological processes, resulting in foliar injury, increased senescence, decreased growth rates (Kress and Skelly, 1982; Sandermann, 1996) and increased production of reactive oxygen species (ROS; Foyer *et al.*, 1994).

The ascorbate-glutathione pathway plays an important role in protecting plants from ROS (Foyer *et al.*, 1994). GSH acts as an antioxidant that can directly scavenge ROS, and also protect thiol-containing enzymes and reduce dehydroascorbate, as it is oxidized to glutathione disulfide (GSSG). The reduced GSH pool is maintained by the activity of GR, and many plant species have exhibited gains in resistance to photo-oxidative stress, herbicides or drought, or a combination, through the up-regulation of GR or superoxide dismutase (Foyer *et al.*, 1994).

When transgenic poplar overexpressing GSS in the cytosol or GR in the cytosol or chloroplast were exposed to various levels of ozone stress, there was no

apparent difference in the phenotypic response between the transgenic and control trees, despite elevated activities of GSS and GR (Strohm *et al.*, 1995). Rather, sensitivity to ozone stress appeared to be directly related to leaf developmental stage (Strohm *et al.*, 1995). Poplar with GR overexpressed in the chloroplast did, however, recover more quickly when exposed to high light levels and low temperatures than did wild-type trees, or trees expressing GR or GSS in the cytosol (Foyer *et al.*, 1995; Strohm *et al.*, 1995).

Plants with increased peroxidase activity also display increased resistance to abiotic stress (Hiriga *et al.*, 2001). Hybrid aspen (*P. sieboldii* × *P. grandidentata*) overexpressing the horseradish peroxidase gene (*prxC1a*) showed increased growth and elevated peroxidase activity. Transgenic callus tissue and plantlets were resistant to oxidative stress imposed by hydrogen peroxide, although the growth rate was decreased (Kawaoka *et al.*, 2003).

Salt stress

Salt stress is an increasingly important issue throughout the world, and it is imposed by two factors: water deficit due to osmotic stress, and the accumulation of ions that negatively affect biochemical processes (Tang, Charles and Newton, 2005). A number of genes have been tested in attempts to increase salt tolerance in trees. Poplar transformed with the *E. coli* mannitol-1-phosphate dehydrogenase gene (*mt1D*) grew faster and had a higher survival rate than non-transformed controls (Liu *et al.*, 2000). Hu *et al.* (2005) found that the up-regulation of *mt1D* in poplar led to increased mannitol levels, and, under salt stress, all lines had higher stomatal conductance, transpiration and photosynthetic rates. Under non-salt-stressed conditions, transgenic plant growth was about 50% that of controls (Hu *et al.*, 2005). Other species have also demonstrated gains in salt tolerance when transformed with *mt1D*, glucitol-6-phosphate dehydrogenase (*gutD*), choline dehydrogenase (*betA*) and choline oxidase (*codA*) genes (see Table 4-3).

Drought stress

Drought stress is primarily osmotic stress, which causes the disruption of homeostasis and ion distribution in the cell (Serrano *et al.*, 1999; Zhu, 2001). Poplar transformed with a pine cytosolic GS (GS1) was shown to be more tolerant to drought stress than wild-type trees (El-Khatib *et al.*, 2004). At all levels of water availability, the transgenic trees had higher photosynthetic assimilation rates and stomatal conductance than the corresponding controls. All GS1-containing lines also showed an irreversible decline in photosystem II (PSII) antennae transfer efficiency after drought and during recovery, but the increased photo-assimilation capacity of the transgenic poplar allowed more resources to be allocated to photoprotective mechanisms. Gains in drought stress were also reported in hybrid eucalypts (*Eucalyptus grandis* × *E. urophylla*) transformed with the DREB1A transcription factor (Kawazu, 2004).

Efforts have been made to engineer tolerance to multiple stresses. Hybrid larch (*Larix* × *leptoeuropaea*) expressing a pyrroline 5-carboxylate synthase gene

TABLE 4-3
Summary of genetic modifications in trees, targeting resistance to abiotic stress

Gene	Modification	Effects	Reference
GSS	Overexpression in *Populus tremula* × *alba*	No changes in response to ozone stress; ozone sensitivity related to leaf development stage	Strohm *et al.*, 1995
FeSOD	Overexpression in *Populus tremula* × *alba*	No change in response to high light and photo-inhibition of PSII; suggests rate of conversion of superoxide to hydrogen peroxide is not a rate limiting factor in protection against or repair of photo-inhibition	Tyystjarvi, 1999
mtlD	Overexpression in *Populus* sp.	Transgenic plants grew significantly better with a higher survival rate	Liu *et al.*, 2000; Liu *et al,*, 2002
Bet-A	Overexpression in *Populus* sp.	Conferred salt resistance	Yang *et al.*, 2001
Phospholipase D	Antisense expression in *Populus* sp.	Conferred salt tolerance	Liu *et al.*, 2002
PsG6PDH and *PsAFP*	Overexpression in *Populus* sp.	Freezing resistance tests under way	Lin and Zhang, 2004
mt1D and *gutD*	Overexpression in *Pinus taeda*	Increased salt tolerance at both calli and plantlet stage; accumulated mannitol and glucitol	Tang, Peng and Newton, 2005
codA	Overexpressed in *Eucalyptus camaldulensis*	Increased salt stress tolerance	Yamada-Watanabe, Kawaoka and Matsunaga, 2003
mt1D	Overexpression in *Populus tomentosa*	Increased mannitol; increased salt tolerance *in vitro* and in hydroponic culture; decreased growth in the absence of salt	Hu *et al.*, 2005
prxC1a	Overexpression in *P. sieboldii* × *P. grandidentata*	Increased growth rate; elevated peroxidase activities; calli resistant to oxidative stress imposed by hydrogen peroxide	Kawaoka *et al.*, 2003
vhb	Overexpression in *P. alba*	No change in growth pattern, or chlorophyll and protein contents; no change in stress resistance	Zelasco *et al.*, 2006
dreb1a and citrate synthase	Overexpression in *Eucalyptus grandis* × *E. urophylla*	Conferred resistance to drought and acid soil tolerance	Kawazu, 2004
GS1	Overexpression in *P. tremula* × *P. alba*	Higher photosynthetic assimilation and stomatal conductance at all levels of water availability; increased photo-assimilation allows increased allocation of resources to photoprotective mechanisms	El-Khatib *et al.*, 2004
P5CS	Overexpression in *Larix leptoeuropaea*	Increased proline; increased resistance to cold, salt and freezing stress	Gleeson, Lelu-Walter and Parkinson, 2005
CaPF1	Overexpressed in *Pinus strobus*	Increase in tolerance to drought, freezing and salt stress	Tang, Newton and Weidner, 2007

(*P5CS*), which functions as a rate-limiting step in proline synthesis, had increased proline content and were shown to be more resistant to cold, salt and freezing stresses (Gleeson, Lelu-Walter and Parkinson, 2005). Proline, which is produced via the glutamic acid pathway (Delauney and Verma, 1993; Kavi Kishor *et al.*, 1995), has been shown to play a role in protecting trees against stresses (Gleeson, Lelu-Walter and Parkinson, 2004). When *P5CS* was up-regulated in tobacco, rice, lettuce and wheat, increased proline and biomass production were observed, despite environmental stresses (Kavi Kishor *et al.*, 1995; Zhu *et al.*, 1998; Pileggi *et al.*, 2001; Sawahel and Hassan, 2002). Similarly, Tang *et al.* (2007a) overexpressed

paprika (*Capsicum annuum*) pathogen and freezing tolerance-related protein 1 (CaPF1) in eastern white pine (*Pinus strobus*). The result was a dramatic increase in tolerance to drought, freezing, and salt stress. This was related to polyamine biosynthesis, as putrescine, spermidine and spermine levels were maintained in the transgenic lines, while they decreased in stress-treated controls (Tang *et al.*, 2007).

Phytoremediation

The use of plants to remove contaminants from the environment is known as phytoremediation (Schnoor *et al.*, 1995). This technology has recently been applied to several environmental problems, including disposal of municipal wastewater, biofiltration of farm and industrial runoff, and the remediation of soils spoiled by industrial processes (Che *et al.*, 2003, 2006; Lee, Isenhart and Schultz, 2003; Strand *et al.*, 2005). Because this technology is less costly, less invasive, more aesthetic, and often yields a usable product (e.g. biomass), it has many advantages over traditional, engineering-based methods. Phytoremediation plantings can provide additional environment benefits, such as a means to sequester carbon (i.e. carbon credits), erosion control, wildlife habitat maintenance and the creation of buffers against noise, garbage and harmful dust (Rockwood *et al.*, 2004).

Using transgenic plants for improved phytoremediation is a relatively new, but highly successful, technological advance. For example, transgenic plants have been developed that are more effective in translocating arsenic (Dhankher *et al.*, 2002) and selenium (LeDuc *et al.*, 2006) from soil to the plant. Similarly, transgenics have been generated that are more tolerant of and better able to degrade explosives (French *et al.*, 1999; Hannink *et al.*, 2001), and that can detoxify sites contaminated with mercury (Bizily, Rugh and Meagher, 2000; Meagher, 2000).

Heavy metal contamination is a major problem globally. Anthropogenic uses of mercury, zinc, cadmium, selenium, lead and arsenic have led to problems in many terrestrial and aquatic ecosystems. These harmful pollutants are generated from numerous sources, and although many have recently been the target of stricter regulation, historically, contaminated areas have not been reclaimed due to extremely high cost and the destructive nature of available methods. Many metallic compounds can be taken up by plants or microbes and thus enter the food chain, potentially causing significant problems for animals and humans. Although the concept of using plants to remediate contaminated sites is not new (Baker and Brooks, 1989), heavy metals are multisite inhibitors of several metabolic pathways and are therefore generally phytotoxic. Genetic manipulation can overcome these obstacles. Several plant species have been considered for phytoremediation efforts, but trees have most recently been identified as particularly useful vehicles because they produce large amounts of biomass, have far-reaching roots and are perennial, although leaves may need to be collected for incineration (Bittsanszky *et al.*, 2005). There is the added benefit of employing the woody biomass for fuel or value-added products. Despite the development of successful transformation techniques for numerous tree species, research in dendroremediation is relatively new, but results are consistent with what has been seen in other plant species.

The volatilization of mercury by tobacco and *Arabidopsis* transformed with the mercuric ion reductase (*mer*) genes from bacteria inhabiting contaminated sites has been particularly successful (Summers, 1986). The *mer* gene catalyses the conversion of ionic mercury Hg(II) to its volatile derivative Hg(0). Yellow-poplar (*Liriodendron tulipifera*) was transformed with *merA18* from *E. coli* (Rugh *et al.*, 1998), and the resulting plantlets grew vigorously on a medium containing levels of mercury that were roughly ten fold those known to be toxic. They also released elemental mercury at ten times the rate of wild-type trees over a six-day trial in mercury vapour sampling tubes, with no apparent affect on growth.

Eastern cottonwood (*Populus deltoides*) has been identified as a key dendroremediant, as its native growth habitat includes riparian areas, which are similar to many contaminated sites. In 2003, Che *et al.* transformed eastern cottonwood with the *merA9* and *merA18* genes from *E. coli*. The transgenics trees grew normally and rooted in medium containing 25 µM Hg(II), while wild-type trees did not survive. When exposed to lower levels of Hg(II), which were not lethal to wild-type trees, the transgenic lines emitted two- to four-fold more Hg(0) than the corresponding wild-type trees. Transgenic plants (*merA18*) also accumulated significantly higher biomass than wild-type trees when grown in soil containing 40 ppm Hg(II). However, in soils with lower levels of contamination, there was no difference in biomass accumulation, and in soil that was not contaminated, wild-type trees grew faster (Che *et al.*, 2003).

More recently, eastern cottonwood harbouring both *merA* and *merB*, which encode for organomercury lyases, were produced for use in mercury-contaminated soils (Lyyra *et al.*, 2007). *In vitro*-grown plants were highly resistant to phenylmercuric acetate, and were able to detoxify organic mercury compounds at two to three times the rate of controls or trees containing one of the two *mer* genes. Only trees transformed with both genes were capable of rooting in media supplemented with mercury, although their roots were shorter and thicker than in mercury-free media. The plants expressing both transgenes probably convert mercury first to Hg^{2+} and then to elemental mercury, with lower toxicity in a coupled reaction, as was previously shown in both *Arabidopsis* and tobacco (Bizily, Rugh and Meagher, 2000; Ruiz *et al.*, 2003). It is expected that when grown in soil with no mercury contamination, the *merA*/*merB* poplar will be less productive than the wild-type trees, based on a previous report of *merA* alone in poplar (Che *et al.*, 2003).

Another heavy metal of interest is zinc, which can cause reduced foliage and dry-mass accumulation (Di Baccio *et al.*, 2003). When the cytosol and chloroplasts of *P. canescens* were transformed with the *E. coli gsh1* gene encoding for γ-glutamylsysteine synthetase (γ-ECS), the resulting trees clearly contained elevated levels of glutathione (Bittsanszky *et al.*, 2005). It is expected that higher GSH levels will result in enhanced phytochelatin production (Cobbett, 2000). When these transgenic and wild-type trees were subjected to varying levels of zinc, similar results were observed. At 10^{-1} M, the symptoms were necrosis and severe phytotoxicity, while at 10^{-2} M the leaves bleached, but continued growing.

At lower levels (10^{-3} to 10^{-5} M), there were no toxic effects. Leaf zinc content increased with increasing treatment concentration, but did not significantly differ between transgenics and controls. However, trees expressing γ-ECS in the cytosol accumulated significantly more Cd, Cr and Cu than the wild-type or other transgenic lines, which is consistent with previously published results in poplar and *Arabidopsis* (Koprivova *et al.*, 2002). While GST activity in the wild-type and chloroplast-expressing lines increased, there was no observable change in lines with cytosolic expression, suggesting a lower stress response. GST is known to possess GSH peroxidise activity and can contribute to detoxification of active oxygen species (AOS). Therefore, increased GST levels are thought to contribute to the improved detoxification capacity in Zn-treated poplar leaves, but the mechanism is still unknown (Bittsanszky *et al.*, 2005).

Cadmium, another significant pollutant, can accumulate in soils and be phytotoxic due to its reactivity with O-, N- and S-containing ligands. In short, it inhibits photosynthesis and increases respiration, as carbohydrate metabolism (e.g. TCA cycle) is induced by increased leaf Cd content. In addition, Cd induces the synthesis of phytochelatins, which form complexes with Cd that can then be sequestered in the vacuole (Arisi *et al.*, 2000).

Phytochelatins are synthesized by γ-glutamylcysteine (γ-EC) dipeptidyl transpeptidase from reduced GSH, which is formed by two sequential reactions catalysed by γ-ECS and GS in the chloroplasts and cytosol of plant cells (Arisi *et al.*, 2000). Cd tolerance is related to GSH accumulation in leaves and increased capacity for GSH synthesis. Hybrid poplar (*P. tremula* × *P. alba*) overexpressing bacterial γ-ECS in the cytosol displayed 30-fold increases in foliar γ-ECS activity in the absence of Cd (Arisi *et al.*, 2000). Foliar γ-EC was increased ten fold and foliar GSH accumulation increased 2.5 to 3.5-fold relative to controls grown in the absence of Cd. All transgenic trees also had higher leaf cysteine concentrations in the absence of Cd. In the presence of Cd, foliar γ-ECS, γ-EC, glutathione and cysteine all increased in both transgenics and control trees. However, the transgenic lines were able to accumulate more Cd than control at all concentrations, and there was also less GSH accumulation in the leaves of controls. Cd-induced changes in enzyme activities were less pronounced in the leaves of transgenic lines. Despite being able to accumulate more Cd in the leaves, the transgenic plants did not have a greatly increased tolerance to Cd (Arisi *et al.*, 2000). Koprivova *et al.* (2002) also examined the effects of overexpression of a bacterial γ-ECS on Cd accumulation in poplar. They initially looked at CdCl2 in a hydroponic system, and the transgenics were able to accumulate significantly more Cd in root tissue than plants overexpressing GS or wild-types. At low Cd concentrations there was no difference in accumulation, but at higher Cd concentrations the transgenics were able to accumulate 2.5 to 3 times more Cd in the leaf tissue than the wild-type trees (Koprivova *et al.*, 2002).

Many sites with heavy metal contamination are also contaminated with organic components such as trichloroethylene (TCE; Meagher *et al.*, 1998). Chlorinated compounds, such as TCE, are among the most widespread groundwater

contaminants in the United States of America. TCE was commonly used as a dry cleaning solvent and as a metal-degreasing agent, especially by the military and the electronics industry. The USEPA classifies TCE as a suspected human carcinogen and gives high priority to its clean-up (Doty *et al.*, 2000). About 40% of all its Superfund sites are contaminated with this substance. It has also been shown that exposure can result in depression of the central nervous system (Costa, Katz and Ivanetich, 1980). Moreover, TCE persists in the environment for decades. Existing remediation techniques (e.g. pumping or air-stripping) are labour-intensive, expensive and wasteful. While some plants have an innate ability to absorb and metabolize TCE, those that have been genetically engineered to contain the appropriate gene possess enhanced ability to metabolize TCE. For example, tobacco plants transformed with the gene encoding a mammalian cytochrome P450 2E1 (CYP2E1) are able to metabolize TCE at a rate that is 640-fold greater than plants without this gene (Doty *et al.*, 2000). The encoded enzyme also metabolizes a wide range of other harmful pollutants, including ethylene dibromide (used as a gasoline additive and as a soil fumigant to control nematodes), carbon tetrachloride, chloroform and vinyl chloride (Doty *et al.*, 2007). This gene is a prime candidate for genetic engineering trees to remediate contaminated sites.

As is the case with heavy metals, a disadvantage of phytoremediation is that when the plants remove pollutants from the groundwater and soil, they either transpire them, unaltered, into the atmosphere, or sequester them in various tissues. Thus, plants that could metabolize the pollutant would be more desirable. Although significant gains in the phytoremediative capacity of trees have been shown in controlled environments, limited field-testing has been conducted to date.

Hormones

Much research has been carried out in lignin modification, flowering, and abiotic and biotic resistances. Genes that control hormone synthesis and sensitivity are potential candidates for producing trees that have altered wood properties and other desirable characteristics. These include reduced axillary bud break, high density, long fibres, better rooting and improved growth rate, all of which are influenced by hormones.

Cytokinins are important as mediators of growth and differentiation in plants. The isopentenyltransferase gene (*IPT*) from *Agrobacterium tumefaciens* catalyses the conversion of adenosine-5'-monophosphate and isopentenylpyrophosphate to isopentenyladenosine-5'-monophosphate (Akiyoshi *et al.*, 1984), which is then converted to isopentenyl- and zeatin-type cytokinins (Von Schwartzenberg *et al.*, 1994). Poplar overexpressing *IPT* showed increased formation of branching, with short internodes that were unable to root. The calli were able to regenerate buds in the absence of exogenous cytokinins, and contained high concentrations of zeatin, zeatin riboside and isopentenyladenosine (Von Schwartzenberg *et al.*, 1994). The effects of the transformation were noticeable even at a very early stage, as transgenic explants developed green calli with significantly more buds than

non-transformed controls on medium lacking thidiazuron (TDZ). When grown on media containing TDZ, fewer buds formed on the transgenic explants. Isolated transgenic shoots showed reduced apical dominance with frequent branching, shorter internodes and the inability to root (Von Schwartzenberg *et al.*, 1994).

Another phytohormone, indole-3-acetic acid (IAA), is important for maintaining the structure and integrity of the vascular cambium. Exogenous application of auxins or auxin transport inhibitors affect many aspects of cambial growth, including xylem production, cell size and thickness, and reaction wood and vessel density (reviewed by Little and Savidge, 1987). Enzymes encoded by *iaaM* (trp-2-mono-oxygenase) and *iaaH* (indole-3-acetamide hydrolase) catalyse the two-step formation of IAA from tryptophan, and the overexpression of these two genes in plants has led to increased IAA levels (Sitbon *et al.*, 1991, 1992). Poplar transformed with *Agrobacterium iaaM* and *iaaH* showed phenotypic alterations (Tuominen *et al.*, 1995), as might be expected. All transgenic lines were smaller than controls, but there was variation in extent of stunting. Some lines showed elevated levels of free and conjugated IAA in the mature leaves and roots, relative to controls. Decapitated transgenic lines had a lower bud release rate than controls, with some plants showing no release at all. Many of these plants survived and continued to grow, with large increases in the growth of the lower trunk. Transgenics also had alterations in xylem formation, with decreased width and altered structure, and also had large fibres and small vessels, which appeared more uniform in size and rounded.

Tuominen *et al. (*2000) engineered poplar (*P. tremula* × *P. tremuloides*) to overexpress *iaaM* fused to the *GUS* reporter gene under the control of the cambial region-specific *Agrobacterium rhizogenes rolC* promoter. While IAA levels were increased, the radial distribution pattern remained unchanged, and no changes were seen in the developmental pattern of cambial derivatives or in cambial second growth, suggesting that the distribution pattern of IAA holds a more important role in wood formation than changes in the amount of IAA (Uggla *et al.*, 1996). Despite a 35% increase in IAA (with only minor changes in conjugate pools), phenotypic changes were relatively minor. Transgenic lines had increased internode length, and decreased occurrence of axillary bud break following decapitation. Some lines also showed decreased leaf size or height, but this response was not consistent.

Plants overexpressing the *rolC* gene showed significant alteration in growth and development. This has been observed in various species, including tobacco (Nilsson *et al.*, 1993) and potato (Fladung, 1990). The plants are dwarfed and have reduced apical dominance, shorter internodes and smaller leaves, suggesting that *rolC* is related to an increase in cytokinin activity (Nilsson *et al.*, 1996). Hybrid poplar transformed with *rolC* resulted in an increased level of free cytokinins (Nilsson *et al.*, 1996). Transgenic trees had reduced apical dominance with more axial shoots. When the side shoots were removed, the trees showed normal growth and apical dominance with a single shoot (Nilsson *et al.*, 1996). Additional lines exhibited fasciation, enlarged shoot apices and revealed the apical meristem in some cases.

The fasciated apices of the transgenic lines were smaller and more numerous, and the leaves were also smaller and thicker, with larger palisade cells than wild-type leaves. The transgenics also had flattened stems as a result of fasciation (Nilsson *et al.*, 1996). Transgenic lines had less free IAA in the upper leaves and apical meristem, but the conjugated IAA level was not changed, or only showed a slight increase in the apex. Gibberellic acid (GA) activity was markedly lower in the upper regions of the transgenic trees, while cytokinins were unchanged relative to control plants, despite increases in zeatin riboside levels. It appeared that cytokinin levels were regulating conjugation (Nilsson *et al.*, 1996). Additional studies with *rolC* genes have consistently shown changes in hormone levels and growth morphology, as well as changes in timing of dormancy and bud flush (Table 4-4).

Poplar overexpressing *rolC*, characterized by reduced shoot growth and early bud break, have also been examined for changes in wood properties. Wood formation started at the same time as control trees, and there were no changes in wood structure. These observations suggest that the dwarfism was due to a decreased number of cells, as a result of slower cell differentiation rates. However, when compared with controls, cells in transgenics lacked secondary walls and normal lignification, and had discoloured wood and tyloses. In addition, ring borders were not easily identifiable because the transgenics lacked thick-walled fibres associated with latewood (Grunwald *et al.*, 2000). In the control trees, the reactivation of wood formation coincided with bud break, but in the transgenic lines it coincided with full leaf expansion. Given these results, it is possible that

TABLE 4-4
Summary of genetic modifications in trees, targeting hormone regulation

Gene	Modification	Effects	Reference
ipt	Overexpression in *Populus tremula* × *P. alba*	Increased branching shoots, short internodes, unable to root	Von Schwartzenberg *et al.*, 1994
iaaH and iaaM	Overexpression in *P. tremula* × *P. tremuloides*	Smaller trees with elevated free and conjugated IAA; decreased axillary bud release following decapitation	Tuominen *et al.*, 1995
rolC	Overexpression in *P. tremula* × *P. tremuloides*	Reduced apical dominance, increased axillary shooting, fascinated apices	Nilsson *et al.*, 1996
rolC	Overexpression in *P. tremula* × *P. tremuloides*	Alterations in hormone levels with a decrease of ABA in pre-dormant buds and during resting; earlier flushing	Fladung, Grossmann and Ahuja, 1997
OSH1	Overexpression in *P. nigra* L. var. *italica*	Alterations in phenotypes with three relatively distinct phenotypes identified: I - slender leaves, II - dwarfed, III - multiple shoot apices with tiny leaves	Mohri *et al.*, 1999
rol genes	Overexpressed in *P. tremula*	Shorter, but more numerous internodes; axillary shooting; decreased shoot:root ratios; delayed dormancy	Tzfira, Vainstein and Altman, 1999
GA-20 oxidase	Overexpressed in *P. tremula* × *P. tremuloides*	Increased height and diameter; increased internode length; longer, broader leaves; increased number of cells; significantly longer xylem cells	Eriksson *et al.*, 2000
rolC	Overexpressed in *P. tremula* × *P. tremuloides*	Reduced shoot growth; early bud break; no change in timing of wood formation; more numerous tyloses formed; lacked thick walled late wood	Grunwald *et al.*, 2000
iaaM	Overexpressed in *P. tremula* × *P. tremuloides*	35% increase in IAA, but no change in radial distribution pattern; increased internode length; decreased occurrence of axillary bud break following decapitation	Tuominen *et al.*, 2000

the transgenics produced lower amounts of auxin or other factors that are required for cambial division (Grunwald *et al.*, 2000). Discoloration and tyloses may be associated with wounds because, in aspen, tyloses are formed when air enters the wood (Grunwald *et al.*, 2000). Thus, the *rolC* trees may be more susceptible to damage than the controls. The transgenics also did not have fully formed and lignified cell walls, possibly as a result of slower differentiation, causing incomplete development of cells formed at the end of the growing season, or a lack of a signal for latewood maturation (Grunwald *et al.*, 2000).

Gibberellins influence growth and development in plants, including effects on shoot growth, leaf growth and shape, flowering and seed germination. GA also plays a role in cell division and elongation (Kende and Zeevaart, 1997). They are formed through the isoprenoid pathway from mevalonic acid, and are regulated by transcriptional control (Hedden and Proebsting, 1997). GA20 catalyses the production of the immediate precursors to the active gibberellins GA4 and GA1. Overproduction of GA in hybrid poplar resulted in improved growth rates. Transgenics had increased height and diameter growth; increased internode lengths; and longer, broader leaves with longer petioles. The transgenic lines also showed an increase in the number of cells, and the xylem cells were significantly longer than those found in control plants (Eriksson *et al.*, 2000).

Poplar transformed with a gene encoding the rice homeodomain protein *OSH1* also showed morphological abnormalities in the leaves and stems (Mohri *et al.*, 1999). This is similar to results observed previously in rice (Kano-Murakami *et al.*, 1993), *Arabidopsis* (Matsuoka *et al.*, 1993) and tobacco (Kusaba *et al.*, 1998). There were three major phenotypes identified: type 1 had slender leaves; type 2 were dwarf plants with limited life spans; and type 3 had multiple shoot apices and tiny leaves. The expression level was highest in type 3 and lowest in type 1. The phenotype is thought to be the result of a disruption in the balance of plant hormones, as seen in other plants (Mohri *et al.*, 1999).

WOOD TRAITS

As a consequence of a rapidly growing human population, the world's forests are experiencing increasing pressures to meet demands for wood products, fuel and agricultural land. Moreover, these efforts are being met with more stringent environmental regulations and an increasing interest in sustainability. Clearly, there are huge opportunities for forest and tree biotechnology research, particularly that focused on making wood products available faster, of better quality, and with fewer negative effects on native forests and the environment in general (Boerjan, 2005). The following section highlights traits related to wood fibre chemistry, ultrastructure and growth.

Directed genetic modifications altering the quality and quantity of wood and cell wall components have been pursued by researchers for nearly two decades. Numerous modifications have been reported in model plants such as *Arabidopsis* and tobacco, but they are not useful for the study of wood. *Populus* is not only a valuable model tree, but it is also commercially important, particularly in

the Northern Hemisphere. Because of this and the ease with which it can be manipulated, the vast majority of the tree genetic modification efforts have been with species in this genus. Less frequent, but equally important, are the reports on *Eucalyptus* and industrially important conifers, such as spruce and pine. Genetic modification of genes involved in cell-wall biosynthesis fall into two categories: lignin and non-lignin cellulosic material. The altered expression or regulation of representative genes in both categories has resulted in a range of changes to the tree cell walls, from extreme to no quantifiable difference. The efforts include attempts to up- and down-regulate gene expression, and have employed both endogenes and novel sequences and promoters. Nearly all of the enzymes implicated in the currently accepted lignin biosynthetic pathway have been targeted or modified in some manner (see Table 4-5). Although not as common heretofore, modifying the expression of genes involved in cellulose biosynthesis is a rapidly growing area of interest in tree biotechnology research (see Table 4-6).

TABLE 4-5
Summary of genetic modifications in plants, targeting lignin biosynthesis

Gene	Modification	Effects	Reference
PAL	Downregulated in tobacco	Reduced lignin content, slightly increased S:G	Sewalt *et al.*, 1997
	Downregulated in tobacco	Reduced phenylpropanoid compounds in leaves and stems	Blount *et al.*, 2000
C4H	Downregulated in tobacco	Minor reduction in lignin content, minor changes to S:G	Blee *et al.*, 2001
	Downregulated in tobacco	Decreased lignin content, decreased S:G	Sewalt *et al.*, 1997
	Downregulated in tobacco	Altered *PAL* activity, decreased phenylpropanoid compounds	Blount *et al.*, 2000
COMT	Downregulated in *Populus tremula* × *alba*	4-yr field trial; no dramatic ecological/biological impacts	Halpin *et al.*, 2007
	Downregulated in *P. tremula* × *alba*	Decreased lignin content, decreased S:G	Jouanin *et al.*, 2000
	Downregulated in *P. tremula* × *alba*	Increased G, lower pulping efficiency	Lapierrer *et al.*, 1999
	Downregulated in *P. tremula* × *alba*	4-yr field trial; normal growth	Pilate *et al.*, 2002
	Downregulated in *P. tremuloides*	No change in lignin content, S:G decreased, more coniferaldehyde	Tsai *et al.*, 1998
	Downregulated in *P. tremula* × *alba*	No change in lignin content, decreased S, increased G units	Van Doorsselaere *et al.*, 1995
	Downregulated in *P. tremula* × *alba*	Lignin contains 5-hydroxyconiferyl alcohol and benzodioxane units	Ralph *et al.*, 2001
F5H	Overexpressed in *P. tremula* × *alba*	Increased S units	Franke *et al.*, 2000
	Overexpressed in *P. tremula* × *alba*	No change in lignin content, increased S:G, decreased kappa	Huntley *et al.*, 2003
4CL	Downregulated in *P. tremuloides*	Decreased lignin content, no S:G changes	Hu *et al.*, 1999
	Downregulated in *P. tremuloides*	Decreased lignin content, S:G increase,	Li *et al.*, 2003
	Downregulated in *P. tomentosa*	Decreased lignin content	Jia *et al.*, 2004
	Downregulated in *P. tremuloides*	Decreased lignin content, no S:G changes	Hancock *et al.*, 2007

TABLE 4-5 (CONTINUED)

Gene	Modification	Effects	Reference
HCT	Downregulated in *Pinus radiate*	Decreased lignin content, altered monolignol composition	Wagner *et al.*, 2007
C3H	Downregulated in *P. alba* × grandidentata	RNAi down regulation, reduced lignin, increased H units, decreased G units	Coleman *et al.*, 2007
CCR	Downregulated in tobacco	Some reduced lignin, decreased kappa	Chabannes *et al.*, 2001
	Downregulated in tobacco	Changes in transcriptome and metabalome; decreased phenylpropanoid pathway genes	Dauwe *et al.*, 2007
	Downregulated in *P. tremula* × *alba*	5-year field trail; decreased lignin content, decreased S:G, improved pulping efficiency	Leple *et al.*, 2007
	Downregulated in tobacco	Decreased lignin content, some increased S:G	O'Connell *et al.*, 2002
	Downregulated in *Picea abies*	5-yr field trial; slightly decreased lignin content, decreased H units, slightly decreased kappa	Wadenback *et al.*, 2008
Cald5H	Downregulated in *P. tremuloides*	Decreased lignin content, S:G increased	Li *et al.*, 2003
	Downregulated in *P. tremuloides*	No change in lignin content, increased S:G	Hancock *et al.*, 2007
CcoAOMT	Downregulated in *P. tremula* × *alba*	Decreased lignin content, slightly increased S:G	Meyermans *et al.*, 2000
	Downregulated in *P. tremula* × *alba*	Decreased lignin content	Zhong *et al.*, 2000
	Downregulated in *P. tremula* × *alba*	Decreased lignin content	Wei *et al.*, 2001 in Lin *et al.*, 2006
CAD	Downregulated in *P. tremula* × *alba*	Slightly decreased lignin content, increased aldehydes	Baucher *et al.*, 1996
	Downregulated in tobacco	Some reduced lignin, decreased kappa	Chabannes *et al.*, 2001
	Downregulated in tobacco	Changes in transcriptome and metabalome; decreased phenylpropanoid pathway genes	Dauwe *et al.*, 2007
	Overexpressed in tobacco	No change in lignin content; thicker cell walls	Goicoechea *et al.*, 2005
	Overexpressed in tobacco	Slightly decreased lignin content; more easily extracted	Halpin *et al.*, 1994
	Downregulated in *P. tremula* × *alba*	4-yr field trial; no dramatic ecological or biological impacts	Halpin *et al.*, 2007
	Downregulated in *P. tremula* × *alba*	Decreased lignin content, higher free phenolics, easier pulping	Lapierre *et al.*, 1999
	Downregulated in tobacco	Increased S, more easily extracted	O'Connell *et al.*, 2002
	Downregulated in *P. tremula* × *alba*	4-yr field trial; normal growth, lower kappa, higher yield, no change in insect interactions	Pilate *et al.*, 2002
	Downregulated in *Eucalyptus camaldulensis*	No change in lignin content, quality, composition	Valerio *et al.*, 2003
	Downregulated in *P. taeda*	Reduced lignin, brown wood, lignin contains dihydroconiferyl alcohol, increased aldehydes	MacKay *et al.*, 1997; Ralph *et al.*, 1997
Laccase	Downregulated in *P. trichocarpa*	No change to lignin content or composition, deformed xylem, increased phenolics	Ranocha *et al.*, 2002
Peroxidase	Downregulated in *Populus sieboldii* × *grandidentata*	Decreased lignin content, increased S:G ratio	Li *et al.*, 2003

Notes: Modifications are in trees, and some references review results in tobacco. Tree lignin comprises two main forms, guaiacyl (G) and syringal (S), and their ratio determines many characteristics of the organism. The S:G ratio is therefore an important indicative parameter of suitability for particular uses.

TABLE 4-6
Summary of genetic modifications in plants targeting cell wall biosynthesis

Gene	Modification	Effects	Reference
Xyloglucanase	Expressed in *Populus alba*	Increased cellulose content, decreased xyloglucans, decreased lignin	Park *et al.*, 2004
UDP-GD	Downregulated in tobacco	Decreased xylose-containing polymers, increased glucose/xylose content in cell walls	Bindschedler *et al.*, 2007
4CL	Downregulated in *P. tremuloides*	Compensatory increase in cellulose content due to lignin reduction	Hu *et al.*, 1999
Invertase	Expressed in tobacco	Increased cellulose content, reduced growth, increased biomass	Canam *et al.*, 2006
SuSY/UGPase	Overexpressed in tobacco	No change in cellulose content, increased biomass	Coleman *et al.*, 2006
UGPase	Expressed in *P. alba*× grandidentata	Increased cellulose, decreased lignin, increased s units in lignin, reduced growth	Coleman *et al.*, 2007
CCR	Downregulated in *P. tremula*× alba	Proportional increase in cellulose due to lignin reduction, reduced hemicellulose content	Leple *et al.*, 2007
4CL/CAld5H	*4CL*-downregulated, *CAld5H* overexpressed in *P. tremuloides*	Increase in cellulose content, reduced lignin content	Li *et al.*, 2003
AtCelA1	Overexpressed in *P. tremula*	Increased cellulose, increased hemicellulose	Shani *et al.*, 2004

Lignin content

Hu *et al.* (1999) were among the first to demonstrate the potential of genetic engineering for modifying lignin in trees for industrial applications. *Populus tremuloides* was transformed with antisense 4-coumarate:coenzyme ligase (4CL) constructs that resulted in a 45% reduction in lignin content. This dramatic decrease in total lignin, with no concurrent changes to lignin monomer composition, is advantageous to several industries, including the manufacture of pulp and paper, because lignin removal consumes large amounts of energy and reagents. Pilate *et al.* (2002) conducted a four-year field trial with hybrid poplars (*P. tremula* × *P. alba*) engineered for lower caffeic acid O-methyltransferase (COMT) and cinnamoyl alcohol dehydrogenase (CAD) activity. CAD-reduced trees demonstrated greater ease of delignification and superior yield, whereas COMT-altered trees required more energy for lignin removal. By contrast, in similar efforts with transgenic *Eucalyptus*, reduced CAD expression (antisense) resulted in no change in lignin quality and composition, or pulp yields (Tournier *et al.*, 2003). Importantly, the lignin composition changes seen in the field-grown transgenics studied by Pilate *et al.* (2002) were maintained over the four-year trial. More recently, it was shown, using these same trees, that there does not appear to be dramatic changes in the local insect and soil microbe communities surrounding the transgenic plots. This suggests that, depending on what modifications are done, there may be little or no negative ecological impacts of growing transgenic trees (Halpin *et al.*, 2007). However, it must be emphasized that ecological studies are complicated and critically needed in order to assess more fully the impact of transgenic trees. Furthermore, longer-term trials will be required to fully appreciate the potential for unexpected changes and effects.

Measuring changes in the transcriptome and metabolome of CAD- and cinnamoyl CoA reductase (CCR)-modified tobacco plants has revealed that altering one gene in the lignin biosynthetic pathway affects the expression of other genes within the same pathway, as well as genes involved in detoxification, carbohydrate metabolism, and photorespiration (Dauwe *et al.*, 2007). Although the effect of genetic modifications to forest trees will surely differ from those of tobacco, this research provides an example of the changes that can occur. In fact, more recently, the metabolism and transcript changes in response to CCR down-regulation in *P. tremula* × *P. alba*, which had decreases in total lignin and an increase in G monomer units, suggest that a stress response was elicited (Leple *et al.*, 2007). Also, a general decrease of transcripts related to non-cellulosic cell-wall polymers was observed. Although pulping efficiency was increased for wood from these transgenics, the trees in this five-year-old field trial were stunted. These reports emphasize the importance of long-term field trials and the need to assess non-target effects. Although over 200 field trials exist throughout the world (FAO, 2004; Boerjan, 2005), no published reports have evaluated transgenic trees modified for wood traits over the normal rotation of a forest plantation. This is a critical hole in bridging the gap between tree biotechnology and practical uses, and in gaining public acceptance.

Although the technology for genetic modification of conifers has existed for several years (Ellis *et al.*, 1993), the production and growth of genetically modified conifers is slow and lags behind similar work in angiosperms. Recently, however, Wadenback *et al.* (2008) reported a slight reduction in lignin content (8%) in five-year-old antisense CCR Norway spruce (*Picea abies*). In comparison, down-regulation of CCR activity in tobacco and poplar has demonstrated as much as a 50% reduction in lignin content (Chabannes *et al.*, 2001; Leple *et al.*, 2007). As was reported with the CCR-altered tobacco and poplar, the down-regulation of CCR in spruce led to narrower stems: a form of stunting. Although the reduction in lignin content of the modified spruce is at the lower limit of biological variation (Wadenback *et al.*, 2008), it is important to note that these findings are in an economically important conifer. These results also suggest that it may be necessary to target multiple genes to achieve the desired lignin modifications in trees.

Lignin composition

Equally important to reducing lignin content for downstream processing is altering the composition of lignin monomers to improve the overall delignification process. An increase in the lignin S:G monomer ratio has been clearly shown to improve the manner and the efficacy of pulping wood (Chang and Sarkanen, 1973; Stewart, Kadla and Mansfield, 2006; Mansfield and Weiniesen, 2007). Over the last decade, substantial effort has been devoted to altering monomer composition (Table 4-5). A significant reduction in total lignin content and a concurrent decrease in S monomers has been achieved by sense-suppression of COMT under the regulation of the 35S promoter (Jouanin *et al.*, 2000). Alternatively, the

overexpression of COMT under the regulation of the *Eucalyptus* CAD promoter resulted in only slight increases in COMT activity in some lines, but the increased COMT activity did not result in altered S:G ratios (Jouanin *et al.*, 2000). This result is interesting because, based on the currently accepted lignin biosynthetic pathway, one would expect that increasing COMT would drive biosynthesis toward S units. The results achieved to date point to the need for careful promoter and gene selection when making targeted genetic modifications.

By down-regulating caffeoyl-coenyzme A O-methyltransferase (*CCoAOMT*), Meyermans *et al. (*2000) were able to generate transgenic lines of hybrid poplar displaying an 11% increase in the S:G ratio, along with a 12% decrease in lignin content. Alternatively, the successful expression of the *Arabidopsis* ferulate-5-hydroxylase (*F5H*) gene under the regulation of the cinnamate 4-hydroxylase (C4H) promoter, employed by Franke *et al. (*2000), led to a substantial increase in the composition of syringyl monomers in *P. tremula* × *P. alba*, at greater than 90% mol S lignin (Huntley *et al.*, 2003). The pulping efficiency of these trees clearly showed dramatic decreases in the energy required for chemical pulping (delignification), with a 23 kappa unit decrease compared with control trees. In addition, pulps had a higher ISO brightness value. In combination, these transgenic trees provide an excellent opportunity to decrease the energy and chemicals required for extracting lignin and obtaining a high-quality pulp.

In an effort to decrease total lignin content while also altering the lignin monomer ratio, Li *et al. (*2003) used a combinatorial approach by simultaneously decreasing 4CL activity and increasing coniferaldehyde 5-hydroxylase (CAld5H) expression in *P. tremuloides*. The authors reported lignin content reductions as high as 52% and concomitant increases in the S:G ratio. This work highlights the potential benefit from concurrent augmentation and reduction of different gene products within the lignin biosynthesis pathway.

Efforts to modify cell wall polysaccharides

Genetic modification in trees has resulted in increased cellulose content, both directly and indirectly (Table 4-6). For example, efforts to engineer trees with altered lignin composition has demonstrated the added advantage of indirectly improved cellulosic quantities per unit mass, as demonstrated by Hu *et al. (*1999) and Li *et al. (*2003). Park *et al. (*2004) have successfully increased cellulose and decreased xyloglucan contents in *P. alba* by expressing a fungal xyloglucanase gene. Similarly, *P. tremula* transformed with an *Arabidopsis* endoglucanase (*cel1*) were shown to have a 10% increase in cellulose content (Shani *et al.*, 2004). More recently, Coleman *et al. (*2007) have shown that transgenic *P. alba* × *P. grandidentata* trees expressing a bacterial UDP-glucose pyrophosphorylase (UGPase) gene have substantially increased cellulose content, and decreased lignin. However, these trees grew more slowly than the controls. Alternatively, in tobacco, the expression of yeast-derived invertases has been shown to result in decreased growth rates, but some plants accumulated more biomass and up to 36% more cellulose (Canam *et al.*, 2006). These genetic modifications indicate

that altering carbon allocation is possible, but optimizing cellulose production and growth requires further work.

The identification of genes and enzymes involved in cellulose and hemicellulose biosynthesis is ongoing. For example, Suzuki *et al.* (2006) identified a xylem-specific mannan synthases from *P. trichocarpa* by comparing orthologous genes from *Arabidopsis* and subsequently measuring mannan synthase activity *in vitro*. Looking globally at the genes and proteins involved in cellulose-rich G layer production in poplar, Andersson-Gunneras *et al.* (2006) also revealed some potential targets to alter cellulose production, or perhaps cellulose extractability. The identification of functioning xyloglucan transglycosylases (XETs) in developing secondary xylem in aspen (Bourquin *et al.*, 2002) and its involvement in the formation of the G layer in tension wood (Nishikubo *et al.*, 2007) suggests that XET may be a potential target to modify cellulose properties. If XET is involved in establishing cross-linkages between cellulose microfibrils, altering its expression could greatly affect fibre structure.

IMPLICATIONS AND FUTURE DIRECTIONS

Although not all efforts have led to an improvement in industrial processes, they have contributed significantly to our understanding of the fundamental mechanisms of cell wall synthesis and formation. For example, a 90% reduction in *CCoAOMT* activity in transgenic poplar only led to an 11% decrease in lignin content (Meyermans *et al.*, 2000), suggesting that *CCoAOMT* has minimal control over the flow of carbon through the lignin pathway (Anterola and Lewis, 2002). Very recently, Wagner *et al.* (2007) revealed a functional hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (HCT) in *Pinus radiata* tracheary elements. This gene had not previously been implicated in lignin biosynthesis in gymnosperms, and may be a new target to genetically modify lignin for forestry and biofuels.

Understanding the role of genes involved in cellulose biosynthesis lags behind the progress that has been made with phenylpropanoid biosynthesis. Along with the CesA complex itself, the roles of genes such as sucrose synthase (*SuSy*), sucrose phosphate synthase (*SPS*), invertase, *UGPase* and korrigan (*KOR*), to name a few, are examples of genes or gene families that are currently under investigation (for a review, see Joshi and Mansfield, 2007). As information from these studies becomes available, it is very likely that new opportunities for modifying cellulose production, cell wall architecture and growth in trees will become apparent.

The identification of other genetic elements, such as transcription factors, is also an area of tree biotechnology research that affects wood traits. Legay *et al.* (2007) identified a MYB transcription factor, EgMYB1, in Eucalyptus xylem cDNA that represses lignin biosynthetic genes. This discovery could lead to new approaches for modifying wood. For example, Goicoechea *et al.* (2005) overexpressed EgMYB2 in tobacco and showed that the plants had slightly higher S:G, and thicker cell walls. Although no change in total lignin was reported, there could have been an increase that was offset by the additional cell-wall mass (perhaps because of

other cell-wall components). This requires further investigation. Tobacco was also used as a model to study the function of a pine MYB, PtMYB4 (Patzlaff *et al.*, 2003). The authors found that PtMYB4 expression in tobacco caused an increase in overall lignin. Also, identification of transcription factors that affect secondary cell wall deposition in *Arabidopsis* (e.g. Ko *et al.*, 2007) may provide yet another avenue to genetically modify wood traits. Undoubtedly, understanding the role that transcription factors play in lignin and cellulose biosynthesis will be necessary for downstream modifications to be effective.

Other biotechnology applications, such as activation tagging (Weigel *et al.*, 2000) in poplar may also reveal useful and novel modifications that affect cell-wall quality and quantity. Phenotypes from activation-tagged trees have been reported (Busov *et al.*, 2003) and will continue as thousands of activation-tagging transgenic lines are evaluated in the greenhouse and in the field (Harrison *et al.*, 2007; Arborea project, www.arborea.ulaval.ca/). Finally, the identification of microRNAs involved in tree-specific mechanisms, such as tension wood formation (Lu *et al.*, 2005), is yet another area of research that will soon contribute greatly to our understanding of cell-wall biosynthesis and regulatory mechanisms of wood formation, all of which will be important for identifying future targets of tree biotechnology.

GROWTH

One of the major goals of plant research is to increase yield, primarily in the form of increased stem biomass through an increase in height and diameter. Numerous, indirect approaches have been used (Table 4-7), some of which were mentioned in previous sections of this chapter. Here we focus on transformations that have resulted in increased growth.

One approach has been to transform poplar with the gene encoding uridine diphosphoglycosyl-transferase, *ugt*, which catalyses the conjugation of IAA with

TABLE 4-7
Summary of genetic modifications in trees affecting growth

Gene	Modification	Effects	Reference
4CL	Antisense inhibition in *Populus*	Increased plant growth; structural integrity maintained	Hu *et al.*, 1999
GS1	Overexpressed in *Populus*	Increased node and leaf number, larger leaves; increased growth; enhanced nitrogen assimilation and increased growth under both high and low nitrogen conditions	Fu *et al.*, 2003, Man *et al.*, 2005
Xylo-glucanase	Overexpression in *Populus*	Increased stem length and internode length	Park *et al.*, 2004
cel1	Overexpression in *Populus tremula*	Increased growth, larger leaves; increased stem diameter	Shani *et al.*, 2004
ugt and *acb*	Overexpression in *Populus*; sense and antisense expression of *acb*	*ugt* plants show increased growth; *ugt* and *acb* lower growth than *ugt* alone; sense *acb* show increased growth; antisense *acb* show decreased growth	Salyaev *et al.*, 2006
vhb	Overexpression in *Populus*	Increased height and stem diameter	Zhang *et al.*, 2006
PttEXPA1	Overexpression in *Populus*	No change in height; increased internode length, fibre diameter and vessel element length; increased leaf expansion	Gray-Mitsumune *et al.*, 2007

glucose, allowing for a larger pool of IAA for transport. A second gene, *acb*, encoding acyl-CoA binding protein was also used in both sense and antisense directions. Its function is not known, but it is thought to help in the stabilization of membranes (Salyaev *et al.*, 2006). Transgenics overexpressing *ugt* and *acb* showed faster growth, along with elevated IAA concentrations. Transgenics also showed rapid bud and branch development. The height growth of the resultant *ugt* transgenics was about three times that of the control plants, and root elongation was greatly enhanced. Transgenics containing both *ugt* and *acb* had lower height growth than those with *ugt* alone. In contrast, poplar with sense *acb* grew faster than controls and those with *acb* antisense. The increased growth caused by *ugt* was either reduced or cancelled by the effects of *acb* mis-regulation (Salyaev *et al.*, 2006).

Poplars overexpressing an expansin gene, *PttEXPA1*, were recently shown to have increased stem internode length, increased leaf expansion, and larger cells in its leaf epidermis. Fibre diameter growth was increased, as was vessel element length (Gray-Mitsumune *et al.*, 2007). Additionally, poplar overexpressing the *Vitreoscilla* haemoglobin gene showed no significant morphological differences, but three lines had noticeably higher height and diameter growth rates (Zhang *et al.*, 2006). Although gains have been made in the area of increased biomass, more work is required as the trade-off between increased growth and fibre quality in trees is paramount to commercial end uses.

Flowering control

Before genetically engineered trees can be commercialized, governing bodies will probably require a solid strategy to mitigate the risk of transgene spread and persistence in the environment. One way to satisfy this need is to control flowering (Meilan *et al.*, 2001). The manipulation of flowering can provide many benefits, such as development of a strategy to genetically engineer reproductive sterility. This may help alleviate, or at least reduce, public and regulatory concerns over the commercialization of transgenic trees. Sterility can also reduce genetic pollution from non-transgenic plantations, promote vegetative growth, and eliminate nuisance tissues (e.g. pollen, seed pods). In addition, flowering control may lead to shorter juvenile periods, resulting in shorter breeding cycles.

It is assumed that trees engineered for flowering control will re-direct photosynthate to harvestable products while, at the same time, minimizing gene flow to wild populations. Different types and degrees of sterility may be obtained via polyploidy (e.g. triploids or aneuploids), by genes specifically controlling male or female floral development, or genes controlling the onset of maturation. Ideally, flowering control should be reversible, so that with appropriate stimulus, the tree can be used for conventional breeding.

While each strategy for engineering sterility has advantages, it is unclear which method will work best with trees. Hence, tests are under way that involve a variety of techniques, such as tissue-specific ablation, dominant negative mutations and post-transcriptional gene silencing, including RNA interference. Employing the first approach, Skinner *et al.* (2003) successfully used the promoter from PTD, the

Populus trichocarpa homolog of the *Arabidopsis APETALA3* gene, to drive the expression of reporter and cytotoxin genes in floral tissues of *Arabidopsis*, tobacco and poplar.

Recently, RNA interference (RNAi) was used to reduce expression of the poplar ortholog of CENTRORADIALIS (*PtCENL1*), a gene that plays a key role in maintaining trees in a juvenile state (Mohamed, 2006). When transgenic poplars containing this RNAi vector were grown under field conditions, four of the most strongly silenced lines produced inflorescences or floral buds within two years of planting, which was several years earlier than that observed in wild-type trees. Surprisingly, overexpression of *PtCENL1* also resulted in delayed vegetative budbreak (Mohamed, 2006). Based on this work, it appears that *PtCENL1* is involved in regulating release from winter dormancy and resumption of growth. Hopefully, this work will ultimately lead to the development of methods for shortening breeding cycles, as well as possibly informing further research on flowering control.

Despite indications that one or more of the strategies involving flowering control can be successfully employed to engineer transgene confinement, no single method fulfils the basic requirements for long-term commercial use. Researchers are continuing to determine whether sterility can be complete and stable over several rounds of propagation and growing seasons, successfully identified in juvenile trees, and lack negative growth impacts.

CONCLUSIONS

The modulation of complex traits such as tree growth, yield, chemical composition, morphology, and health is of vital interest to the plant biotechnology community. These characteristics are influenced by a multitude of environmental and genetic factors. Availability of the full *Populus* genome sequence, along with recent advances in transcript, protein and metabolomic profiling, will continue to lead to a better understanding of genetic modifications and regulation in trees. They will also provide new insights that will be needed to resolve uncertainties concerning the molecular processes that underlie wood formation, growth and plant-environment interactions. These advances, coupled with an improved understanding of the genes and enzymes involved in key metabolic pathways, should enable the genetic manipulation of trees so they will possess the desired properties and produce sufficient volume to satisfy society's ever-increasing demand for forest products.

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5. Integrating genetically modified traits into tree improvement programmes

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Forest tree breeding programmes have typically been based on the progressive population improvement that accrues in breeding populations (White, Adams and Neale, 2007). Seed orchards or clonal stool beds serve as the means of delivering genetic gain using current top-ranked selections from within breeding populations, rather than representing the actual breeding process. A breeding population is usually initiated by selecting superior phenotypes that are both intermated and subjected to evaluation (Namkoong, Kang and Brouard, 1988). After the first generation, the breeding population undergoes recurring cycles of selection, intermating, evaluation, selection and so on, to build up frequencies of favourable alleles of additive effects that will confer cumulative genetic gain. For intermating and evaluation, various crossing designs and field testing schemes may be used (Namkoong 1979; White, Adams and Neale, 2007). Of central importance here, however, is that selection has almost always been based on phenotypic data from individual candidates or relatives, or both.

There are many variations of this process of tree breeding based on cumulative population improvement. For instance, it can be implemented within a single base population, or it can involve hybridization of populations (Fins, Friedman and Brotschol, 1992; White, Adams and Neale, 2007). But, whatever the detailed form that it takes, this process is termed conventional breeding. It depends on naturally occurring genetic variation, unless supplemented by deliberate mutagenesis. And it has the limitation that, with the sexual reproduction that it entails, half of any parent's genes get passed on to offspring. That means that, along with the parent's desirable genes, many unwanted ones will almost certainly be passed on. Eliminating the undesirable genes, by further crossing and selection, is a slow and cumbersome process that will typically take many generations even to approach completion.

Genetic modification, often termed genetic engineering, removes both these limitations. It can be used to introduce DNA sequences (not necessarily complete genes) that are not available in populations of sexually compatible material, or even in nature, and the sequences can be introduced without introducing the unwanted 'genetic baggage' that sexual reproduction brings (El-Kassaby and Mansfield, 2007).

Despite these additional capabilities, genetic modification is seen not as a replacement for conventional breeding but more as a complement to it. To address

the complementarity, the technical options for genetic modification are first reviewed briefly, followed by a review of what has been entailed in conventional breeding, and an outline of how the process can be enhanced by an array of new gene technologies. The appropriate nature of the complementarity must be governed by the fact that conventional breeding entails not only the breeding proper, but also the systems for mass production of either seed from seed orchards, or of planting stock through vegetative propagation as rooted cuttings or by means of tissue culture techniques, such as organogenesis or somatic embryogenesis (El-Kassaby and Krakowski, 2004; Sutton *et al.*, 2004). These aspects are involved in a complex interplay, which must be examined.

GENETIC MODIFICATION OPTIONS

Genetic modification relates in this context to asexual technologies for generating new genetic combinations. This may take several forms:

- Genetic transformation, involving the insertion of short and specific sequences of DNA into chromosomes, to incorporate new, functioning structural genes or to modify activity levels of 'resident' genes that are already present. Collectively, the various classes of inserted DNA sequences are all referred to here as transgenes. Genetic transformation is now very much the preferred approach for genetic modification.
- Somatic hybridization by fusing cells containing different genomes (each containing n or 2n chromosomes), and regenerating whole plants from such fusion events (Ma *et al.*, 1998).
- Production of 'cybrids', containing the nuclear genome of one species or population with either or both of the organelle genomes of another (Pelletier *et al.*, 1983).

Further discussion in this chapter will focus on the case of genetic transformation, given its overwhelmingly preferred status.

CONVENTIONAL BREEDING

Choice of appropriate base populations will remain an essential platform for any genetic improvement. It can be based on general adaptation or basic economic worth, or on special attributes that can be incorporated into domesticated stocks. The choice, once made, sets the base for the recurrent cycles, already referred to, of selection, intermating, evaluation, selection, and so forth.

The phenotypic information used in conventional breeding can be extended beyond expression of field performance. It can include performance under special screening conditions (e.g. response to frosting in growth rooms (Aitken, 2004), or response to inoculation by pathogens in laboratory conditions (Kinloch and Libby 1997). Such specific phenotypic information is effectively a more direct expression of genes of interest. However, there are various ways of screening on the basis of more immediate gene expression, such as using tissue conductance as a measure of frost resistance (Benowicz, L'Hirondelle and El-Kassaby, 2001), or assays for metabolites that are crucial steps towards phenotypic expression (Robinson *et al.*,

2007). The latter approach, however, can be bedevilled by strongly non-linear relationships, or the fact that metabolite fluxes can be more important than the concentrations that are easier to measure, or a combination.

An ideal has long been to locate, and better still identify, genes of desired effects (Neale and Ingvarsson, 2008). Locating polymorphic chromosome regions (termed quantitative trait loci (QTLs)) that exercise detectable phenotypic effects can be exploited by marker-assisted or marker-based selection (collectively, MAS). The actual genes concerned, if they can be characterized, are identified as quantitative trait nucleotides (QTNs), but this again depends on the individual genes having appreciable phenotypic effects (González-Martínez *et al.*, 2006). Past tree breeding has proceeded on the assumption that genetic control of traits of interest in forest trees is strictly polygenic, with allelic differences at individual loci exerting infinitesimal phenotypic effects (Fisher, 1941). With imperfect heritability, however, genes of quite large phenotypic effects may be present without being evident from purely phenotypic data, yet experience with various crop species indicates that such genes of quite large effect are often lurking (Thoday, 1961, 1976), undetected until recently developed genomic analysis has become possible. Such genes, however, may have been mutations favoured in a very long history of domestication (e.g. in maize; Szabó and Burr, 1996), or have been major points of differentiation among populations or even species that have been fused in the course of domestication. For forest trees, detecting such genes of substantial phenotypic effects has often been problematic, with many reported QTLs proving to have been false positives due to the hidden genetic admixture of the studied population (Pritchard, Stephens and Donnelly, 2000; Pritchard, Wen and Falush, 2007). Disease resistance, however, is a case where some major-gene effects can evidently operate in essentially wild, undomesticated populations (Wilcox *et al.*, 1996; Amerson *et al.*, 2005).

Even if new gene technologies are used to identify QTLs or QTNs that thence can serve as selection criteria, this will remain fundamentally an enhancement of classical breeding, rather than an actual application of genetic modification.

Exploiting interactions between genes, in the form of non-additive gene effects (allelic dominance or epistasis between loci, or a combination), generally falls outside the framework of long-term tree breeding. A possible exception is use of reciprocal recurrent selection (or variations thereof), which may have applications in forest trees if different species or populations are bred to produce F_1 hybrids for commercially deployed stocks. Possible examples include interspecific hybrids of poplar (e.g. White, Adams and Neale, 2007), or an intervarietal hybrid of *Pinus radiata* (Low and Smith, 1997). Nevertheless, exploiting non-additive gene effects has been part of the classical breeding tradition, most notably in intercrossing distinct populations (which may even represent different species, e.g. poplars), or in hybrid maize breeding (Simmonds, 1979), which has depended on exhaustive searches for strong heterotic effects in crosses between highly inbred lines. For forest trees, only part of the total non-additive gene effects can be captured by making specific pair-crosses. Identifying the appropriate crossing combinations

can be very difficult and costly but, if this is achieved, vegetative multiplication of offspring may greatly reduce the effective costs of controlled crossing. Clonal forestry in principle offers full capture of non-additive gene effects, but they must be captured afresh each generation rather than contributing over time to cumulative genetic gain.

Where disease resistance is sought, one needs durability against pathogen-strain shifts, through either fresh mutations or changes in frequencies of existing strains. Since durability evidently depends on the presence of multiple resistance mechanisms, we have a situation where epistatic gene effects can be extremely important, yet only targeted readily if resistance-gene effects are quite large.

Population improvement, along the lines of conventional breeding, remains very much the method of choice for exploiting polygenic variation, which will probably be large for many traits in forest trees and yet virtually impossible to characterize as either QTLs or QTNs. Moreover, large populations might be needed to prevent loss of valuable alleles, especially for traits like disease or pest resistance. While genetic modification may have potential for incorporating pathogen resistance, it can face the practical difficulty that many biotic risks may not be identifiable in advance (e.g. pitch canker in Monterey pine).

In many crops, notably cereals, phenotypic uniformity can be crucial to commercial success. This has doubtless led, to varying degrees, to crops shifting to an inbreeding system with pollination being predominantly self-fertilization. Because the inbreeding leads to major purging of genetic load, there is often no obvious inbreeding depression, quite unlike the typical situation with forest trees.

COMBINING GENETIC MODIFICATION WITH CONVENTIONAL BREEDING

Genetic modification can be combined with conventional breeding in two ways:

- Confining the application of genetic modification to clones produced by the breeding programme; in other words, confining the application to a context of clonal forestry. This basically superimposes genetic modification on the population improvement that is at the core of properly constituted tree-improvement programmes.
- Applying genetic modification to members of the breeding population, such that the inserted DNA sequences are included in the genes that are subject to the genetic recombination that naturally occurs during sexual reproduction. This would be true integration of genetic modification with conventional breeding.

Prevailing opinion (e.g. Burdon and Libby, 2006) is that, for the time being, any pursuit of operational use of genetic modification in forest trees would be based on the former option. In it, transformation would be done afresh on clones selected from untransformed and largely traditional breeding populations.

By contrast, the second option is the way in which genetic modification has been widely applied for annual crops, notably cotton, soybean, canola and maize. In it, transgenes are incorporated into what are effectively the breeding populations, and are delivered into commercial crops by sexual reproduction. It is

appropriate to examine critically the reasons for this contrast, and several reasons stand out:

- The very short generation time for annuals makes it much easier to select effectively for stable expression without adverse side effects on field fitness (although generation intervals can be greatly reduced in some tree species), and to fix the transgenes.
- With the relatively small plants of annuals it is also easier to achieve high intensities for such selection (which could be partly achievable with those forest trees in which generation intervals can be greatly foreshortened).
- Seed propagation is effectively obligate in the annuals, in contrast to many forest trees.
- The only way to achieve crop uniformity with such annuals is by use of intensive inbreeding, in contrast to clonal propagation of forest trees.
- With such inbreeding, the purging of genetic load can be a help in breeding.
- With clonal propagation there is no need to fix transgenes.
- In some of the annual crops, polyploidy would confer some protection against the genetic damage associated with insertional events, whereas the domesticated forest tree species are mostly diploid.
- In annual crops the requirements for field fitness may be less stringent than they are for forest trees.

SUPERIMPOSING GENETIC MODIFICATION UPON POPULATION IMPROVEMENT

Operational use of genetic modification on preselected clones, in conjunction with appropriate risk-management protocols (below), is seen as the precautionary approach. If mass clonal propagation is possible for a species, there will be no need to have any transgenes in a homozygous state, provided they behave as dominant alleles whose presence and activity in the heterozygous state should be easy to confirm. When once identified as containing the desired transgene(s) candidate clones from the breeding population can then be subjected to the usual testing, which should confirm transgene function and lack of adverse side effects of the insertional event(s) and/or the trangene(s), as well as evaluating general performance.

If use of transformation is confined to deployed clones, the requirements for control of flowering may be limited to sterility conferred by inserted transgenes. While the feasibility of conferring complete sterility is debatable, sterility may be a regulatory requirement to assure transgene containment. If conferred successfully, it would have the bonus of avoiding diversion of important resources from producing wood, with the expectation of an increase in wood production (Burdon and Libby, 2006). Yet with genetic modification confined to commercially deployed clones, there would be no need to reverse sterility for flowering on command.

The model described above implies a tandem process of testing candidate clones for basic merit and then testing them after transformation for effect of

transformation. Simultaneous genetic transformation beginning at the seed-embryo stage or soon after it, on multiple candidate clones, to be tested jointly for both successful transformation and overall genetic merit, would pose logistical and technical challenges. There should be scope for prompt initiation of a multistage culling process, eliminating many candidates before even beginning field tests. However, testing both transformed and untransformed ramets of the same clones would seem indicated.

FULL INTEGRATION OF GENETIC MODIFICATION INTO POPULATION IMPROVEMENT

To apply genetic modification in a context of mass propagation by seed would certainly entail full integration of genetic modification into population improvement. This, however, appears problematic, at the least. To achieve fixation of transgenes without expression of genetic damage from gene insertion, yet with stable vertical transmission through generations assured, could be very slow and difficult – much more so than achieving stable integration without adverse side effects in the heterozygous state in deployed clones. Problems of transgene containment would loom very large, given that the breeding population depends on sexual fertility, creating a strict requirement for flowering 'on command and command only' (Brunner *et al.*, 2007).

A context of clonal forestry would be more consistent with the higher level of domestication entailed in use of genetic modification. In this situation, requirements for integrating genetic modification with population management would seem less, but still stringent. It would not be essential to fix transgenes, provided individual offspring can be screened cheaply and early for successful vertical transmission.

An advantage of using genetic modification within a breeding population is that if a transgene is known to show stable vertical transmission, without adverse side-effects, it can be used indefinitely without risks attendant upon re-creating the transformation. At the same time, availability of new transgenes may render it obsolete.

With clonal forestry for deployed crops, only a subsample of a total breeding population would have to be subjected to genetic modification. Choice of subsample, however, might not be straightforward. On present knowledge, choice of subsample may be guided by a need to provide for some failures of vertical transmission through seed. Also, transformation will be needed in sufficient parental clones to assure an appropriate genetic base in the deployed population. The requisite number will depend, as with transformation on deployed clones, on the risk-spread needs for the operation. The needs will be governed, *inter alia*, by the status of the species concerned in any broader frame of risk spread. However, even for a large, stand-alone operation, further risk-spread protection becomes limited as numbers of deployed clones exceed 15 to 20 – the standard error of performance varies according to the inverse of the square root of the effective number of clones, but the confidence limits about expected performance would

decrease more rapidly than that. However, additional provision should be made for transformed parents not contributing to the pool of selected clones. If a breeding population is structured so as to contain specialized breeds, representing different breeding goals (cf. Jayawickrama and Carson, 1990), the particular transformations may be confined to particular breeds, to serve the specific breeding goals.

Note that with multiple transgenes, integration with the breeding population is likely to be less straightforward than with single or very few transgenes.

RISK-MANAGEMENT ISSUES

Accepting that use of genetic modification for forest trees will be based on transformation of pre-selected clones, the existing risk-management protocols for clonal deployment, which are based largely on risk spread (Burdon and Aimers-Halliday, 2006) would remain essentially in place. However, these protocols would need to be supplemented by protocols for managing risks specifically associated with genetic modification (e.g. Burdon, 1999; Burdon and Walter, 2004). A lesson learned from the Southern corn blight epidemic that occurred in the United States of America in 1970 is seen as being relevant to use of genetic modification with forest trees. A massive reliance on a single organelle gene, the Texas male sterile cytoplasmic factor, which was used to simplify the production of 'hybrid maize', contributed greatly to the severity of the epidemic. This was because the gene concerned destroyed the resistance to a mutant strain of the Southern corn blight pathogen (Levings, 1990). The side-effect took many years to become manifest, but was not totally disastrous because the epidemic did not spread quickly enough to destroy crops over the entire United States of America Corn Belt, while there were sufficient stocks of unaffected varieties to meet sowing needs in the following year. The long delay in the side-effect becoming evident, if it involved forest trees, could be disastrous, as it could involve many years' plantings, instead of just one year's sowings. Admittedly, the parallel with genetic modification is by no means exact (Burdon and Walter, 2004). Whereas the cytoplasmic modification involved an organelle genome, genetic transformation is directed at the nuclear genome, although the male sterility was similar to what is often sought now through genetic modification. And, while pathways of gene action can now be traced much more readily, thus improving the chances of predicting unwanted side-effects of transgene action, massive reliance on any single transgene and any single insertion event may still pose some risks. While such risks may involve low probability events, they can be significant because they could involve extreme consequences. Achieving risk spread not only among clones, but also among transgenes to achieve a given objective and among insertion events, would pose challenges, challenges that could be much greater in the context of practising genetic modification within a breeding population.

NUMBERS OF GENES INVOLVED IN GENETIC MODIFICATION

For various reasons the breeder may want to insert multiple transgenes, a measure termed stacking. Engineering sterility (if possible) has already been mentioned as

both a potential regulatory need and a likely boon to commercial productivity. There might also be cases where several other transgenic attributes are sought. However, intensive stacking has its own potential risks. Even if it can be achieved without increasing genetic damage through multiple insertion sites, the number of potential interactions between different transgenes multiplies dramatically as transgene numbers increase (Burdon, 1999). It would only take occasional significant adverse interactions to be disastrous. Speculatively, incorporating multiple transgenes into single constructs, while it may reduce insertion sites, could accentuate interactions among the transgenes.

A specific case where stacking may eventually be strongly indicated is with disease resistance, for which multiple resistance mechanisms may be needed in order to ensure durability of resistance in the face of genetic shifts on the part of a pathogen. This would achieve the same result as 'pyramiding' multiple resistance factors by classical breeding, which can be difficult without gene discovery because some resistance genes can mask the expression of others. Indeed, a 'hybrid' approach could be used, supplementing naturally occurring resistance factors with transgenic ones in order to achieve effective pyramiding (Burdon and Wilcox, 2007; Wilcox, Echt and Burdon, 2007).

Of special interest is a move to incorporate resistance to chestnut blight in American chestnut by genetic modification, with the eventual aim of restoring the species to its former ecological status (Burdon and Libby, 2006). This is a notable exception to the usual rule of genetic modification being reserved for a context of intensive domestication.

Different classes of transgenes, which include up-regulating, down-regulating and gene-silencing sequences, as well as complete alien genes, will doubtless incur different risks of adverse side-effects. A provisional categorization of risk levels for different transgene categories was given by Burdon and Walter (2004), but better knowledge of such risks will surely come with time.

A precautionary approach based on avoiding intensive stacking of transgenes should not greatly impede the application of genetic modification, because transformation is used to incorporate DNA sequences of large phenotypic effect, which is likely to mean a focus on small numbers of transgenes in any one breeding programme. Capturing polygenic variation conditioned by factors that are widely dispersed through the genome is clearly not a realistic option for genetic modification.

DEPENDENCE ON PROPAGATION TECHNOLOGIES

Successful use of genetic modification is enormously dependent on adequate propagation technologies. Transformation itself depends on a suitable platform of *in vitro* propagation technology. In many species, major technical challenges remain in achieving transformation with recipient genotypes of choice. And even if successful transformation is achieved, the genotypes for commercial deployment need to be amenable to mass multiplication from *in vitro* culture or more conventional vegetative propagation, or both (e.g. nursery cuttings). If

amenability to *in vitro* culture, transformability and mass-multiplication from *in vitro* culture have to become selection criteria, gain in breeding-goal traits will tend to be eroded or the genetic base of deployed material will be compromised.

THE SIGNIFICANCE OF GENE DISCOVERY

Detection and use of QTL is a vexed issue for forest trees (e.g. Wilcox, Echt and Burdon, 2007), because the typical outbreeding behaviour means that there is very limited general linkage disequilibrium (Brown *et al.*, 2004), which means in turn that QTL-marker associations are mostly pedigree-specific. Gene discovery has promise for making pervasive contributions to intensive, long-term genetic improvement of forest trees (Burdon and Wilcox, 2007; Wilcox, Echt and Burdon, 2007). Admittedly, it is likely to be relatively slow to achieve with forest trees, especially with the very large genomes of conifers. The use of association genetics with trees can be slow and expensive, since it will involve either causal DNA sequences or linkages over very limited segments of chromosomes (Neale and Savolainen, 2004). Thus, for gene discovery, it will almost certainly need to be heavily supplemented (if not supplanted) by identifying candidate genes from other plants and then confirming their roles in the trees. As indicated earlier, gene discovery, if achieved, can allow selection on the basis of genes of large effect (if they are present) within the framework of classical breeding. Alternatively, such genes when once identified can be cloned and then used for transformation. Moreover, genetic modification followed by observation of phenotype can serve to verify gene discovery.

POTENTIAL IMPACTS OF FUTURE ADVANCES IN GENE TECHNOLOGY

Engineering sterility, preferably as complete suppression of reproductive structures, has been mentioned as an ideal. Its feasibility, though, is still controversial, but will surely be intensively researched. Gene discovery, apart from the inherent difficulties with forest trees, especially conifers, could pose major challenges (Burdon and Wilcox, 2007; Wilcox, Echt and Burdon, 2007). Some of the challenges arise from the fact that large phenotypic effects can be governed by gene regulatory sequences and possibly sequences that code for RNA with poorly understood roles in developmental processes (see references in Burdon and Wilcox, 2007).

Luo *et al.* (2007) report a technology that has promise for excising transgenes during sexual reproduction, to avoid transmission in seed or pollen. Its potential for forest trees, however, is very problematic. It would not prevent unwanted diversion of resources into reproduction, nor would it favour full integration of transgenes into breeding populations without adding a process to the actual breeding operation.

ALLOCATION OF RESOURCES AMONG TECHNOLOGIES

In any genetic improvement we now have an array of different technologies that include genetic modification. While different technologies can be used

complementarily, and even synergistically, they can often make strongly competing demands on available resources for research and development. This will create new organizational challenges for optimizing allocation of resources among the various technologies, new and old. Whatever the potential for gene discovery, phenotypic expression will remain a necessary benchmark, if only for calibrating new screening technologies. Achieving the requisite phenotypic expression with typical forest trees will remain slow and expensive, such that intensive genetic improvement cannot in the foreseeable future be undertaken lightly.

CONCLUSIONS

The topic of this chapter has so far been addressed only very tentatively, if at all, in operational tree breeding programmes. How the integration will actually occur will doubtless depend on many technical developments that are still very uncertain. Accordingly, much of the emphasis has been on reviewing the issues and the possibilities that will need to be explored. Much of the requisite technical knowledge is likely to advance rapidly, but a precautionary approach remains indicated on both technical and political grounds. Achieving such a precautionary approach, however, is a challenge in itself.

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6. Research, deployment and safety management of genetically modified poplars in China

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China's population is the world's largest, and under demographic pressure from unprecedented expansion coupled with rapid social and economic development. This is exerting severe pressure on its forest resources. This scarcity of forest products is mainly caused by past and contemporary extensive deforestation and forestland degradation. While great efforts are being dedicated to reforestation and afforestation to increase forest resources, the gap between timber supply and demand remains large. To alleviate this shortage, the development of fast-growing, high-yielding short-rotation plantations has been identified as a national top priority.

During the past decade, the country has embarked on and implemented six major national forest programmes, one of which was the development of short-rotation industrial timber plantations. The fast growing nature of poplars and their responsiveness to cultivation made them an ideal species for the establishment of industrial, high-yield short-rotation plantations. Particularly in northern China, poplar is being widely used in large-scale plantations and currently plays an irreplaceable role in both commercial and ecological plantations.

In China, the total area covered by poplar trees is approximately seven million hectares, exceeding the total of other poplar plantations globally (Lu and Hu, 2006). However, the major poplar species in China are often subject to attacks by pests and diseases, which become the major barriers to the development of large-scale poplar plantations (Zhang and Li, 2003; Lu and Hu, 2006; Wang and Lu, 2002). The same problem was also identified for other crops of great economic potential, such as fruit trees and ornamental plants (Cheng *et al.*, 1999; Fang and Wang, 2000; Han *et al.*, 2000, 2006; He *et al.*, 2004). Moreover, China has large areas of saline land currently unsuitable for growing trees, but which might be utilized if trees are bred for salinity tolerance. Thus, the development of trees with improved pest and disease resistance as well as tolerance to stress conditions is paramount for the successful deployment of poplars as high-yield fibre plantations.

Compared with traditional breeding methods that often require a long time, and the inherent difficulty of meeting predetermined breeding objectives, such as improved pest and disease resistance and stress adaptabilities, modern technologies such as genetic engineering, if successful, appear to be very efficient (Lu and Hu, 2006; Su *et al.*, 2003a, b). Additionally, the rapid technological development and the progress accomplished further encourages the application of genetic

engineering in breeding poplar trees, and significant progress has been achieved in genetically modified trees, particularly poplar, in China.

This chapter summarizes the status of genetically modified poplars in China, the research associated with their development, release and commercialization, as well as associated biosafety issues.

CURRENT STATUS AND PROGRESS OF GENETICALLY MODIFIED TREE APPLICATIONS IN CHINA

Tree species in genetic modification studies

Poplars have been the most successful trees for genetic transformation studies due to their predisposition to clonal propagation and high transformation efficiency, with the added benefits of poplar being the model tree species for genomic research (a relatively small and completely sequenced genome) and the vast number of molecular and biotechnological studies coupled with its fast growth rate and short rotation period making it amenable to genetic engineering.

The first genetically transformed poplar produced in China was obtained in 1989, where the Bt insect resistance gene was introduced into the *P. nigra* genome (Tian *et al.*, 1993). Following this introduction, several genetically modified trees were placed in field trials and displayed significant improvement in insect resistance (Lu and Hu, 2006). The success of these trials prompted the approval of genetically modified tree release and the establishment of the first genetically modified commercial tree plantations in 2004 in China. Following this, several successful transformations have been achieved in poplar species: *P. tomentosa*, *P. alba*, *P. nigra*, *P. deltoides*, *P. euramericana, P. xinjiangensis, P. tremuloides*, *P. xiaozhannica* and hybrid poplars of *P. deltoides* × *P. simonii*, *P. alba* × *P. glandulosa*, *P. deltoides* × *P. cathayana*, *P. euramericana* × *yunnanensis*, *P. simonii* × *P. nigra* (Table 6-1).

Other trees used for genetic modification studies were mostly fruit trees of economic importance, such as apple, walnut, orange, date and cherry (Cheng *et al.*, 1999; Fang and Wang, 2000; Han *et al.*, 2000, 2006; He *et al.*, 2004). These activities focused on improving insect resistance, nutritional condition, and fruit taste (Han *et al.*, 1999, 2000, 2006; He and Han, 2000; He *et al.*, 2004; Shi *et al.*, 2000; Zhan *et al.*, 2001; Zhou *et al.*, 2005). Of interest was the development of infertility (i.e. inhibition of the development of reproductive organs) through transformation of *Liquidambar formosana* trees to maximize vegetative growth (Qiao *et al.*, 2007) and the use of the ornamental tree Bergamot (*Citrus medica* var. *sarcodactylis*) to develop a model for a genetic transformation system.

Target traits for improvement

The main drive for genetic transformation of trees in China was to improve resistance to diseases and pests and the acquisition of pesticide and herbicide resistance. Tolerance to salt and reduction of lignin content were also of interest and have been receiving increased attention as major target traits for improvement (Table 6-1). However, variable extents of improvement were obtained in various target traits under different genetic controls. So far, pest resistance seems to be the

TABLE 6-1
Summary information on genetically modified poplars in China

Species used	Method	Transferred genes	Target traits	Receptor	Test stage	Reference
P. deltoids × *P. simonii* 'NL-80106'	*Agro-bacterium*	*Bt*	Insect resistance	Stem disc, leaf	Field test	Rao *et al.*, 2000
P. deltoids × *P. simonii*	*Agro-bacterium*	*AaIT*	Insect resistance	Leaf, petiole	Lab	Wu *et al.*, 2000
P. xinjiangensis	*Agro-bacterium*	*cpti*	Insect resistance	Leaf	Lab	Zhu *et al.*, 2003
P. alba × *P. glandulosa*	*Agro-bacterium*	*SacB*	Drought resistance	Leaf	Lab, Greenhouse	Zhang *et al.*, 2005; Li *et al.*, 2007a, b, c
P. alba × *P. glandulosa* '84K'	*Agro-bacterium*	*BtCry3A/OC-I*	Resistance to coleopteron	Leaf	Lab	Zhang *et al.*, 2005
P. deltoides × *P. cathayana*	*Agro-bacterium*	*mt1D/gutD*	Salt resistance	Leaf	Lab	Fan, 2002
P. deltoids	*Agro-bacterium*	*Bt*	Insect resistance	Leaf	Lab	Chen *et al.*, 1995
P. deltoids, *P. euramericana*, *P. nigra*	*Agro-bacterium*	Antibacterial peptide *Lcl*	Insect resistance	Leaf	Field test	Li *et al.*, 1996
P. euramericana	*Agro-bacterium*	*cpti*	Insect resistance	Leaf	Lab	Zhao *et al.*, 2005
P. euramericana	*Agro-bacterium*	*CrylA(C)*	Insect resistance	Stem, leaf	Lab	Wang *et al.*, 1997
P. euramericana 'Guariento'	Gene gun	*SacB/vgb/ BtCry3A, OC-I/JERF36/ NPT II*	Insect, drought and salinity resistance	Leaf	Field test	Wang *et al.*, 2006
P. euramericana, *P. euramericana* × *yunnanensis*	*Agro-bacterium*	*CrylA(C)*	Insect resistance	Stem disc, leaf	Lab	Wang *et al.*, 1997
P. nigra	*Agro-bacterium*	*CrylA(c)*	Insect resistance	Leaf	Commercial production	Tian *et al.*, 1993
P. nigra	Gene gun/ leaf disc	*TA29-BARNASE*	Male infertility	Leaf	Lab	Li *et al.*, 2000a, b
P. simonii × *P. nigra*	*Agro-bacterium*	*Bet-A*	Saline resistance	Leaf	Lab	Yang *et al.*, 2001, Liu *et al.*, 2006
P. tomentosa	Polyethylene glycol (PEG)	*GUS*	Transient expression	leaf	Lab	Wang *et al.*, 1991
P. tomentosa	*Agro-bacterium*	Antisense *CCoAOMT*	Reduction of lignin content	Leaf with petiole	Field test	Zhao, Wei and Lu, 2004
P. tomentosa	*Agro-bacterium*	*cpti*	Insect resistance	Leaf	Field test	Hao and Zhu, 2000
P. tomentosa	*Agro-bacterium*	*Bt*	Insect resistance	Leaf	Lab	Zheng, Liang and Sun, 1996
P. tomentosa	*Agro-bacterium*	*NP-1*	Anti bacteria	Leaf	Lab	Zhao *et al.*, 1999
P. tomentosa '741'	*Agro-bacterium*	*BtCrylAc/Api*	Insect resistance	Leaf	Commercial production	Zheng *et al.*, 2000
P. tomentosa '741'	*Agro-bacterium*	*Bt*	Insect resistance	Leaf	Lab	Zheng *et al.*, 2000
P. tomentosa (Triploid)	*Agro-bacterium*	*Dγ*	Saline resistance	Leaf	Lab	Liu *et al.*, 2002
P. tremula × *P. alba*	*Agro-bacterium*	Antisense *CCoAOMT*	Reduction of lignin content	Leaf	Field test	Wei *et al.*, 2001
P. tremuloides	*Agro-bacterium*	Antisense *4CL*	Reduction of lignin content	Leaf	Lab	Jia *et al.*, 2004
P. xiaozhannica 'Balizhuangyang'	*Agro-bacterium*	*mt1D*	Saline resistance	Leaf	Field test	Liu *et al.*, 2000
Populus euramericana	*Agro-bacterium*	*BG2*	Resistance to bacteria	Leaf	Lab	Han *et al.*, 2004

most successful trait attained through genetic transformation, probably attributed to its simple genetic control (i.e. single or major gene resistance) in comparison with other target traits that are likely to be controlled by multiple genes. Salt tolerance is another trait that has shown evidence of improvement in trees using salt-tolerance genes (Sun *et al.*, 2002; Yin *et al.*, 2004; Yang *et al.*, 2001).

Performance of genetically modified trees

By far, pest resistance in poplars has proven to be the most successful genetically modified trait in China. Transformation of Bt genes significantly improved poplar tree pest resistance. Due to the excellent improvement in pest resistance, two genetically modified trees *P. nigra* and hybrid poplar clone 741 [*Populus alba* × (*P. davidiana* + *P. simonii*) × *P. tomentosa*] have been approved for environmental release and *P. xiaozhannica* 'Balizhuangyang' was placed in field tests. The pest resistance of these genetically modified trees was assessed and verified by feeding experiments.

Test feeding of gypsy moths with leaves from genetically modified *P. nigra* trees showed 70–95% mortality on 15% of the trees, and over 50% mortality on 50% of the trees. The genetically modified trees with higher insect mortality rate also suppressed development of the surviving insects. Seven days after start of feeding, the leaves of genetically modified trees were showing evidence of resistance to the insects, with almost no damage, while most leaves of the control trees were eaten.

The genetically modified hybrid poplar clone known as '741' was tested with *Clostera anachoreta*, and insect mortality rate was monitored during the entire development period (23–25 days corresponding to 1–2 days after hatching to cocooning). The results showed that the genetically modified trees induced significantly higher mortality than non-transgenic trees with 91.8% mortality on 14.3% of the genetically modified trees, 40–70% on 23.8 of genetically modified trees, and <20% mortality on 61.9% of the genetically modified trees, while the non-genetically modified '741' clone induced very low mortality (3.4%). Additionally, the insect-resistant genetically modified trees also delayed the development of living insects by 2–8 days from instars I to II, 2–6 days from instars II to III, and 2–8 days from instars IV to V. The average weight of individual insects fed on genetically modified trees was reduced by 41–49% compared with those fed on non-genetically modified trees, providing further evidence of the impact of genetically modified trees on the target insect's development.

The *P. xiaozhannica* 'Balizhuangyang' transformed with the *mtl-D* gene has shown significantly increased saline resistance. Field tests in Shandong and Tianjin indicated both a 70% increase in survival rate of genetically modified trees in soils with 0.3–0.4% salt content and increased growth rate compared with control trees. It should be noted that the upper limit for soil salt content reached 0.43% for the genetically modified trees in the trial.

Although resistances of the genetically modified poplar trees were significantly improved, their growth performance was found to show no notable improvement compared with non-genetically modified trees. However, some trees showed

better survival at these experimental sites, and this could be attributed to the improved pest resistance or to improved tolerance of saline soil conditions.

METHODS FOR GENE TRANSFORMATION

Agrobacterium-mediated transformation is the most widely used method to obtain genetically modified trees in China, with almost all genetically modified trees so far obtained thus. This method was used in most cases for transformation of single genes (Table 6-1).

Genetic transformation by particle bombardment with gene gun was only reported on *P.* × *euramericana* 'Guariento') and *Malus micromalus* (Wang *et al.*, 2006, 2007), where up to five exogenous genes were transformed into the genome of *P.* × *euramericana* 'Guariento', which acted as recipient plant. Genetic transformation by gene gun was considered to cause multiple copies, leading to genome re-arrangement and instability, and silence of the exogenous genes. However, not all these shortcomings were observed in the study (Wang *et al.*, 2006). An advantage of this method is that it can be applied to any type of plant material. The gene gun method appeared to have greater potential in transformation of multiple genes.

Other methods of genetic transformation were also tried, with a few tree species, but application has been limited due to various constraints (Table 6-2).

RELEASES AND COMMERCIALIZATION

In China, genetically modified trees have to go through multiple steps to satisfy biosafety assessment before release for commercial production. These steps include laboratory tests, field tests, environmental release assessment and productivity trials, before final commercialization.

TABLE 6-2
Comparison of different methods of genetic transformation of trees

	Transformation method						
	***Agro-bacterium* mediated**	**PEG**	**Electrical shock**	**Micro-injection**	**Ultrasonic mediated**	**Gene gun**	**Pollen-tube pathway**
Receptor	Complete cell	Protoplast	Protoplast	Protoplast	Any part of plant	Any part of plant	Oocyte
Hosts	Yes	No	No	No	No	No	Sexually reproduced plant
Conditions for tissue culture	Simple	Complex	Complex	Complex	Simple	Simple	Tissue culture not needed
Transformation rate	10^{-2}–10^{-1}	10^{-5}–10^{-4}	10^{-4}–10^{-3}	10^{-2}–10^{-1}	10^{-2}–10^{-1}	10^{-2}–10^{-1}	10^{-1}–10^{0}
Chimera DNA	Presence	Absence	Absence	Absence	Presence	Presence	Absence
Operation	Simple	Simple	Simple	Complex	Simple	Simple	Simple
Equipment required	Cheap	Cheap	Expensive	Expensive	Cheap	Expensive	Cheap
Efficiency of transformation	High	Low	Low	High	High	High	Low
Application in monocotyledons	Few	Applicable	Applicable	Applicable	Applicable	Applicable	Applicable

To date, eight poplar lines of seven different crosses have been transformed (Table 6-1), but only *P. nigra* and the hybrid clone '741' of the native *P. tomentosa* have been approved for commercial production, while seven genetically modified poplar species are in the field testing stage. The first genetically modified poplar trees (*Populus nigra*) were obtained in 1989 and entered field testing in 1994, and finally used in commercial plantation in 2002 (Tian *et al.*, 1993). Plantations of this genetically modified poplar have been established on eight sites in seven provinces or municipalities (Beijing, Jilin, Henan, Shandong, Xinjiang, Shaa'nxi and Jiangsu) (Su *et al.*, 2003a, b). The hybrid clone '741' (*Populus alba* × [*P. davidiana* + *P. simonii*] × *P. tomentosa*) that was transformed with two genes was approved for commercial planting in 2002 (Tian *et al.*, 2000; Zheng *et al.*, 2000; Su *et al.*, 2003b). The genetically modified poplar clones were granted plant variety rights by the National Plant Variety Rights Protection Agency. The approvals for commercial production of the two genetically modified poplars were given after the completion of all biosafety assessment requirements organized by the National Forestry Biosafety Management Authority in the State Forestry Administration. Seven additional genetically modified poplars, including *Populus xiaozhannica* 'Balizhaungyang' genetically modified with salt-tolerant genes have been approved for field testing (Sun *et al.*, 2002; Yin *et al.*, 2004). Many other genetically modified trees are still restricted to the laboratory testing stage (Table 6-1). However, every year an increasing number of applications for field testing and environment release are being submitted for safety assessment.

RISK ASSESSMENT AND SAFETY MANAGEMENT

Concerns with biosafety issues of genetically modified trees

The increasing research and deployment of genetically modified trees has heightened public concerns regarding the safety of genetically modified trees. Worries aroused concern aspects such as pollen contamination of genetically modified trees of other, related species; impacts on non-target insects and other organisms in the soil; and other possible impacts of genetically modified trees on the biotic and abiotic aspects of the ecosystem.

Biosafety of genetically modified trees

With the rapid development of genetic engineering technologies and the increasing number of released and planted genetically modified trees, increasing attention has been given to biosafety, with the deployment of a number of regulations. In 1993, "Rules for Safety Management of Genetic Engineering" were formulated by the Ministry of Science and Technology (MST, 1993), followed by publication of "Rules for Implementation of Safety Management of Genetic Engineering in Agriculture" in 1996 by the Ministry of Agriculture (MOA, 1996), and the issuance of "Regulations for Biosafety Management of Agricultural GMOs" (State Council, 2001). More recently, regulations were developed to cope with more specific biosafety issues associated with forest trees, including "Rules on Administration of Examination and Approval for Genetic Engineering Activities

of Forest Trees" issued in 2006, followed by "Technical Codes for Biosafety Assessment of Transgenic Forest Plants and Products" in 2007 (State Forestry Administration, 2007). All these regulations and rules have paved the way for managing the biosafety of genetically modified trees.

According to the rules and technical codes, genetically modified trees are classified into three classes based on an evaluation of the risks, and they must pass through a series of tests (laboratory, field, environment release and productivity). Only when these tests are completed and the results are satisfactory can the genetically modified trees be deployed for commercial production. A biosafety assessment must be carried out for each of these tests before granting approval for next step.

Technical standards for safety assessment and long-term monitoring of genetically modified trees

As we enter the twenty-first century, biosafety of genetically modified trees should be given increasing attention. Studies have been initiated to investigate the potential impacts of genetically modified trees on elements of the ecosystem, such as impacts on soil micro-organisms, target and non-target insects, and gene flow to non-genetically modified trees (Hu *et al.*, 2004; Gao, Li and Liu, 2003; Zhang *et al.*, 2006). Early results from these studies have shown that genetically modified products did not produce any significant changes to the natural ecosystem. However, it may not be feasible to assess many impacts of genetically modified trees during the early stages of tree growth and development due to their long life cycle, so long-term continued monitoring of these impacts is needed to obtain reliable information and reach reliable conclusions. A number of monitoring studies on the long-term impacts of genetically modified trees on soil micro-organisms have been established (Hou, Zhang and Su, 2006) and more information on genetically modified tree impacts on the ecosystem at large will become available in future years.

FUTURE PROSPECTS

Since the first genetically modified *P. nigra* development, a large number of studies have been carried out with the aim of developing genetically modified trees for different purposes. Although two of the genetically modified poplars are being commercialized and a couple of the genetically modified poplars are at the field testing stage, most genetic modification studies are still at the laboratory stage (Lu and Hu, 2006). Further information on the performance of genetically modified trees of other poplar species still needs to be collected and analysed to develop reliable conclusions.

In addition to pest-resistant genetically modified poplars, progress has been made in saline tolerance in genetically modified trees of several poplar species. Given the large areas of saline land in China, saline-tolerant genetically modified poplar trees appear to be of great potential in afforestation in such areas. Moreover, genetically modified trees of *P. tomentosa* transformed with *4CL* and *CCoAOMT* genes showed great potential for significantly reducing lignin content (Wei *et al.*, 2001; Zhao, Wei and Lu, 2004).

With rapid development of poplar-based industries in China, the plantation area under poplar will be further expanded. Breeding of poplar trees with high resistance to pests and diseases as well as tolerance to saline soils will greatly facilitate the expansion of poplar plantation. As already shown by some genetically modified poplar trees, genetic modification appeared to be a promising tool to achieve this goal.

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Part 2

ETHICAL AND SOCIO-ECONOMIC DIMENSIONS

7. Theoretical and practical considerations of gene flow

J.J. Robledo-Arnuncio, S.C. González-Martínez and P.E. Smouse

Gene flow, defined as the incorporation of genes from one gene pool into another, is at the core of the transgenic plant debate. In particular, a widespread societal perception of genetically modified plants is that of a hazardous material with high 'pollution' potential for the environment. The transfer of engineered genetic sequences (transgenes) from genetically modified plantations into natural populations of wild relatives via propagule dispersal is the natural vehicle for the feared 'pollution'. From a scientific risk assessment perspective, proper evaluation of the environmental implications of genetically modified plants involves both hazard and exposure assessments (Johnson *et al.*, 2006). Hazard assessment targets the identification and quantification of potential adverse effects of transgenic plants for the environment. Exposure assessment evaluates the probability of the environment being exposed to the hazards. Gauging the probability of transgenic incorporation into natural plant populations is the key step of exposure assessment.

It must be stressed that the detection of transgene flow into natural populations is not a demonstration of the risk of genetically modified organisms, which would require evidence of the transgenes being hazardous for the environment. This chapter deals solely with the role of gene flow in the genetically modified-plantation debate, without additional consideration of hazard assessment. The chapter is structured along four lines, describing the main contributions of gene flow researchers to exposure assessment of genetically modified trees:

- characterization of propagule dispersal patterns in non-genetically modified tree populations, which provides general insights into transgene flow potential and quantitative measurements for model parameterization;
- elaboration of theoretical models of gene flow from genetically modified tree plantations into natural populations, essential for predictive inference over large spatial and temporal scales;
- detection of transgene flow into natural populations, necessary for real-time monitoring, decision-making and management;
- formulation of transgene flow limitation practices.

There are several specific features of trees that are relevant in the genetically modified forest risk assessment context, which will be reiterated throughout this chapter. First, trees are long-lived perennials, a fact that has three important consequences:

- propagules will be dispersed from genetically modified plantations recurrently for many years before harvesting;

- it is very difficult to establish empirically the multiple-generation fate of these propagules in natural ecosystems;
- induced-sterility containment measures have increased chances of failing, due to temporal instability.

Second, trees disperse pollen and seed over broad spatial scales, increasing the probability of long-distance transgene movement and hampering its effective containment and accurate monitoring. Third, trees have typically very high fecundities, translating into large numbers of dispersed propagules, which are expected to increase the longest realized dispersal distance, particularly for fat-tailed dispersal distributions (Clark, Lewis and Horvath, 2001; Klein, Lavigne and Gouyon, 2006). Fourth, genotypes used for genetic modification are often taken from undomesticated tree stands and grown in similar locations, so cross-mating with natural populations of the same species (or close relatives) is likely to be common (González-Martínez, Robledo-Arnuncio and Smouse, 2005). Lastly, trees are the dominant life form of many terrestrial ecosystems, so introgression of transgenes into natural tree populations might have long-term and large-scale impacts on ecosystem function.

DISPERSAL PATTERNS IN NON-GENETICALLY MODIFIED TREE POPULATIONS

Given the absence of dispersal data for genetically modified trees and the legal and social restrictions on genetically modified-tree field trials, dispersal studies in non-genetically modified tree populations provide a necessary surrogate to investigate transgene flow potential. Assuming that no particular containment measures are taken and that genetic transformation for the target trait does not significantly alter the dispersal function, the available data on propagule dispersal patterns in natural tree populations, seed orchards and commercial plantations should reflect the potential scale of propagule flow from genetically modified plantations. Note that this section refers to the arrival of transgenes via pollen and seed dispersal into natural stands, and not to the long-term persistence of transgenes once they have arrived in the wild, which is discussed in the next section, on predictive models.

There are several statistical methods that have been developed for estimating gene movement within and among populations. Some of these methods provide historical estimates of gene flow, under various assumptions about evolutionary equilibrium, based on the spatial genetic structure of populations (Wright, 1931; Slatkin, 1985; Rousset, 1997; Beerli and Felsenstein, 1999) or individuals (Hardy and Vekemans, 1999; Rousset, 2000). Other methods yield contemporary gene flow estimates, inferred either from parentage analysis (Meagher, 1986; Devlin, Roeder and Ellstrand, 1988; Adams, Griffin and Moran, 1992; Smouse, Meagher and Kobak, 1999; Burczyk *et al.*, 2006) or from the spatial genetic structure of propagules (Smouse *et al.*, 2001; Austerlitz and Smouse, 2001; Robledo-Arnuncio, Austerlitz and Smouse, 2006). Several reviews on gene flow and transgenic trees have already extensively reported the main assumptions, statistical properties, pros and cons of each of these different estimation procedures (Ellstrand, 2003; Slavov,

DiFazio and Strauss, 2004; DiFazio *et al.*, 2004; Smouse, Robledo-Arnuncio and González-Martínez, 2007). The reader should refer to these previous works for detailed technical reference. Here, some results that are particularly relevant for genetically modified flow are summarized:

- Within-population mean dispersal distance estimates range from a few tens to several hundred metres (most frequently <1000 m in temperate forest trees), both for pollen (Dow and Ashley, 1998; Streiff *et al.*, 1999; Lian, Miwa and Hogetsu, 2001; Schuster and Mitton, 2000; Sork *et al.*, 2002; Robledo-Arnuncio and Gil, 2005; Goto *et al.*, 2006; Hardy *et al.*, 2006; Hardesty, Hubbell and Bermingham., 2006) and seeds (Clark *et al.*, 1999; Jones *et al.*, 2005; Goto *et al.*, 2006; Hardesty, Hubbell and Bermingham, 2006; González-Martínez *et al.*, 2006; Robledo-Arnuncio and García, 2007; Hardy *et al.*, 2006; Jordano *et al.*, 2007). Both insect- and wind-pollinated tree species show a similar range of mean dispersal distances in published studies, although there are large differences among species. It is noteworthy that estimates of the mean dispersal distance based on parentage analyses are likely to be downwardly biased, since the distribution of observed dispersal distances is usually truncated by the sampling plot boundaries, and propagules immigrating into the plot are usually discarded to compute this quantity.
- Yearly pollen immigration rates into forest fragments or stands are typically very high (>30%), and remain high (>5%) even with isolation distances of a few kilometres from the nearest conspecific stand (Kaufman, Smouse and Alvarez-Buylla, 1998; Adams and Burczyk, 2000; Schuster and Mitton 2000; Plomion *et al.*, 2001; Stoehr and Newton, 2002; Robledo-Arnuncio and Gil, 2005; Hanaoka *et al.*, 2007; O'Connell, Mosseler and Rajora, 2007).
- Seed immigration rates into sampling plots embedded within large forests (Jones *et al.*, 2005; González-Martínez *et al.*, 2006) and into isolated forest fragments (García, Jordano and Godoy, 2007) are both typically high (>10%). Secondary dispersal by fruit and seed predators, not always accounted for in seed migration estimates, is expected to increase the range of seed dispersal (Vander-Wall, 2001; Valbuena-Carabaña *et al.*, 2005).
- The estimated pattern of seed and pollen dispersal is very leptokurtic, i.e. there is a rapid decline in dispersal probability over short distances but non-negligible probability maintained beyond distances of several hundred metres (Clark *et al.*, 1999; Austerlitz *et al.*, 2004; Robledo-Arnuncio and Gil, 2005; Jones *et al.*, 2005; Goto *et al.*, 2006; Robledo-Arnuncio and García, 2007).
- Although empirical evidence for long-distance propagule dispersal in trees is abundant, its accurate probabilistic description remains a daunting challenge (Nathan, 2005). The usual procedure of parentage-based studies is to fit probability distributions to dispersal data collected on a small spatial scale and extrapolate the fit to the unobserved range of the distribution. Quantitative predictions established in this way should be considered with extreme caution, since functions with profoundly different tail-behaviour often fit observed data about equally well.

The general pattern is that while a substantial proportion of dispersal events occur over short distances, the potential for long-distance gene movement among tree populations or stands is quite high, though difficult to predict. The probability of seed or pollen from genetically modified tree plantations effectively reaching natural populations located even a few kilometres away should be considered non-negligible, *a priori*, especially when dispersal episodes accumulate over several years or decades. For instance, a low (say $p = 0.01$) yearly probability of transgene dispersal from a genetically modified plantation can translate into a substantial ($1 - 0.99^{20} = 0.18$) probability over a period of 20 years (Haygood, Ives and Andow, 2004; Smouse, Robledo-Arnuncio and González-Martínez, 2007). Similarly, a low probability of escape from a single genetically modified stand can translate into substantial risk of spread if there are multiple genetically modified plantations.

Observed dispersal patterns in natural populations provide a rough idea of the rate and spatial scale of transgene dispersal. Obtaining more precise estimates of transgene escape rate by direct extrapolation of these patterns, however, may not be adequate: most empirical studies report seed or pollen immigration rates into small study plots or small populations, surrounded by widespread conspecific forests, while source genetically modified tree stands (especially experimental plantations) may be small relative to wild recipient populations. This demographic scenario would result in transgene escape being less frequent than observed migration rates among natural stands, since increasing population size is expected to decrease immigration rates (Ellstrand and Ellam, 1993). But even if probably lower than reported immigration rates into small natural stands, potential rates of gene movement from small genetically modified tree stands into large wild populations may still be significant, as suggested by the observed low levels of gene flow from hybrid poplar plantations into wild populations of interfertile congeneric species (reviewed in Slavov, DiFazio and Strauss, 2004), and by the available estimates of transgene spread from genetically modified agricultural crops (Rieger *et al.*, 2002; Beckie *et al.*, 2003; Watrud *et al.*, 2004). Moreover, a very low rate of gene flow may be sufficient for eventual transgene fixation in the wild if it occurs recurrently or if it confers a selective advantage over conventional trees (Haygood, Ives and Andow, 2004; see next section).

Overall, considering an appropriately large temporal scale, the available evidence strongly suggests that the efficient dispersal systems of trees render the movement of transgenes from genetically modified plantations into conventional forests highly probable. But although it is reasonable to assume a very high likelihood of occurrence of a certain amount of transgene flow, predicting the rate at which it will happen, especially over very long distances, requires further empirical and theoretical analysis.

Predictive models

Thoroughly assessing the long-term exposure of natural forests to genetically modified trees through gene flow can hardly be accomplished without theoretical

modelling. There are numerous challenging aspects of the problem for which field trials, though highly desirable, are not really feasible. The most difficult and critical factor is that the relevant spatial and temporal scales are very large, with serious implications for many aspects of the assessment of exposure through gene flow. One should be ready to imagine a mosaic landscape of genetically modified tree plantations and natural stands, in more or less close proximity, spreading over thousands of hectares of land, eventually across different properties or even national territories. One would like to be able to predict the expected rate of transgene movement into a particular natural population and the probability of long-term persistence and eventual fixation of the transgene in this population.

Long distance dispersal models

A first consequence of the large spatial scale of the problem is the need to quantify the frequency and range of long-distance transgene dispersal, so that one can make predictions about the expected rate of transgene dispersal in particular spatial and demographic scenarios. Measuring rare long-distance dispersal events is very difficult in practice and, as mentioned above, extrapolating phenomenological functions beyond the experimental range of real data does not constitute a reliable approach to predicting long-distance dispersal. As pointed out earlier, phenomenological model predictions are quite sensitive to model selection, which in turn is highly dependent on sampling scale (Kuparinen *et al.*, 2007a). Moreover, the dispersal process is expected to be highly dependent on environmental variation, and thus extrapolating case-specific dispersal patterns to different environments may lead to misleading predictions (Kuparinen, 2006). Mechanistic dispersal models, by quantitatively describing the relationship between dispersal and the underlying physical factors causing particle movement (mainly propagule terminal velocity, release height, canopy structure and air flow statistics), may be more adequate to infer solutions outside the spatial and environmental domain for which observed data are collected, providing a wider range of predictive relevance. It must be noted, however, that mechanistic models are not so easily applicable to animal-dispersed species.

Mechanistic wind dispersal models are especially suitable to model long-distance propagule transport because they can emulate stochastic turbulent transport processes, such as updrafts above the forest canopy, considered a major determinant of long-distance seed and pollen transport (see Kuparinen, 2006 for a review of mechanistic wind dispersal models). For instance, in a study involving laboratory and field experiments with five tree species in a deciduous forest in North America, Nathan *et al.* (2002) fitted a Eulerian-Lagrangian model that was able to predict the proportion (1–5%) of seeds collected at different heights of a 45-m tower, a proportion considered as an upper bound on the probability of their long-distance transport. Given the typical high seed fecundity of wind-dispersed trees (roughly 10^3–10^5 per tree per year; Clark *et al.*, 1999), this would represent substantial numbers of potential long-distance dispersal events. Using similar coupled Eulerian-Lagrangian simulations, parameterized for *Pinus taeda*, Williams

et al. (2006) predict 0.007% to 0.1% of seedlings establishing beyond 1 km from 16–25-year-old plantations, or about 40–60 seedlings per year, assuming a 10-ha genetically modified stand, a conservative annual fecundity of 10^5 seeds/ha, and a 6% germination rate.

More recently, Kuparinen *et al.* (2007b) have developed a specific mechanistic approach to airborne dispersal of propagules in forested areas that explicitly addresses long-distance transport by modelling complex turbulent flows in upper parts of the atmospheric boundary layer. Consistent with previous studies, their simulations suggest that large amounts of light pollen, and small but significant proportions of heavier particles like seeds, may easily disperse over several kilometres. Lower propagule terminal velocities, higher release heights and changing wind conditions significantly increased the predicted rate and range of long-distance transport. They also point out, however, that further work is needed for better understanding of implementing release and deposition processes and within-canopy turbulences, which are critical for effective seed and pollen dispersal.

Population dynamics models

Once estimates of the frequency and spatial range of transgene escape are available, the next step is to investigate the long-term demographic dynamics of immigrant transgenes in natural populations, in competition with wild genotypes, and under a range of environmental conditions, including the presence or absence of the agent that the transgene may have been engineered to mitigate (Farnum, Lucier and Meilan, 2007). Only in this way will it be possible to predict the degree and duration of the exposure of natural forests to transgenes, which will range between fixation of the transgene in the recipient natural population or its quick elimination by natural selection. Given the long lifespan of trees, and taking into account that the relative fitness of transgenes may have multiple components expressed at different life stages, the necessity of theoretical models to examine multiple-generation transgene population dynamics becomes evident.

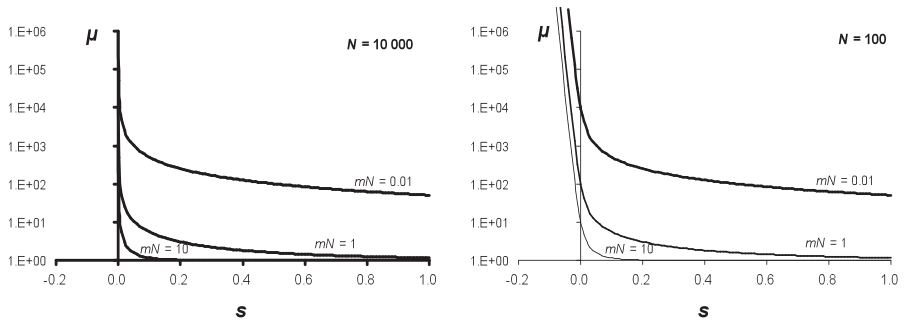
Simple demographically and spatially unstructured models can provide a first insight of transgene population dynamics. As an example, Williams and Davis (2005) use deterministic phenomenological simulations to investigate the fate of transgenes in a small escaped genetically modified-tree colony, with assumed initial transgene frequency of 50%, under different selective and demographic scenarios. Although their quantitative predictions are not easily interpretable, because of the absence of stochastic drift in the model and because of the artificial assumption that any immigrants arriving into the colony after its foundation had the same transgene frequency as colony residents, they illustrate the intuitive idea that both the relative abundance and relative fitness of the escaped genetically modified individuals are critical for transgene spread. Specifically, transgenic alleles in the escaped genetically modified-tree colony will tend to fixation if the transgene confers a net fitness advantage relative to wild-type alleles, but this process may be retarded if the genetically modified colony is embedded within a relatively large natural forest.

A more straightforward and formal description of the probability of transgene escape in a spatially and demographically unstructured model is provided by the analytical treatment of Haygood, Ives and Andow *(*2004). They define transgene escape into a wild population as the arrival of a transgene whose descendants will eventually take over the population, i.e. the descendant lineage of which will be destined for fixation, showing that the probability distribution of escape time (not time to fixation), defined in this way, is approximately geometric, with mean equal to the inverse of the probability of transgene escape.

Here, we derive the probability of transgene escape in a similar fashion to Haygood, Ives and Andow *(*2004), but considering a diploid transgenic locus and allowing for negative selective coefficients for the transgene, in order to illustrate the interplay between the probability of transgene escape, the transgene migration rate, the recipient population size and the adaptive value of the transgene. Let N be the number of mature individuals in the wild population, m the fraction of gametes in the wild population that flow from the genetically modified plantation per generation ($m < 0 < 1$), and s the selection coefficient for the transgene under wild conditions. From standard population genetics theory (e.g. eqs. 3.31 and 5.47 in Ewens, 2004), the probability that a newly arrived transgenic lineage is destined for fixation is approximately $\pi = (1 - e^{-s})/(1 - e^{-2Ns})$, assuming there is no dominance. The first generation after gene flow begins, we have Nm transgenes in the wild population, and the probability that at least one of them is destined for fixation, i.e. the probability of transgene escape, is given by $p = 1 - (1 - \pi)^{Nm}$. If the transgene does not escape in the first generation, we assume (following Haygood, Ives and Andow, 2004) that the situation is essentially the same in subsequent generations, until the transgene escapes or gene flow ends. That is, we assume that in these subsequent generations the amount of transgenes produced in the wild population and the number of individuals in transgenic lineages destined for extinction are small enough that p remains approximately the same until a transgene escape event occurs. That leads to a probability distribution of escape time, in generations, that is approximately geometric with mean $\mu = 1/p$ (Haygood, Ives and Andow, 2004).

Using this model, we examined (Figure 7-1) the estimated value of the mean escape time (μ), in generations, for different values of wild population size (N = 100 and 10 000), transgene selective value (s = -0.1 to 1.0) and number of transgene migrants per generation (Nm = 0.01, 1 and 10). A first interesting result is that the escape time becomes virtually independent of N as soon as the transgene has a relatively small selective advantage ($s > 0.01$ in our examples). If the transgene is neutral ($s = 0$), by contrast, the mean escape time is greatly reduced for small population sizes, assuming a fixed number of transgene migrants per generation (Nm). For instance, if $Nm = 10$, we have $\mu \approx 10$ for $N = 100$ and $\mu \approx 1000$ for $N = 10\,000$ (since, as expected under our model assumptions, the fixation probability of the transgene becomes approximately m for $s = 0$). Now, if the transgene is maladaptive in the wild ($s < 0$), escape time becomes enormous for large populations (the probability of transgene escape becomes negligible),

FIGURE 7-1
Expected mean transgene escape time in generations (*μ*) into a wild population of size *N* as a function of the selective coefficient of the transgene under wild conditions (*s*) and for different numbers of transgene immigrants per generation (*mN*)



irrespective of the number of migrants, while it can be relatively short if the wild population is small and the number of migrants relatively large (e.g. $\mu \approx = 100$ for $N = 100$, $s = -0.02$, and $Nm = 10$) (Figure 7-1). This is because stochastic drift reduces the efficiency of selection in small populations. Finally, for any given value of the selective coefficient (with $s \geq 0$), escape time increases as the number of transgene migrants decreases. Interestingly, however, escape time becomes fairly short ($\mu < 100$) as soon as the number of migrants per generation is not too small ($Nm \geq 1$) and the selection coefficient of the transgene is very slightly positive ($s > 0.001$). We believe that the arrival of at least one transgene migrant per generation ($Nm \geq 1$) can be considered a minimal working rate for genetically modified tree populations (given that this is a per generation rate and that trees may have a generation time of several decades), and thus that the probability of transgene escape will be generally very large for any transgene that is even slightly favoured by selection.

Spatial distribution, demographic structure and environmental variation may influence population dynamics in real systems, interacting with population genetic processes. Therefore, predictive models for the spatial and temporal dynamics of escaped transgenes need to be spatially, ecologically and demographically realistic. An early example of long-term spatial simulation modelling of transgene spread is provided by the STEVE model (DiFazio *et al.*, 2004), aimed at investigating potential invasiveness of transgenic poplars in the northwest United States of America. This stochastic model tracks transgenic and conventional genotypes in a virtual landscape that includes topographical and ecological information, with population dynamics being governed by modules simulating growth, reproduction, seed and pollen dispersal, and competition. The authors performed sensitivity analyses to study the consequences of different dispersal and selective conditions, several deployment and flowering control scenarios, and contrasting

selective values for the transgene. The main results highlighted by the authors (Slavov, DiFazio and Strauss, 2004) are:

- transgenic introgression into conventional stands was insensitive to the slope of local dispersal curves, but highly sensitive to changes in the proportion of long-distance dispersal;
- an imperfect, but tightly linked, sterility gene could dramatically slow the spread of a transgene that provided even a strong selective advantage;
- the spread of neutral transgenes could be greatly reduced by sterility levels whose effectiveness was of the order of 95%.

Perhaps the most elaborated and realistic spatial simulation model of transgene escape to date is that of Kuparinen and Schurr (2007). The model, which can be run in deterministic or stochastic form, includes: modules for seed dormancy; seedling establishment; tree growth; individual mortality; ovule, pollen and seed production; and pollen and seed dispersal. Many of the relevant demographic and reproductive processes are density-, genotype- and size-dependent. Seed and pollen dispersal are simulated using a mechanistic Lagrangian stochastic model especially configured to account for long-distance dispersal events. As an application, the authors examined the sensitivity of transgene escape to demographic differences between genetically modified and conventional trees, to the expression (dominant or recessive) of the transgene, and to the initial genotype of the genetically modified plants at the engineered loci (homozygous or heterozygous). After 100 years, a neutral transgene had diffused through short distance dispersal from the plantation into a contiguous conventional stand, with declining frequency with distance. Additionally, small numbers of transgenes had escaped the plantation via long-distance dispersal to distances beyond 1000 m. Decreased density-dependent mortality and increased growth, relative to conventional trees, were the two demographic factors of transgenes that resulted in a higher increase of escape rate into natural populations. The expression of transgenes only affected the probability of escape when they had demographic effects, with markedly reduced escape for recessive transgenes. Escape rate was also reduced for dominant transgenes if the initial genetically modified population consisted of heterozygous individuals.

Despite the utility of modelling, it must be kept in mind that theoretical models lacking realistic calibration will only provide qualitative insights on the sensitivity of transgene escape to particular factor effects. Quantitative predictions will require adequate parameterization, requiring experimental data, which should be pursued to the extent that model factors are legally amenable to empirical testing. That necessary caveat translates into a pair of serious challenges facing forest geneticists. One is the need to validate long-distance dispersal models empirically, including mechanistic models. The other, most critical, is to quantify the relative fitness of transgenes under different ecological conditions. As has been pointed out (Lee and Natesan, 2006), predictive models will not be really useful for transgene risk assessment if the uncertainty surrounding transgenic fitness impacts is not reduced.

REAL-TIME TRANSGENE FLOW ASSESSMENT

What do we need?

Another front where gene flow researchers can contribute to exposure assessment of genetically modified trees is the development of methods for real-time detection of transgenes. Although field release of genetically modified trees is still uncommon (Van Frankenhuyzen and Beardmore, 2004), there will soon be high demand for tools for field assessment for transgenic presence in natural forests. Many of the available methods for gene flow analysis are not adequate for this purpose. Genetic methods for assigning individuals to populations (Manel, Gaggiotti and Waples, 2005), for instance, require a thorough characterization of the recipient and the genetically modified donor populations, and will be of little help unless there is very strong divergence within the allele frequency spectra of the populations, since otherwise assignment error rates are likely to be larger than the presumably very low transgenic frequency to be estimated. Parentage assignment, in contrast, requires exhaustive genotyping of all potential parents within the study area, which becomes unfeasible over the spatial scales that are relevant for transgene flow detection, being moreover subject to a level of statistical uncertainty that may be unacceptable for decision-making. In fact, parental designation is not necessary for detecting transgenes, which only requires a categorical diagnostic criterion to conclude whether an individual is carrying the engineered sequence or not, for which several more powerful monitoring methods are available (Stewart, 2005).

Transgene monitoring methodologies

The most straightforward detection method is laboratory screening of the transgenic sequence directly. This will require tissue collection and DNA extraction from potentially escaped genetically modified individuals in conventional populations for the examination of a diagnostic DNA segment at the modified region. This can typically be achieved by means of PCR amplification, followed by automated sequencing or by single nucleotide polymorphism (SNP) analysis. European regulatory schemes are already demanding all sequence information of transgenes in applications for authorization for release of genetically modified organisms, including the location of primers used for detection (EFSA, 2006). Ideally, the proposed 'biobarcode' technology (Gressel and Ehrlich, 2002) would permit a standardized procedure for transgene detection. This technology would consist of the inclusion of a non-coding DNA segment in the engineered DNA sequence, flanked by universal PCR primers, which would contain a variable region encoding information on transgene identity and origin.

More elaborated screening procedures, using nanotechnologies, would allow faster and *in vivo* monitoring of transgenes in the field. These techniques, still not implemented for commercial transgene detection in plants, involve developing nucleic acids that are complementary to the target transgenic transcript and that carry a fluorescent label that can be seen by shining an ultraviolet light on the plant (see Stewart [2005] for a detailed description of different methods). There are, however, several barriers to the use of this kind of approach, that may

eventually prevent its implementation, such as safety concerns about fluorescence-based technologies, the additional investment for genetically modified tree re-engineering, and, most importantly, legal restrictions on and social rejection of further transgenic engineering (Stewart, 2005).

An alternative approach for transgene screening is testing for diagnostic phenotypic traits expressed by the transgene, such as herbicide and pest resistance, or some easily detectable protein. This procedure can allow an intensive, low-cost screening, prior to more direct assessment using DNA-PCR analysis. Watrud *et al. (*2004), for instance, used two cycles of herbicide spraying to detect the presence of herbicide-resistance transgenes in progenies collected from conventional populations of creeping bentgrass. Survivors of the second cycle were then tested for the presence of a transgene-encoded protein using commercial test strips. Finally, DNA from herbicide resistant and protein-positive plants was extracted and sequenced for final confirmation of transgene presence. Similar screening protocols might also prove useful for forest trees, as long as the engineered traits are expressed at an early life stage (Smouse, Robledo-Arnuncio and González-Martínez, 2007), which may not be the case for altered fibre quality or growth. Testing for herbicide or pest resistance by spraying progenies collected from seed trees could be feasible for detection of transgene flow via pollen, but similar tests on naturally regenerated seedlings in the wild might be ecologically unacceptable.

Challenges related to sampling

The challenge of categorically detecting the early stages of transgene spread in the wild can be intimidating. Assume that a transgene is present in the natural regeneration of a conventional forest at a frequency of $q = 10^{-3}$. Then, if we wanted to reduce the probability of not detecting the transgene below $\alpha = 0.01$, we would need to screen at least $n = 4600$ seedlings (ensuring that $\alpha = (1 - q)^n < 0.01$). If the introgression rate were as low as $q = 10^{-4}$, we would then need over 46 000 samples to ensure $\alpha = <0.01$. Given the additional advisability of sampling over large spatial and temporal scales, the problem becomes such that some have simply concluded the impossibility of proving that transgenes are absent from a given region (Ortiz-García *et al.*, 2005). Of course, if a transgene were ultimately to reach fixation, its frequency would eventually have to reach levels much easier to detect, but this may only happen after a minimal initial frequency (as low as $1/2N$, N being the recipient population size) and several generations of random drift or positive selection, which probably means several centuries for forest tree species. Nevertheless, early detection is critical if we are to intervene. That being the case, strongly replicated sampling over large spatial scales seems unavoidable, which, if legally enforced, might have implications for the economic payoff associated with genetically modified tree plantations.

The intricacy of accurate early detection of transgenes is illustrated by the intense and publicized scientific debate about the presence or absence of transgenic flow into maize landraces in Mexico, with more than ten studies conducted since

2001 and several replies and counter-replies disputing statistical and sampling issues (see Mercer and Wainwright, 2008 for review and discussion). In fact, it has been argued that too much emphasis is being placed on the rate of transgene flow, when the parameter of greater concern should be the relative fitness of the transgenes (Hails and Morley, 2005; Lee and Natesan, 2006; Chapman and Burke 2006). The reasons for this argument can be summarized as follows:

- it is reasonable to assume that occasional transgene flow into natural populations is unavoidable in practice, even if at very low rates;
- the magnitude of the transgene migration rate may be very difficult to estimate;
- the relative fitness of transgenes is the primary force governing their spread.

One agrees with this view, and stresses the need for a shift towards further empirical research on life-time fitness costs and benefits of transgenes under contrasting ecological conditions, a challenging task for long-living forest trees. It is also likely, however, that any scientific risk assessment protocol and, perhaps more importantly, any political or social debate on the risks of genetically modified trees, will hardly pass without convincing transgene flow estimates.

Transgene flow avoidance

The exposure of natural ecosystems to genetically modified trees could be essentially avoided if effective gene flow from transgenic plantations were interrupted. Since, as discussed above, spatial isolation does not provide an efficient barrier to transgene flow, alternative transgenic containment and mitigation strategies are being developed. Specifically, containment methods use different forms of genetic engineering to prevent transgenes from leaving genetically modified plants, either by inducing sterility or by removing the transgene from gametes before their release (excision techniques). Mitigation procedures intend to reduce the fitness of transgenes by tightly linking it to an engineered gene that is maladaptive in the wild, hence providing a useful complement to the expected leakages in containment strategies. Technical and practical details concerning the development, implementation and efficiency of different containment and mitigation strategies were extensively dealt with in an earlier chapter. Here, it is simply asserted that fully safe transgene containment methods are yet to be developed and thoroughly tested on a case-by-case basis. A recent study reports promising results along this line, with some excision techniques achieving 100% deletion of functional transgenes from pollen and/or seed, as tested on more than 25 000 progeny of tobacco plants for each transgenic event (Luo *et al.*, 2007). Further research is needed, however, to test the temporal and environmental stability of this technique for tree species and different transgenes. Due to the long life cycle of forest trees and the diverse ecological conditions they experience, the stability of any genetically engineered transgene containment strategy remains a matter of concern. It must be kept in mind that containment failure rates much lower than 10^{-3} may be necessary to reduce transgene escape probabilities to acceptable levels (Haygood, Ives and Andow, 2004).

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8. Ethical considerations regarding genetically modified trees

C. Gamborg and P. Sandøe

NON-TECHNICAL LIMITS TO BIOTECHNOLOGY

Until recently, the main limits to modern biotechnology were of a technical type: "What is it possible to do?" However, as the technical difficulties began to be resolved, and as practical applications came within reach, the question increasingly became one of "What is it acceptable to do?" Today, scientists and the biotechnology industry face a growing number of ethical issues and questions relating to the social context in which biotechnology is used. This may mean a growing discrepancy between expert and public views. Public apprehension about gene technology is triggered by a range of concerns: about environmental risks; the patenting of genetically modified organisms; labelling of products; and the possibility of exerting democratic control on the development and application of biotechnology (Holland and Pratt, 1995; Thompson, 2001).

When it comes to genetically engineered trees, systematic silvicultural improvement measures such as selective breeding are, compared with agricultural plant breeding, very much in their infancy (Campbell *et al.*, 2003). The science underlying the genetic engineering of forest trees, i.e. tree and plant genomics, is limited (Adams *et al.*, 2002). Moreover, the first large-scale commercial applications of transgenic trees are only just beginning to appear (Sedjo, 2004). It is clear that ethical discussion of the complex issues raised by the genetic engineering of forest trees needs to be appropriately directed.

Successful adoption of genetically engineered trees will depend not only on the soundness of the technology and science, but also on how these trees are perceived by the public. In public debate, the terms 'genetically modified', 'transgenic' or 'genetically engineered' are used interchangeably for those trees that have been modified using recombinant DNA and asexual gene transfer methods, regardless of the source of DNA employed (Brunner *et al.*, 2007). Potential use of gene technology with forest trees has raised concerns around the world. These concerns are serious in Japan and in Europe; they have also emerged in North America (Owusu, 1999; Strauss, 2004a). The results of silvicultural genetic engineering have been disapprovingly dubbed 'Frankenstein forests' (Warwick, 1999), 'Designer trees' (Rautner, 2001) and 'Frankentrees' (Native Forest Network, 2000) – with clear reference to the term 'frankenfoods' used in the genetic modification food debate. A number of protests, sometimes involving the destruction or vandalism of field trials, have occurred – for example in the United Kingdom in 1999, where

two genetically modified poplar trials owned by AstraZeneca were ruined; in the United States of America in 2001, where a laboratory of the University of Washington was firebombed (cf. Strauss, 2004b); and in Finland in 2004, where Finland's only field study on genetically modified trees was attacked, destroying 400 trees (Hall, 2007). Concern has also been manifested in non-violent protests and campaigns by high-profile environmental organizations. At the same time, genetically engineered trees have been characterized as 'superior' (Merkle and Dean, 2000) and 'highly green new tool[s]' (Valenzuela and Strauss, 2005) by proponents of the use of genetic engineering in forestry. Indeed it has been argued that genetically engineered trees are part of, if not the key to, sustainable silvicultural development (Salwasser, 2001; Doering, 2004).

In the context of the aim for greater sustainability, forests can be viewed according to their underlying management philosophy. Genetic engineering in forestry relates in an interesting way to two currently recognizable and opposing trends in forest management. One trend is technological. It involves an efficient system of tree cropping, advances in tree breeding and the continued use of exotic species. Characteristically, followers of this trend respond to, and control, the prevailing ecological, environmental and economic conditions by employing artificial seeding, planting, breeding and so forth. The other recognizable trend is the 'ecological' or 'back to nature' trend, which in some respects parallels the organic trend in agriculture. Here the aim is not one of exploiting natural forests, but rather of controlled, sustainable harvesting of semi-natural forests. It is also considered important to plant forests and silviculturally treat them so that they resemble the structures and processes of comparable naturally wooded areas (Gamborg and Larsen, 2003). The 'back to nature' approach is gaining a foothold in Europe and elsewhere, as problems are now recognized associated with intensively managed plantations: problems of ecological stability and flexibility, of biodiversity, and of an aesthetic and recreational nature. When it comes to new technology, it is not just a question of the technology itself but of the attitude to the technology and the underlying management philosophy in which the technology is embedded.

The forestry sector can learn lessons from the development and introduction of genetically engineered (food) crops – although, evidently, there are significant differences between genetically engineered forest trees (by which we mean trees without edible fruits) and genetically engineered food crops (cf. Hall, 2007). Some important differences are set out in Table 8-1.

Forestry is essentially different from agricultural plant production, not only in respect of biological factors such as rotation age, but even more so in socio-economic and cultural factors. These differences should be borne in mind when one assesses gene technology in forestry.

The distinctive features of forestry are biological and socio-economic as well as cultural. Strikingly, forest trees for timber, pulp or fuel production are not part of the human food chain. Hence, the use of genetically engineered trees will not be part of the massive food safety discussion that surrounds genetically engineered

TABLE 8-1
Parameters of genetic engineering: non-food forest trees versus agronomic food crops

Biological factors	Socio-economic and cultural factors
Forest trees are far less improved through selective breeding than agricultural crops	Forests are more accessible to the public than agricultural fields
Forest trees evidently have a much longer life than agricultural, even perennial, crops, and the forest persists much longer; rotations may span more than a hundred years	Forests, unlike agricultural production units (fields), encompass everything from natural or semi-natural woodland to tree plantations
As ecological systems, forests are much more structurally and functionally complex than their agricultural counterparts	Forests produce several recognized goods and services at the same time
Forest trees (by definition) do not produce edible goods (but timber, pulpwood, woodfuel and so on)	Forests have conferred upon them a diversity of social, cultural, symbolic and other values

food crops. Initially, genetically engineered trees should have an advantage, as the food discussion has many cultural and ethical connotations. However, some concerns are aggravated, such as environmental concerns and concerns about biodiversity, as uncertainty mounts with ecological complexity and time. Some concerns are specific to forests and forest trees, including concerns about the special cultural and symbolic values attaching to forests, and forest as an important component of the landscape (O'Brien and Claridge, 2002). Nevertheless, many of the features listed above do not alter the fundamental mechanisms underlying our attitudes to, and concern about, genetic engineering.

In examining the potential concerns related to genetically engineered trees, and in assessing the underlying ethical issues, we need to consider two sets of issues. First, what should be on the agenda, and what is considered an ethically relevant concern? Second, how should we address and discuss these concerns, how should we handle conflicts of interest, and how should we take into account the differing opinions? It is to be hoped that early addressal of these issues will help to avoid the problems and controversies connected with the introduction of gene technology in agriculture.

GENETIC ENGINEERING AND RISK ASSESSMENT

Like most new technologies, gene technology gave rise to both huge expectations and widespread worries when it emerged in the 1980s. In Europe, so-called Eurobarometer surveys (CEC, 1992, 1993, 1997, 2000, 2003) have consistently shown that among the general public the use of gene technology in agriculture and other areas of food production has a low level of support relative to other applications. In general, studies have shown a more positive perception of biotechnology among the United States of America public than the European public (Eyck, Thompson and Priest, 2001). Since 1999, approximately 60% of the respondents were of the opinion that biotechnology would provide them benefits within a five-year time frame. However, it is interesting that concern about genetically engineered crops does not involve total opposition to gene technology but instead specifically relates to the application of such technology to food. For example, in a series of qualitative nationwide interviews conducted in the Eurobarometer surveys, it was apparent that most people welcomed medical

progress brought about by genetic engineering (Lassen, Holm and Sandøe, 2003). Consequently, the antagonism is not created by the process of genetic engineering but its application to modern food production. A reasonable assumption is then that other factors must be at stake, and that these factors have to be included in any analysis (Madsen and Sandøe, 2001).

The conceptual framework for dealing with the worries was risk analysis: before the release of genetically engineered organisms a scientific risk assessment should be undertaken to identify and evaluate any potential adverse effects on human health and the environment. The results of the assessment should form the basis for management of the risks by public authorities; it should also provide input to risk-communication efforts directed at the broader public. In some cases this strategy seemed to be a successful model for introducing gene technology in ways acceptable to the public. In Denmark, for example, after an intense public debate, the industrial use of gene technology for the production of enzymes and pharmaceuticals was accepted by the public. In other cases, however, the introduction of gene technology and other forms of modern biotechnology has led to controversies that seem to have no end.

Some of the time, then, rather than putting an end to controversies, risk assessments have appeared to fuel new controversy. This is paradoxical. Considerable sums have been spent on the risk assessment of genetically modified crops. Broadly speaking, no major scandals have occurred, and a number of the plants have been deemed safe both for human health and for the environment. Nevertheless, after more than ten years of, at times, intense debate the crops are as controversial as ever, and in particular, it seems, to the European public. Is there any reason to believe that the same will happen with genetically engineered forest trees?

KEY ETHICAL CONCERNS ABOUT MODERN FOREST BIOTECHNOLOGY: GLOBAL PERSPECTIVES

When it comes to new technology, it is not just a question of the technology itself but of the management philosophy in which the technology is embedded and the underlying values. Here, concerns specific to forests and forest trees play an important role. Using genetically modified trees in silviculture is not exclusively a technical issue. Ethical assumptions relate to what kind of nature is wanted and the means considered acceptable (List, 2000). In answering these questions, we should be looking at what are the likely consequences of genetic engineering. Should we be trying to improve on nature? (Reiss and Straughan, 1996). 'Ethics', as the term is here understood, has as its main function to reflect and clarify. Reflection may for example concern the complex trade-offs between conservation and the consumption of renewable resources. The output may be a better understanding of various ways of looking at such trade-offs and thereby making room for dialogue about the goal of forest management. In general, ethical reflection may help to formulate and discuss the relative importance of potentially conflicting concerns and values.

Modern forest biotechnology has brought the techniques of silviculture and plant development before the public eye in a way that is unprecedented in recent times (Thompson, 2001). Yet, in forestry, biotechnology has not (yet) been subject to anything like the intensity of debate it has received where agricultural products, and in particular genetically engineered crops such as soybean, are concerned. There are several reasons for this.

One reason is that, at present, no large-scale commercial production is taking place. For gene technology in agriculture, serious debate first started when genetically modified crops were produced commercially. In forestry, commercialization has begun in China and is imminent in South America (Strauss, 2004a). In Europe, genetically engineered trees are unlikely to appear for the time being. Despite the fact that the number of trees tested has risen substantially in recent years, timber trees still make up only a small proportion of the total number of field trials with plants. Thus, between 1987 and 2001, timber trees were involved in just 1.2% of field tests (Sedjo, 2004). Consequently, data on the ecological effects, and of any unintended potential side-effects, of genetic modification in this field are currently scarce. Moreover, we cannot necessarily transfer the abundant number of more available results from studies of genetically modified plants used in agriculture because trees are essentially undomesticated, have intrinsically long life spans and host a wide variety of organisms.

A second reason, as already mentioned, is that trees are not food crops, and therefore strongly held beliefs about genetically modified foods do not carry over to them. The worry about genetically engineered trees is often portrayed as relating to the natural environment. This worry seems to be intensified by the longevity of trees (as compared with agricultural crops), since this makes it harder to anticipate potential implications.

In general, the concerns we are dealing with here have to be seen in connection with the ways in which forests are perceived by the public. Forests have an emotive value for many people, which does not apply to agricultural crops like wheat. Trees have a place in history, mythology and identity. And as a North American study reported by Hall (2007) claims, genetically modified trees could "come into conflict with a socio-psychological need found throughout Western history, for the forest to remain apart from civilization, uncultivated and untamed". Forests, unlike agricultural fields, are seen as 'uncultivated' – even though they are, in fact, in many cases both cultivated and intensively managed. So concerns about genetic modification may be rooted in an unacknowledged disapproval of the management of forests as such. Three main sets of concern about genetic engineering may be distinguished and separately discussed: risk-centred, socio-economic and cultural. These are set out in Table 8-2.

According to Lassen and Jamison (2006), it is a characteristic of the concerns of the public that these concerns are framed in ways that go beyond risk to the environment and (in the case of genetically engineered food crops) health. Socio-economic concerns deal with gene technology as a way of achieving economic development while looking at the socio-economic costs and benefits, and the

TABLE 8-2
Matrix of public apprehensions concerning genetic engineering in the context of risk-centred, socio-economic and cultural concerns

Concerns for discussion	Central themes	Key concepts
Risk-centred	Environment, health	Risk, uncertainty
Socio-economic	Profitability, production	Cost/benefit, power
Cultural	Moral, religious aspects	Ethics, rights, integrity

Source: After Lassen and Jamison, 2006.

power determining the distribution of these costs and benefits. They also examine intellectual property rights in relation not only to economic profits, but also to democratization. And, finally, cultural discussion of gene technology changes our understanding of ourselves and our capabilities, and indeed the borders between the natural and the unnatural.

Across large sections of the general public, there is limited understanding of biotechnology and its requirements (BEPCAG, 1997). A major problem is, though, that greater knowledge does not *per se* lead to less scepticism towards biotechnology; in some cases, indeed, quite the contrary occurs. Where information about biotechnology is provided, both the overall proportion of people with a more positive attitude towards biotechnology and the proportion of sceptics increase, but the net result is that the number of sceptics rises. Improved understanding and knowledge puts one in a better position to take a stance, but it does not necessarily lead one to sympathize with the technology.

PUBLIC ACCEPTANCE? LESSONS FROM GENETICALLY MODIFIED AGRICULTURE

In reality, the attitudes of the general public and other stakeholders to genetically engineered forest trees are far from as well known, as demonstrated by the worries that agricultural crops provoke (cf. Hall, 2007). Results from studies on perception of plantation forestry have been used to gain a greater understanding of how the public reacts to land-use changes (Neumann, Krogman and Thomas, 2007). Studies from Asia (Yap, 2004) and Australia (Barlow and Cocklin, 2003) find that the development of plantation forestry may be accompanied by controversy. A recent study of public perception of hybrid poplar plantations in Canada – although the techniques used to create the trees are different from genetic engineering, the social impacts of going from traditional management, for example, to more intensive tree production may be similar – suggests that landscapes are closely linked to the values and identities of the people living there.

One of the main lessons from genetic engineering in agriculture shows that if modern biotechnology is to stand a chance, three main conditions for public acceptance must be met: utility, low risk, and an assurance that biotechnology is used in a 'decent' way. These three conditions are somewhat interrelated. Many people would accept a certain risk (depending on how risk-averse they generally are) as long as potential utility attaches to the application of the biotechnology (BEPCAG, 1997). Nevertheless, something more seems to be at stake. In surveys, this has sometimes been labelled 'moral doubt', but it has no clear definition.

Earlier results from the United States of America suggest that there moral acceptability is a better predictor of encouragement than risk or usefulness (Eyck, Thompson and Priest, 2001). The important thing about 'moral' is that it seems to override what would otherwise be seen as an acceptable technology in terms of risk and utility.

Utility

A technology or innovation can possess utility in several ways. From an economic perspective a technology is useful if it is competitive in commercial conditions, e.g. through increased productivity. However, more than this will be required if the public are to consider a certain forest tree biotechnology useful: the technology in question has to contribute significantly to mitigation of key human or societal problems. Evidently, what is considered 'significant', and what is a 'key' problem, are debatable issues, but examples could be positive environmental impact or helping to alleviate poverty in developing countries. Moreover, usefulness is gauged not only relative to existing conditions but also in relation to alternative methods of reaching the same level of utility (e.g. insect damage to trees might be reduced through increased insect resistance obtained by conventional breeding practices or altered silvicultural practice).

Risk

Another key factor in the acceptance or rejection of modern biotechnology is risk. A number of studies have consistently shown that the majority of people are willing to run a risk provided there is a proportionate gain. For example, most of us drive a car although this specific activity has a high, well-documented risk. Clearly, people may evaluate risks in incompatible ways and make conflicting proposals for mitigating risks (Thompson and Dean, 1996). In the domain of genetically engineered crops, several risk assessments have failed to show conclusively that there is a ('substantial') danger to the environment or to health. Nonetheless the public, especially in Europe, does not feel comfortable with the use of the technology. The discomfort is partly grounded in a public scepticism about science's ability to judge the long-term, accumulated consequences of applying a new technology. Risk assessments that try to identify hazards and quantify risks will not help in this sense, as they are part of the scientific research that is being questioned.

If risk assessments are to help reduce public apprehension, the public must have more trust in experts and authorities: something currently lacking, especially in Europe. Experience from the introduction of gene technology in agriculture and food production suggests that, to regain or strengthen this trust, it would help to ensure that risk assessments are not seen as a way to relieve decision-makers of their part of the responsibility. Moreover, a fair account of the limitations of the risk assessments should be given, and there should be openness about when and where more than scientific reasoning and assessment are needed (i.e. about when we will accept that we must live with the remaining uncertainty).

Moral doubt

The third condition on the use of modern biotechnology requires us to rebut 'moral doubts' by applying the technologies in a 'right' way. To many people, the whole idea of meddling with the genes in living beings, whether they are animals or plants, is ethically problematic. The challenge here is to formulate these concerns about (broadly speaking) respectful use of nature. An important point in this connection is that the public at large do not necessarily share the biological scientists' conception of nature. Many, for example, see species as stable entities that change only as a result of our technical manipulations. However, for the ecologist, stability is a relative concept and species are constantly changing.

Two types of argument often appear in surveys of attitudes to modern biotechnology (Madsen *et al.*, 2002). Roughly, one – 'nature as a safety mechanism' – is that, by relying exclusively on 'natural' processes (here understood as not using genetic engineering and not crossing natural species barriers), we obtain greater control. The second – 'natural order' – succinctly avoids scientific considerations about such matters as the risks of genetic engineering and presents a fundamental ethical criticism. In brief, the position is that we should not 'tamper with nature', implying that genetic engineering is 'unnatural' and inconsistent with the 'balance of nature'. A traditional scientific rebuttal is that we are already, in conventional breeding practices, changing the make-up of nature, and the use of gene technology is merely an extension of currently known tree breeding practices (Kellison *et al.*, 2007). This type of answer may, however, lead to people drawing a conclusion opposite to what was intended. Instead of encouraging acceptance of gene technology, it may lead to a more critical attitude towards existing breeding practices and methods.

ADDRESSING LOCAL AND GLOBAL CONCERNS: TRANSPARENCY AND STAKEHOLDER PARTICIPATION

Some environmental concerns that seem to cause apprehension in the public, such as biodiversity, soil and water effects, can be assessed through comprehensive risk assessments giving detailed information and recommendations to the best of our knowledge. As pointed out by Strauss *et al.* (2004), given the cold reception of the first generation of genetically engineered crops in many parts of the world, a record of usefulness and safety may well be needed for the acceptance of genetically engineered forest trees. Evidently, the type of knowledge required in the latter case will differ from that needed where agriculture and food are concerned because of the elements of uncertainty in forestry, which result from the lengthy time span between establishment and harvesting and the complex interplay between organisms and the natural environment (Tømmerås *et al.*, 1996).

Environmental risk assessments are based on scientific and technical data. But these data must fit into a normative framework that is *not* scientific in nature. This framework stems from the decision problem of whether or not a given application to release and market a particular genetically engineered tree should be approved. The questions the risk assessment is required to answer depend on the criteria

for approval. These criteria involve assumptions about what kinds of risk need to be assessed. Many of these assumptions rest on value judgements. By 'value judgements' we mean judgements implying that, under certain circumstances, something ought to be the case, or one thing or course of action is preferable to another. Only when these judgements are made explicit will it be possible to conduct an effective debate about the broader issues involved in the approval of genetically engineered trees. Hence, an environmental risk assessment views the world through a 'risk window', and this window only makes visible that which has been predefined as a relevant risk. The size and structure of the window is determined by value judgements about what is considered to be an adverse effect within what is considered to be the appropriate horizon of time and space (Jensen *et al.*, 2003). These points are not new. They have been argued for many years by philosophers and social scientists, and they are clearly acknowledged by important scientific bodies like the United States of America National Research Council and the World Health Organization. Nonetheless, they appear not to have diffused into the field of genetic engineering of plants.

Risk assessments are based on current science. Unfortunately they do not reflect the uncertainties inherent in that science. Problematic aspects of new technologies are understood by the public in ways that are essentially different from the risk approach of the scientist, who focuses on risk as the product of effect and probability. This is a lesson which could have been learned already from earlier debates such as the nuclear power debate, but it has either never been learned or has been forgotten. The process of deliberation about genetically engineered forest trees would benefit from its recollection.

Moreover, as was indicated previously, the wider public does not view risk in isolation from potential benefits and other issues. Therefore, to satisfy the concerns of the public, risks should be discussed and dealt with in connection with an assessment of potential benefits to society and other ethical issues. Forests are often associated with naturalness, wilderness, integrity and authenticity. They may also be culturally important (DEFRA, 2002). For example, individual trees and woodlands may represent ways of marking history, contribute to a sense of place, express intergenerational contrasts, or be symbols that represent a 'raw' and 'immediate' bond between 'man and nature'. These aspects cannot be a meaningful part of a risk or impact assessment. In addition, to meet the worries of the general public, some kind of technology assessment is called for that addresses the broader social and ethical issues and goes beyond ordinary risk assessment. Politicians and the authorities must understand the general need for thorough public debate before new technological methods are introduced in order to avoid public frustration arising from the feeling that things are out of control, or beyond the individual's democratic control (Madsen and Sandøe, 2001). A critical issue in this context may be the patenting of crops that biotechnology's critics find so troubling (Cayford, 2003). In view of all this, there is a very reasonable case for the claim that decision-making concerning risk-prone activities should better cohere with societal views and needs.

The participation of the main stakeholders is important. When decisions are made (e.g. about industrial roundwood plantations or reserves designed to protect biodiversity), the subsequent establishment and management routines should take into account the local people. They can do this through social contracts that have been negotiated through discussion and voluntary agreements, as well as through international, national and local policies (Friedman and Charnley, 2004).

Transparency, although now something of a buzz word, is also important. Transparent decision-making can be defined as "decision-making in which the decision-maker clearly presents to others the normative and factual premises behind his conclusions and explains the reasoning leading him from these premises to the conclusion" (Rasmussen and Jensen, 2005). What transparency involves is the uncovering, describing, documenting and communicating of all the steps of the reasoning and evidential assessment that underlies any decision taken. To do this properly, it is necessary to take into account "limitations, weaknesses and uncertainties, as well as pointing at issues which – even though they might be considered relevant from the perspective of some stakeholders – are not addressed by the decision process". It is clear that, to take account of such factors, new efforts from policy-makers, as well as from the scientific community, will be needed: both parties will need to make the value premises of any given risk assessment known, say what is considered a hazard, what constitutes harm, what are the acceptance criteria, and so on. At the same time, it is worth noting that increased transparency may not come easy, as it leaves the authorities and science as a whole more vulnerable to public scrutiny. Decision-makers may also fear that greater transparency about the limitations of the processes through which decisions are made may lead to more public concern instead of increasing trustworthiness.

CONCLUSIONS

Use of gene technology in forestry has been referred to as a help towards producing more efficient forms of plantation forestry, to generate cost-efficient renewable energy and to solve major environmental problems. However, the very same technology has also been met with initial distrust in several parts of the world, a distrust especially pronounced in Europe and Japan (Herrera, 2005), and a distrust that is already discussed as being a sign of the same resistance and type of polarized debate that occurred regarding genetic modification technology in agriculture (Mayer, 2001); a debate which Merkle and Dean (2000) warn that the research community ignores at its own peril. The question remains whether genetically engineered forest trees will make a difference and contribute to more sustainable silvicultural practices. That is, will the public benefit or will the utility of engineered trees – something which, given the time lag between first proof and commercial application, may be hard to establish – be seen as adequate compensation for the environmental and ecological risks? Will genetically engineered forest trees be considered 'morally' acceptable?

The issue of risks and benefits is viewed by many people as something that should be handled by proper scientific evaluation (cf. Strauss, Raffa and List, 2000)

and, to some extent, regulation. The assessments here have to be developed to suit the specific conditions of forest trees. The appropriate way to prepare regulation to address public concerns is currently unresolved. And it might be that evaluation on a crop-by-crop basis or a trait-by-trait basis would not come without a cost because it can be conceived as something that lends credibility to the idea that all genetically engineered products are more dangerous than conventionally bred crops (Strauss, 2003).

One way of dealing with the question of using technology in the 'right' way, i.e. the question of 'moral' acceptability, is to embark upon more public debate. Stakeholder discussions suggest a call for increased public consultation, and for a more participatory decision-making process (Simosi and Allen, 1998). However, it is important to stress the obvious point that dialogue is no guarantee of consensus. Decision-making and regulation in an area where there is no clear consensus may benefit from transparency of the kind discussed above, namely from clear statements of the choices that are to be, or have been, made and the values upon which they rest (Lassen, Holm and Sandøe, 2003). This way, decision-making and regulation stand a better chance of being respected by all parties and ongoing trench warfare may come to a halt.

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9. Genetically modified trees and associated environmental concerns

M. Fladung, H.-L. Pasonen and C. Walter

Over the past 15 years many techniques such as tissue culture, transformation (gene technology) and genome analysis have been developed for various tree species, including both broad-leaved trees and conifers (Groover, 2007; Henderson and Walter, 2006; Merkle and Nairn, 2005; Giri, Shyamkumar and Anjaneyulu, 2004; Campbell *et al.*, 2003). A very powerful tool in forest tree breeding programmes is gene technology, which can be used to transfer genes of interest into tree genomes. Tree species that have been genetically modified belong to genera like *Populus* (poplars), *Betula* (birches), *Picea* (spruces), *Pinus* (pines) and *Eucalyptus* (eucalypts), and many transgenic lines carrying a variety of gene constructs have been produced and tested in the laboratory and the greenhouse. In addition, a few transgenic tree lines have been tested in the field under natural environmental conditions.

In trees, a range of traits are of general interest as target traits for genetic engineering in trees, such as lignin and/or cellulose modification, disease and pest resistance, tolerance to abiotic stresses, male or female sterility and modification of developmental processes. More recently, traits that make trees more suitable for a bio-ethanol or biomaterials economy are also being considered. Transgenic trees carrying transferred or engineered genes and expressing novel traits may have implications for environmental parameters when grown in scientific field trials or for commercial purposes. Comparative risk analysis is required, considering both direct and indirect environmental effects, including possible gene transfer to wild relatives, weediness, effects on non-target species and other unintended effects of genetically modified or transgenic trees. Any risk identified needs to be compared with accepted practice to achieve the same outcome (the production of wood) and the magnitude of risk evaluated in this context. These risks may result from the deployment of transgenic trees, but they may be similar to risks associated with introduced, non-native trees as well as trees bred by conventional tree breeding methods (Hoenicka and Fladung, 2006).

Major concerns have been raised regarding the introduction of transgenic trees into natural environments. These include potential risks related to the functional stability of the transferred genes in long-lived tree species (operational safety), as well as possible flow of recombinant DNA into the environment through a range of different pathways (Strauss *et al.*, 1995; Strauss, DiFazio and Meilan, 2001; Hoenicka and Fladung, 2006). Direct or indirect environmental effects of

transgene instability or spreading of transgenes are mainly still unknown. For instance, insect-resistant trees have direct effects on the target insect species, but insecticidal GMO pollen may indirectly affect non-target organisms, such as butterflies. A herbicide-resistant tree may have direct effects on ecosystem biodiversity because of fewer weeds following herbicide treatment, but at the same time it may have indirect effects on invertebrates because of lower plant diversity. The possible increased sensitivity of 'low-lignin' trees against fungal and bacterial pathogens is considered as a direct effect, while the escape of genes from lignin-modified transgenic trees into natural ecosystems is considered as an indirect long-term effect. However, it is important for any discussion on risk related to the deployment of genetically modified trees to keep the risk in perspective. This means that risk must be compared with the risk inherent in accepted practice currently used to achieve the same outcome, such as the production of wood. This can, for example, be achieved by using insect-resistant trees or alternatively spraying against insects. Both methods carry a certain amount of risk for the environment, and informed decision-making must take both into account.

On a worldwide scale, several field trials have been established during the past few years to study transgenic trees under natural environmental conditions. Since many genetically modified tree lines are close to or even ready for commercialization, environmental concerns should be discussed and monitoring programmes developed before the release of genetically modified trees to the market. The focus on risk assessment should be on the trait introduced rather than the technology used to achieve genetic gain. This chapter summarizes available information on direct and indirect environment concerns following the release of transgenic trees into natural environments. The focus will be on resistance evolution, vertical and horizontal gene transfer, effects on non-target species, transgene stability, weediness and invasion, and other unintended effects.

FIELD TRIALS WITH TRANSGENIC TREES TO STUDY ENVIRONMENTAL CONCERNS

To date, more than 200 field trials with genetically modified forest trees have been documented worldwide (Robischon, 2006). The majority of those were carried out to test herbicide and insect resistance, lignin reduction or developmental processes, and only few to investigate biosafety issues such as sterility, transgene stability, or vertical and horizontal gene transfer (Valenzuela, Balocchi and Rodriguez, 2006; Robischon, 2006).

In North America, transgenic trees were tested mainly in relation to improvement of plantation forestry (Robischon, 2006). Also, an increasing number of studies in recent years have focused on sterility or altered fertility of forest trees. Reduced fertility can increase the productivity of a tree by redirecting energy and resources to growth rather than production of reproductive structures (El-Kassaby and Barclay, 1992). Further, research in this area could lead to an increasing contribution towards lowering potential environmental risks related to gene flow to interfertile species. It has been demonstrated that sterility strategies

developed for annual plants can be transferred to forest tree species (Wei *et al.*, 2007; Brunner *et al.*, 2007).

In Germany, four field release experiments with transgenic forest trees were established between 1996 and 2002. The initiative of the first field trial was a first step toward the evaluation of the possible risks versus benefits of genetically modified trees (Fladung *et al.*, 2004). Transgenic trees carrying a screenable morphological marker based on the *rolC* gene of *Agrobacterium rhizogenes* were planted. Transgenic trees carrying the 35S::*rolC* gene are characterized by dwarf growth and smaller leaves. This type of marker system has some advantages compared with biochemical markers. First, morphological markers can be detected phenotypically during every stage of the life cycle of the plant, or at least at specific developmental stages, whereas marker genes such as *npt-II* (antibiotic resistance) or *uidA* (detected using a histochemical staining procedure) provide results only at the time of evaluation and not during the lifetime of the organism. Second, it is advantageous to use a cell-specific marker that does not diffuse to adjacent cells and hence is detectable at the cell level. Thus, plants transgenic for the *rolC* gene from *A. rhizogenes* offer an appropriate model system meeting the requirements of a morphological marker.

In this field trial, four research projects related to biosafety issues were carried out. The first project was related to the integration pattern of the foreign gene construct into the genome. Originally it was thought that integration patterns are important for stable transgene expression under changing environmental conditions and during the long life span of trees. Analysis of *rolC*-transgenic poplar revealed that expression of the transgene may vary over time (Kumar and Fladung, 2001). At the same time, data from transgenic radiate pine indicate that once the trees are a couple of months old and still express the transgene, this will not change later on, i.e. they will continue to express (Walter, unpublished results). In an associated project, the mycorrhizal status of the roots in the transgenic and non-transgenic trees, and the conditions for a putative transfer of the foreign gene(s) from the tree roots into the mycorrhizal fungal symbiont (horizontal gene transfer), were analysed. In the two remaining projects, the status of phytopathogenic fungi on leaves was investigated, and correlated with parameters of the carbohydrate and hormonal metabolism of transgenic versus non-transgenic trees.

In a second field trial with genetically modified aspen the horizontal gene transfer to mycorrhiza fungi was investigated. Transgenic aspen trees carrying a fungal-specific promoter controlling the bar gene were planted out in the field. The hypothesis was that following horizontal gene transfer the mycorrhiza fungi living in association with these transgenic trees should become BASTA resistant. Subsequently, large screening programmes were initiated to identify putative BASTA-resistant mycorrhiza fungi. The two remaining field trials with genetically modified aspen were initiated by the University of Freiburg (Germany). Transgenic poplars were produced, modified for higher glutathione content. The trees were more tolerant of increased heavy metal concentrations in soil. In the field trials in Germany and in the Russian Federation, the capacity of the uptake of heavy

metals by the transgenic plants was tested, and the possible pleiotropic effects on morphology, growth parameters and mycorrhization were studied.

In New Zealand, several field trials with transgenic *Pinus radiata* and Norway spruce have been conducted. The trees have been mainly genetically engineered with selection and reporter genes in order to collect basic information on the patterns of gene expression. Some of the trees also contain genes that may have an effect on the reproductive capacity of the trees. The aim of the studies was to gain a better understanding of the operational and environmental risks involved and how to manage them in a plantation forestry context. The public has full access to the data generated and that will enable society to assess the risks of genetic engineering and compare them with the risks of techniques currently in practice, leading to informed decision-making. Researchers are investigating the expression of foreign genes in genetically modified conifers by quantifying reporter protein levels in the GMO greenhouse and field trials. Researchers in New Zealand also generate data on the impact of genetically modified needles on selected native insect species, and effects of roots from modified trees on micro-organism populations, in particular mycorrhizae.

In Finland and elsewhere in northern Europe, silver birch (*Betula pendula* Roth) is the most important deciduous tree species and is used commercially, for example, in plywood, pulp and furniture production. Various genetic modifications have been tested in silver birch by several research groups, either in the lab or under greenhouse conditions (e.g. Keinonen-Mettala, Pappinen and von Weissenberg, 1998; Lemmetyinen *et al.*, 1998, Lemmetyinen, Keinonen and Sopanen, 2004.; Valjakka *et al.*, 2000; Pappinen *et al.*, 2002; Tiimonen *et al.*, 2005). Also, three field trials with genetically modified silver birch have been established in Finland. One of the central aims in the establishment of the field trials has been the evaluation of environmental effects of transgenic birch. The first two field trials were established in 2000. The field trial established by the University of Helsinki included silver birch lines genetically modified for fungal disease resistance. So far, most published research results are available on birch lines carrying a chitinase IV gene from sugar beet. The interactions of chitinase transgenic birch with other organisms, e.g. pathogenic and mycorrhizal fungi, soil micro-organisms and herbivorous insects, have been widely studied. This field trial was harvested in autumn 2003, after three growing seasons.

Another field trial was established at the Punkaharju Research Station by the Finnish Forest Research Institute and contained transgenic silver birch lines altered for carbon and nitrogen metabolism (sense-RbcS and NR lines). Unfortunately, this field trial was destroyed in 2004. The third field trial was established by the University of Joensuu in 2005 with the aim of studying the environmental effects associated with the potential establishment of plantations of silver birch genetically modified for the prevention of flowering, using silver birch lines carrying the BpFUL1::Barnase gene construct. This trial was still in progress at the time of writing.

TRANSGENIC TRAITS AND ENVIRONMENTAL CONCERNS

Resistance evolution

Genetically modified trees have been made tolerant to a broad spectrum of herbicides. These herbicides are used to kill all plants considered as weeds growing alongside the tolerant transgenic tree. These herbicides can also be harmful to animal species, including both vertebrates and invertebrates. Spraying of herbicides on large-scale herbicide resistant tree plantations can have negative effects on nearby natural ecosystems, such as forests and grassland, due to wind distribution.

The primary concern related to herbicide-tolerant trees, however, is the development of plant populations that are resistant to particular herbicides. These wild populations may acquire invasive potential and thus can become 'weeds'. The resistance may develop via gene flow from herbicide tolerant trees to wild interfertile relatives. Also the species mix and population structure of known weed communities may change: weed populations may develop tolerance to certain herbicides, which under selective conditions (continued and regular use of herbicides) may enable them to out-compete weed species or populations without that tolerance. Once such use of herbicide has selected for resistant individuals, continued use of herbicide (i.e. continued selection pressure) favours resistant plants over their susceptible counterparts. Over time, the frequency of resistant plants in a weed population increases, representing a potentially serious long-term weed management problem. This observation has typically been associated with reliance on a single herbicide active ingredient over time, i.e. a high level of herbicide selection intensity (Volenberg, Stoltenberg and Boerboom, 2001; Stoltenberg and Wiederholt, 1995). It is important to consider, however, that in plantation forestry, herbicides will only be used prior to and during the first two to three years of establishment of a plantation. Subsequently the plantation will not need further spraying for protection and hence the selective pressure will no longer be present.

Current commercial transgenic insect-resistant trees are grown in China (Ewald, Hu and Yang, 2006) carrying the gene from *Bacillus thuringiensis* (Bt-gene). Concerns raised relate to insect populations potentially adapting rapidly to this pest-protection mechanism. In the event of establishment of Bt-resistant insect populations, the use of higher toxicity pesticide will become necessary. Also, the Bt-resistant insects can move to other tree stands where classically Bt-toxin is sprayed as a pest control mechanism. Thus severe environmental impacts could be the consequence. Regional or interregional scale plans, rather than local, are needed because insects are highly mobile.

However, a number of studies are already available to discuss this aspect of risk in context. These studies have investigated Bt-transgenic maize and cotton and the development of resistance mechanisms in associated insects. It is documented that Bt-resistant insects have been developed in a Bt-transgenic maize field with a frequency of about 3% (Tabashnik *et al.*, 2000). At the same time, it was reported that field outbreaks of resistance to Bt have not been observed so far (Morin *et al.*,

2003). Following new insect resistance strategies that involve gene stacking, the chance of development of insect resistance with two or three stacked Bt genes is infinitesimally small. Further, it has been described that the Bt protein can be used as supplementary food source that may account for faster development rate of Bt-resistant insects (Sayyed, Cerda and Wright, 2003). However, in contrast, Tabashnik and Carrière (2004) state that Bt crops had adverse affects on resistant insects.

Unfortunately, no information is available for Bt-transgenic tree plantations regarding resistance breaks and ecological implications. The only study available so far regarding insect community structure has been published by Gao *et al.* (2003, cited in Ewald, Hu and Yang, 2006). The authors mention that the presence of Bt-transgenic poplar can reduce the density of individuals of defoliating insects and shift the dominance of individual species. At the same time, the insect diversity was enhanced (Gao *et al.*, 2003, citied in Ewald, Hu and Yang, 2006). In general, complete risk assessment must also consider alternative practices used to protect plants from insects. This may show that the use of transgenic trees may actually be more benign to the environment than the conventional and accepted practice, which might, for example, involve the spraying of Bt protein. Further, Bt transgenics controlled by an inducible promoter that triggers the development of Bt protein only where and when insect damage occurs may have even greater benefit and much reduced risk to the environment.

The possibilities of improving fungal disease resistance in a deciduous tree species by genetic engineering have been tested in silver birch by producing birch lines carrying a sugar beet chitinase IV gene. In many crop plants, the introduction of a transgenic chitinase gene has led to improved disease resistance against the fungal pathogens studied (e.g. Grison *et al.*, 1996; Emani *et al.*, 2003; Vellice *et al.*, 2006). Improved resistance against the leaf spot fungus (*Pyrenopeziza betulicola*) was detected in chitinase transgenic silver birch in a greenhouse experiment (Pappinen *et al.*, 2002) but the improvement in disease resistance could not be confirmed in a field trial after natural infection with *P. betulicola*. However, some transgenic lines showed improved resistance against birch rust caused by the fungus *Melampsoridium betulinum* in the field (Pasonen *et al.*, 2004). The contradictory results from the greenhouse and the field trial in the resistance of chitinase transgenic birch to birch leaf spot disease may be due to the fact that only one isolate of *P. betulicola* was used to infect the plants in the greenhouse, while natural infection in the field is likely to consist of more than one genetically distinct individual of the same pathogen (Paavolainen *et al.*, 2001). Also several biotic and abiotic factors, to which the plants were exposed in the field trial but not in the greenhouse, may have influenced the fungal disease resistance reaction of the birch lines studied. These results actually demonstrate the importance of field studies with genetically modified trees, where conditions are very similar to a commercial plantation situation.

INVASIVENESS OF TRANSGENIC TREES

Weediness

Genetic modification may cause unpredictable change in the fitness of a tree species. Thus, it is important to determine whether newly introduced traits have a potential to make genetically modified trees more likely to be invasive in natural habitats. More invasive means increased weediness that is based on many different characters, and weediness of a plant species plays a more important role than isolated genes used for genetic transformation (Luby and McNichol, 1995). At the same time, Fitter, Perrins and Williamson (1990) and Williamson, Perrins and Fitter (1990) propose that small genetic changes can cause large ecological alterations. The potential impacts of individual transgenes should be determined by evaluating their phenotypic effects (Hancock, 2003). Although current information may be insufficient to rank the relative risk of many transgenes, they can be grouped by the type of impact they have on reproductive fitness. Genes, such as mercuric ion reductase in the absence of heavy metal contamination (Bizili, Rugh and Meagher, 2000) or *rolC* from *Agrobacterium rhizogenes* (Fladung, 1990; Fladung, Muhs and Ahuja, 1996) should be considered detrimental because they reduce plant fitness. In general, genes with detrimental effects will be selected against in the natural environment and will not spread (Hancock, 2003).

Genes improving stress tolerance to detrimental biotic or abiotic factors fall into a group whose incorporation into natural populations could increase fitness. Transgenes already deployed that fall into this category include Bt or chitinase genes for insect or fungal resistance (Genissel, Viard and Bourguet, 2000; Pasonen *et al.*, 2005) or those conferring tolerance against drought, salinity or high temperature (Wang, Vinocur and Altman, 2004). In general, however, it must be considered that conventional tree breeding practice, which can include crossing the species barrier (forced hybridization, embryo-rescue), introduces far greater genetic change than the transfer of a single or a few genes into a species. Further, forest tree breeders frequently breed for increased resistance against specific pathogens or other environmental challenges and the weedy potential of those new genotypes has never been evaluated, nor any risk considered in the context of environmental impacts. Consequently, the consideration of weedy potential of transgenics must take place in the context of accepted breeding and selection practice. This will lead to informed and better decision-making that takes all aspects of a specific practice into account, and ultimately will reduce the environmental impact of forestry practice.

Vegetative spreading

Spread by vegetative means, through root suckering, that is known for a number of tree species is also a very important factor in risk assessment for both transgenic and non-transgenic poplars (Fladung *et al.*, 2003). Root suckers arise from adventitious buds on the extensive lateral root system. Large numbers of suckers from a single tree can quickly develop into a dense colony. Strategies for controlling vegetative reproduction may be necessary for containment of modified trees.

In a field trial using 35S::*rolC* and rbcS::*rolC* transgenic aspen, the appearance of a root sucker was first observed after four years from planting (Fladung *et al.*, 2003). In the following year (i.e. fifth year), a total of 226 root suckers were found within the field trial, and their positions were determined. The determination of the exact origin of root suckers was not possible, because root length of individual trees was found to be up to 10 m (Kaldorf, personal communication). All root suckers derived were phenotypically wild type and hybrid aspen (*P. tremula* × *P. tremuloides*) but not pure aspen (two clones of *P. tremula*) or 35S-*rolC* transgenic (Fladung *et al.*, 2003). To confirm the absence of any 35S::*rolC* gene construct and to determine the portion of the rbcS::*rolC* transgenic plants, PCR analysis was performed to determine the presence of the 35S::*rolC* chimeric construct as well as the single *rolC* gene.

The results clearly indicate that in 124 plants the *rolC* gene was present but in no case in combination with the 35S promoter, and 97 root suckers showed no *rolC* but genomic control amplification (Fladung *et al.*, 2003). From these results it is suggested that more than half of the root suckers analysed originated from rbcS::*rolC* transgenic trees. In the same assessment year (five years from planting), 15 root suckers with wild type phenotype were also observed outside the field trial's borders, reaching 5 m to the margin of the field. From 13 plants investigated, nine plants revealed the presence of the *rolC* gene. Only four plants were characterized as non-transgenic. The results indicate that possible vegetative propagation should also be included in risk assessment research studies.

VERTICAL AND HORIZONTAL TRANSFER OF GENES

Gene flow via pollen and seeds

For good management practices of transgenic tree plantations, knowledge of relevant gene flow parameters is required. Gene flow from transgenic plants to interfertile wild or weedy relatives is often cited as a potential risk in the commercialization of transgenic crops. In a poplar plantation, DiFazio (2002) studied gene flow and its implications for transgenic risk assessment. A combination of large-scale field studies, genetic analysis and simulation modelling was used. Field studies demonstrated low levels of gene flow from existing hybrid poplar plantations (*Populus trichocarpa* × *P. deltoides*) in three settings. Using sensitivity analysis, it was demonstrated that competitiveness and fertility of transgenic trees are important factors determining the extent of modelled gene flow, and that these factors interacted such that effects of enhanced competitiveness appeared to be obviated by cultivation of low-fertility transgenic trees. Disturbance regime, plantation silviculture and characteristics of the landscape surrounding plantations also had a strong influence on the rate of gene flow. It has, however, not been demonstrated so far that gene flow from genetically modified trees presents more risk than that from conventionally bred trees. However, as a precaution, the development of sterility strategies provides a favourable solution to limit gene flow to native species and non-native species or bred taxa. If the production of pollen and seeds is reduced, gene flow can be minimized or even prevented (DiFazio, 2002).

In another study, the percentage and flow distance of reproductively effective poplar pollen was estimated. Seeds were harvested from two female trees growing in the Arboretum of the Institute of Forest Genetics (Grosshansdorf, Germany). By microsatellite-based parental analysis germinated seedlings were investigated with respect to pollen origin (Fladung, unpublished results). It could clearly be demonstrated that only two to three trees from the close neighbourhood contributed as the main pollen donors, and approximately 70% of reproductively effective pollen originated from trees growing in the vicinity of the mother trees. The latter result is surprising, in particular in light of the fact that poplar is a wind-pollinated species. However, the results indicate that gene flow might be a problem when dealing with transgenic trees.

To reduce or even avoid gene flow of transgenes into non-transgenic relatives, incorporation of sterility genes into transgenic trees has been proposed (Strauss *et al.*, 1995). A number of sterility gene constructs have successfully been tested in crop plants, e.g. by expression of deleterious genes, such as barnase (Mariani *et al.*, 1990), stilbene synthase (Fisher, Budde and Hain, 1997), the gene for ribosome inactivating protein (Palmiter *et al.*, 1987), use of dominant negative mutations (Mitzukami *et al.*, 1996), and gene suppression strategies such as antisense suppression, co-suppression and RNA interference (Skinner *et al.*, 2003). Sterility conferring genes, however, need specific floral regulatory promoters (e.g. TA29 promoter from tobacco) to direct expression of genes in reproductive structures (Koltunow *et al.*, 1990; Mariani *et al.*, 1990). Few investigations have been reported for induction of sterility in transgenic *Populus* (Meilan *et al.*, 2001; Skinner *et al.*, 2003), but the effectiveness of the transgenic sterility systems still needs to be demonstrated.

The first poplars transformed with sterility genes showed a lower growth performance compared with control plants (Meilan *et al.*, 2001). Here, the use of heterologous promoters seems to direct the activity of cytotoxic gene expression in non-target, vegetative tissues ('leaky' expression; Meilan *et al.*, 2001). However, when these cytotoxic genes are controlled by other, more specific, promoters, e.g. in optimal case under forest trees floral promoters (Skinner *et al.*, 2003) or other genes are used, 'leaky' expression may be avoided. The expression of stilbene synthase under control of radiate pine male cone promoters or the C-GPDHC from *Cuphea lanceolata* revealed no effects on plant performance (Hoefig *et al.*, 2003; Hoenicka and Fladung, 2006).

Horizontal gene transfer

The exchange of genes between organisms that are sexually non-compatible is called horizontal gene transfer (HGT) and it is a common evolutionary mechanism, mainly found in micro-organisms. The possibility of transfer of a transgene from a transgenic plant into other organisms (mainly bacteria, fungi and viruses) has become an important argument against genetically-modified plants (Stirn, 2000; Peerenboom, 2000). Natural HGT has been detected between bacteria and plants (Brown, 2003), where the gene transfer system by *Agrobacterium* species is one of

the best characterized examples of HGT (Chilton *et al.*, 1977; Schell *et al.*, 1979). So-called *Ngrol* genes that are similar in sequence to genes in the left transferred DNA (TL-DNA) of *Agrobacterium rhizogenes* have been found in the genome of untransformed plants of *Nicotiana glauca* (Aoki and Syono, 1999). This implies that this HGT has occurred very early in the evolution of the genus *Nicotiana* (Aoki and Syono, 1999).

Sequence homologies between plant genes and the respective genes in bacteria have indicated that HGT is also possible from plants into bacteria. For instance the glucose-6-phosphate-isomerase gene in *Clarkia ungulata* is similar to the one in *E. coli* (Schlüter and Potrykus, 1996). The mechanisms underlying such prokaryote-eukaryote gene transfer or vice versa, excluding that between *Agrobacterium* and angiosperms, as well as conditions by which HGT takes place, are broadly unknown (Kondo *et al.*, 2002; Won and Renner, 2003). So far, researchers have been able to demonstrate HGT from genetically modified plants to micro-organisms like plant-associated fungi (Hoffman, Golz and Schieder, 1994) or bacteria (Nielsen, van Elsas and Smalla, 2000), but only under optimized laboratory conditions or in soil microcosms. Several experimental studies have failed to demonstrate HGT from transgenic plants to bacteria (Bertolla and Simonet, 1999; Gebhard and Smalla, 1999; Nielsen *et al.*, 1997, 1998) and, to our knowledge, HGT from transgenic plants to other organisms has not been detected in field conditions. In the light of present knowledge, HGT can occur but at such low frequencies that detecting it is extremely difficult, mainly due to the huge sampling efforts needed and the non-cultivable nature of most bacteria (Heinemann and Traavik, 2004; Nielsen and Townsend, 2004).

Since the availability of free DNA in soil is a limiting factor for HGT (Gebhard and Smalla, 1999), and because some fungi grow in intimate contact with trees (ectophytic fungi) or even within plants (endophytic fungi), uptake of plant DNA by these fungi might be more likely. Mycorrhizae are highly evolved, mutualistic associations between soil fungi and plant roots. Many forest tree species are largely dependent on ectomycorrhizal fungi for the uptake of mineral nutrients (Smith and Read, 1997). Up to now, two different approaches have been used to study HGT from trees to fungal hyphae in ectomycorrhizas. In the study of Kaldorf *et al. (*2004), transgenic aspen lines, containing the *rolC* gene from *Agrobacterium rhizogenes* under the control of the light-inducible plant rbcS promoter (Fladung, Großmann and Ahuja, 1997.), were used. The occurrence of HGT was analysed by the amplification of the fungal DNA with nested *rolC* gene primers. No single *rolC* signal was detected in any of the samples analysed (Kaldorf *et al.*, 2004). Unfortunately, only a few replicates were tested in this study.

In a second approach, transgenic aspen containing the *Streptococcus hygroscopicus* bar gene conferring herbicide (BASTA) resistance under the control of a fungal GPD promoter were field tested (Nehls *et al.*, 2006). Mycorrhizae were formed under axenic conditions between transgenic aspen and wild type hyphae of *Amanita muscaria* using a Petri dish system. To detect HGT events, a total of 35 000 ectomycorrhizas were dissected and tested for BASTA resistance.

From these, 102 fungal colonies were formed under BASTA selective conditions. However, since these fungal isolates stopped growth when transferred to fresh selection plates, and no bar gene could be amplified from fungal DNA by PCR, these fungal colonies were characterized as false positives (Nehls *et al.*, 2006).

Another method to determine the frequency of HGT from a tree species to associated micro-organisms may become applicable with increased genome sequence information available for an increasing number of tree species and associated organisms. Any historical HGT of a tree gene into such associated organisms could easily be detected simply by *in silico* analysis and comparison of the genomes. Such study might actually confirm the view of many authors that the frequency of HGT, if it exists between higher eukaryotes and prokaryotes, is infinitesimally small. Further, discussion of risk related to HGT from transgenic plants to other organisms tends to ignore the fact that genes used for transgenic plant production mostly originate from the natural environment, and hence have been available for transfer to other organisms over evolutionary time frames. It is hard to imagine why the HGT of a particular gene from a transgenic plant into a micro-organism should be of higher risk potential than the HGT of the same gene from its natural source into a new organism.

IMPACTS ON NON-TARGET SPECIES

All living organisms including trees are part of the ecological food chain, and thus many non-target species are in contact with transgenic ones expressing the foreign gene and synthesizing its product (Mullin and Bertrand, 1998). Genetically modified trees transformed with the intent of conveying greater resistance to pathogens have been of particular concern because ecotoxic effects on other organisms such as insects or soil organisms have been assumed (Myhr and Traavik, 2003). The expression of broad-spectrum antimicrobial components by genetically modified plants may not only suppress target pathogens, but may also affect plant symbionts such as mycorrhizae and rhizobia, as well as other micro-organisms involved in decomposition and nutrient cycling (Morra, 1994; Glandorf, Bakker and Van Loon, 1997). We need to consider, however, that this is not intrinsic to genetically modified trees and that trees bred and selected for increased resistance to insects or pathogens may have similar effects.

Mycorrhiza fungi

In boreal soil ecosystems, forest trees form symbiotic associations with a number of ectomycorrhizal fungi that facilitate nutrient supply and provide protection against pathogens (Smith and Read, 1997). Mycorrhizal and saprophytic fungi contain chitin in their cell walls and may be highly sensitive to transgenic chitinases or to overexpression of a plant's own chitinases. In a preliminary study, the ability of eight chitinase transgenic birch lines showing varying levels of sugar beet chitinase IV expression to form ectomycorrhizae with the common ectomycorrhizal fungus, *Paxillus involutus* (Batsch) Fr., was tested *in vitro*. All tested transgenic birch lines were able to form normal ectomycorrhizae containing

distinctive mantles and Hartig nets, and the level of sugar beet chitinase IV expression was not detected to have an influence on mycorrhizal colonization. Two transgenic lines showing high chitinase expression had a lower percentage of mycorrhizal root tips than the other transgenic lines or the control plants, but the difference was not statistically significant (Pasonen *et al.*, 2005).

Root samples were also collected from chitinase transgenic and wild-type plants grown in the field, and mycorrhizal colonization as well as mycorrhizal species diversity of the roots of different types of the plants were studied. The roots of all the chitinase transgenic and control plants were well colonized by ectomycorrhizas expressed as the percentage of mycorrhizal root tips. Seven lines showing varying levels of sugar beet chitinase IV expression and total endochitinase activity were selected for detailed analysis of fungal species diversity. Fungal species were separated in denaturing gradient gel electrophoresis (DGGE), and sequencing of the DGGE bands have so far revealed that all the plants were colonized by a variety of fungal genera (Pasonen *et al.*, 2008). Although the transgenic lines were slightly less colonized by mycorrhizae than the control plants, the differences were so minor that the ecological consequences are difficult to estimate. In particular, the influence of environment will most probably be stronger and lead to more variation compared with the results of this experiment. This has been shown in a field trial experiment studying the mycorrhizal populations around genetically modified versus non-genetically modified radiata pine roots in a field test. Population differences between genetically modified and non-genetically modified trees could be detected; however, they were smaller than seasonal differences, and also smaller than differences between non-genetically modified individual trees (Walter *et al.*, in prep.).

The mycorrhizal colonization was also investigated in field-released 35S::*rolC* and rbcS::*rolC* transgenic aspen trees over a 15-month period (Kaldorf *et al.*, 2000, 2002). Arbuscular mycorrhizae were unambiguously identified in root samples from all aspen lines investigated. Arbuscular mycorrhizae formation was rare, with an average of less than 10% of the root length colonized. Quantitative differences between the transgenic and non-transgenic aspen trees were small and not significant. However, the majority of the fine roots were colonized with ectomycorrhizae. Taking all types of ectomycorrhizae together, again no significant differences in the quantity between the different aspen lines could be detected, including all transgenic and non-transgenic lines.

Within the release area of the transgenic aspen in Grosshansdorf, Germany, four fungal species were found to be dominating the ectomycorrhizal community. These four species formed more than 90% of all mycorrhizae, but a further eleven ectomycorrhizal types were found occasionally. The average of different ectomycorrhizal types found in each single sample was 5.1 for Esch5 (untransformed control), 4.9 for E14-4 (rbcS::*rolC* transgenic line) and 4.7 for E2-5 (35S::*rolC* transgenic line). These small differences were not statistically significant and indicate similar ectomycorrhizal diversity between transgenic and non-transgenic aspen (Kaldorf *et al.*, 2000). When investigating the structure of the mycorrhizal

community, a significant difference was found only for one transgenic line. In roots of the transgenic aspen line E2-5 one of the mentioned four common ectomycorrhizal morphotypes was rare and poorly developed when compared with other transgenic lines and with non-transgenic controls (Kaldorf *et al.*, 2002). It is suggested that this effect is clone specific, as the formation of this ectomycorrhizal type was not affected by the transgene expression in the other transgenic line carrying the same construct.

Soil micro-organisms and decomposition rates

The decomposition process of the leaf litter derived from sugar beet chitinase IV transgenic silver birch and the effects on the decomposer populations were studied in a field trial by Vauramo *et al. (*2006). It was hypothesized that the expression of the chitinase gene in transgenic birch would influence chitin-containing saprophytic fungi and fungal-feeding microfauna, thereby affecting the decomposition rate of the litter. The influence of the transgenic leaf litter on the decomposer community was studied by analysing the living fungal biomass and the nematode community structure. Of the soil fauna, nematodes are considered as potential indicators of the function of the decomposer food web because of their high abundance, diversity and close relationship to soil processes via their food specificity. Functional (trophic) group analysis on the abundance of different feeding groups – bacteriovores, fungivores, omnivores and predators – can provide a quick source of information of the available resources, since nematodes can respond rapidly to environmental changes (e.g. Ritz and Trudgill, 1999).

An indication of negative effects of chitinase transgenic leaf litter on the number of nematodes was previously obtained in a microcosm experiment (Kotilainen *et al.*, 2005). The decomposition experiment was established in a field close to the field trial of transgenic birch trees. The experiment included leaves from chitinase transgenic birch lines that showed low, intermediate or high transgene expression. Only the highly expressing transgenic lines and the control plants were included in the nematode and microflora assays. The leaves were collected from birch trees, placed into litter bags and buried in the soil at a depth of 5 cm. Half of the leaves were allowed to decay in the field for eight months and the other half for 11 months. The decomposition rate of the litters was expressed as litter mass loss, fungal biomass as litter ergosterol content, and total microbial biomass as substrate induced respiration, which is a measure of respiratory response of soil microbes to the addition of glucose (Vauramo *et al.*, 2006).

The decomposition rate of any of the leaf litters from the chitinase transgenic plants did not differ from that of the control plants. Also, no differences in fungal biomass, total microbial biomass or activity (basal respiration) were detected between the litters. In the nematode assay, the total number of nematodes and the abundance of bacterial feeding nematodes varied significantly with the decomposition time, but the litter type had no influence on the number of nematodes or on the relative abundance of the different feeding groups. However, pair-wise comparisons revealed that after eight months of decomposition the

transgenic litter from one line showing high chitinase IV expression contained significantly more nematodes than the control litter, while after 11 months the situation was reversed. No differences in the community structure of the nematodes between the transgenic lines and the control plants were detected (Vauramo *et al.*, 2006). These results indicate that the chitinase transgene *per se* had no influence on the decomposition of the transgenic litter or the microbial content of the litter. The negative effect of the transgenic litter on the number of nematodes in the microcosm experiment (Kotilainen *et al.*, 2005) and in the field trial between one transgenic line and the control plants after 11 months of decomposition (Vauramo *et al.*, 2006) may indicate sensitivity of nematodes to the transgenic chitinase or to some other chemical change occurring in the transgenic line(s). The experiment also shows that an effect is not necessarily a negative effect: it may in fact be positive.

Phytopathogenic fungi

The phytopathological status of the leaves and stems of 35S::*rolC* and rbcS::*rolC* transgenic aspen trees were studied in a field trial and infection studies were initiated under controlled conditions. The objective was to determine the influence of the *rolC* gene on infection as well as on the spectrum of fungal pathogens. First, the diseased foliage of the aspen crown was assessed. The assessment of leaf spot disease (*Pollaccia radiosa*) and poplar rust (*Melampsora* spp.) was made by iterative estimation of the symptoms (Gieffers and Fladung, 2000). The infestation patterns of both fungi can be identified with the different transgenic lines. Resistance reactions were not found. Former infection studies showed similar results for 35S::*rolC* transgenic potatoes (Fladung and Gieffers, 1993). The infestation level of the poplar rust was higher than that of *Pollaccia radiosa*. Both fungi showed a similar infection, which is confirmed by a high correlation coefficient ($r > 0.9$).

In addition, infection levels of phytopathogenic fungi and, simultaneously, the content of important metabolites (sucrose, glucose, fructose, starch) were determined. Positive correlations were found between the level of diseased foliage of the aspen crowns and the contents of glucose and fructose in the leaves. Measurements of carbohydrate contents were made on the same dates as phytopathological investigations were done. Again, similar results were obtained with 35S::*rolC* transgenic potatoes.

Insects

The most extensively studied examples of engineered resistance are based on the use of delta-endotoxins of the bacterium *Bacillus thuringiensis*. *B. thuringiensis* is a naturally occurring ubiquitous soil bacterium that produces a toxin (Bt toxin) lethal to certain insects (Dale, Clarke and Fontes, 2002). There is no doubt that Bt-transgenic plants will kill the target pest species, but there is no serious scientific report available describing that non-target pest species are affected as well. The evaluation of possible environmental damage due to insect-resistant Bt trees should take also into account the environmental damage caused by the use of

pesticides. It is argued that millions of birds and billions of insects are killed each year in the United States of America alone as a result of pesticide use (Dale, Clarke and Fontes, 2002). Advantages and disadvantages of Bt trees, therefore, should be carefully considered in the context of accepted practice.

It is also noteworthy that sprays containing living *B. thuringiensis* are broadly accepted as an alternative for pest management, even in organic farming. Release of these living bacteria or proteins into the environment may represent a similar or probably higher risk than genetically modified plants (Brimner and Boland, 2003; Boland and Brimner, 2004), particularly when transgenic plants express the Bt gene only when and where insect damage occurs. However, conventional Bt use has not been raised as a concern with similar implications to those discussed for Bt-toxin-carrying transgenic plants (Bt trees). Further, these conventional Bt pesticides are freely available in many countries and are used frequently in all types of agricultural practice and in forest protection against lepidopteran defoliators, and have been for many years (Bauce *et al.*, 2004; Kouassi *et al.*, 2001; Cadogan and Scharbach, 2003).

In the Finnish field trial using chitinase IV transgenic silver birch, the composition and density of insect populations and leaf damage caused by insects were monitored three times during one growing season, and compared between transgenic lines and wild-type birch clones. The composition of insect populations was studied at order level, and temporal, horizontal and vertical variations in insect density and species composition were recorded. Different types of leaf damage were classified as leaf chewing, leaf mining, gall, leaf roll, web formation, leaves glued together and sucking damage. The level of the leaf damage was expressed as the proportion of the branches studied in which any type of leaf damage was observed. No clear differences between the transgenic and control trees were found in the species composition, but the total insect densities were generally higher among the chitinase transgenic plants than among the corresponding control plants. Also only minor differences in the composition of different types of leaf damage were found between the transgenic and control plants. The results indicated that the expression of the transgenic chitinase gene in birch did not have clear harmful effects on insects (Vihervuori *et al.*, unpublished results).

TRANSGENE STABILITY AND EXPRESSION

Stable integration of foreign genes into plant genomes and predictable transgene expression are important, in particular when transgenic plants are considered as basic material for plant breeding programmes. Depending on the introduced trait, transgene stability may be required for the whole life cycle of plants, including their vegetative growth (mitotic cell divisions in somatic tissues) as well as during the formation of generative cells following meiosis. However, many investigations using annual crops have shown that expression of transgenes is less stable than had originally been thought.

Most of these events reported are homology-dependent gene silencing phenomena that function at the level of transgene transcription, or post-

transcriptionally (reviewed in Paszkowski, 1994; Meyer, 1995). In transgenic trees, gene silencing has been reported in those transformed with the *rolC* gene (Fladung, 1999; Kumar and Fladung, 2001). Plants transgenic for the 35S::*rolC* gene construct show an altered plant phenotype (Fladung, Muhs and Ahuja, 1996.) that was used for morphological screenings of transgene instability under *in vitro* cultivation, in the greenhouse as well as under field conditions (Kumar and Fladung 2001).

Under *in vitro* conditions, long-term (five to six years) morphological observations for *rolC* expression have so far revealed a stable *rolC* phenotype in 15 hybrid aspen (*Populus tremula* × *P. tremuloides*) transgenic lines analysed. Out of the 16 wild aspen (*P. tremula*) lines obtained, however, only seven lines survived the long-term *in vitro* culturing. Among these seven lines, variable morphological expression of the *rolC* gene was detected in three lines (Kumar and Fladung, 2001). More lines revealing alterations in *rolC* expression were observed after transfer from *in vitro* conditions to the greenhouse or field (Kumar and Fladung, 2001). Out of the 15 Esch5-based transgenic lines transferred to the greenhouse, incomplete suppression of the transgene expression was observed in three lines (Fladung, 1999; Kumar and Fladung, 2001). Among five wild aspen transgenic lines transferred to the greenhouse, three lines were observed with altered or reverted transgene expression.

Reversion of leaves or single shoots of a *rolC*-transgenic aspen plant to wild type was observed for the line Esch5:35S::*rolC*#1 (Fladung, 1999; Kumar and Fladung, 2000). In two other hybrid aspen-based transgenic lines (Esch5:35S::*rolC*#2 and Esch5:35S::*rolC*#12), the phenotypically visible *rolC* expression decreased gradually over a period of three to four years of cultivation in the greenhouse (Kumar and Fladung, 2001). The loss of the *rolC* expression seems stable in these lines, thus the plants, once reverted, maintain the changed features in the following years. Similar stable complete *rolC* suppression was observed in two wild aspen transgenic lines (W52:35S::*rolC*#9 and W52:35S::*rolC*#3; Fladung and Kumar, 2002).

Compared with the lines showing completely suppressed *rolC* phenotypes, the alterations in morphological expression of transgene were more complex and variable in other wild aspen-based transgenic lines planted in the field. The altered plants from Brauna11:35S::*rolC*#2 showed morphological features different from both the control and *rolC* phenotype (Kumar and Fladung, 2001). The length and width of the leaves collected from the reverted plants were intermediate between the control and 35S::*rolC* phenotypes. The reverted morphological expression was confirmed by northern experiments, which clearly showed very weak *rolC*-specific transcripts from the leaves of reverted plants grown under field conditions. The *rolC*-specific transcript was, however, present in leaves collected from the plants maintained in the greenhouse, or from *rolC*-expressing plants in the field.

However, other reports for transgenic trees claim that there is no evidence that expression of transgenes under vegetative propagation is more variable than expression of most endogenes (Strauss *et al.*, 2004). Analysis of GUS expression of 35S:*uidA* transgenic poplar grown in a field trial in France revealed that all transgenic

plant lines showed stable expression of the transgene (Pilate, Ellis and Hawkins, 1997). Hawkins *et al. (*2003) evaluated the transgene expression in a hybrid poplar (*Populus tremula* × *P. alba*) clone transformed with constructs carrying the *uidA* reporter gene under the control of either a constitutive or a vascular-specific promoter. While important variations in expression levels occurred, the transgene appeared to be stably expressed throughout a six-year period. Similar results were reported for hundreds of different poplar transformants carrying various gene constructs and tested under field conditions (Strauss *et al.*, 2004). Even when 35S::*uidA* and rbcS::*uidA* transgenic trees are treated with stress conditions (high temperature, UV-light) no stress-related transgene silencing could be observed for poplar, larch or fir (InfoNet-Umwelt SH, 2004). During a field trial of transgenic radiata pine in New Zealand, expression stability of the introduced and non-selected *nptII* gene could be demonstrated. It was observed that, while transgenic radiata pine tissue shows frequent silencing, those transclones that still express reliably when trees are regenerated usually continue expressing the new gene (Walter, in prep.).

Silencing in *35S*::*uidA* transgenic poplar was detected only for lines that were probably silenced from the beginning, i.e. shortly after the transformation process (Hawkins *et al.*, 2003; Strauss *et al.*, 2004; InfoNet-Umwelt SH, 2004). However, due to the destructive nature of the GUS activity test or other enzyme measurement procedures, only a small part of the plant at a given time can be screened with respect to transgene stability. As shown by Kumar and Fladung (2000) and Fladung and Kumar (2002), inactivation of the phenotypic marker gene construct 35S::*rolC* is a very rare event and occurs in an unpredictable manner. Thus, transgene silencing can happen at a single branch of a single plant among a high number of clonal ramets, and in the next year disappear in the same shoot (Fladung and Kumar, 2002). Such silencing events remain undetectable with destructive reporter genes and can only be monitored when non-destructive reporter gene assays are being used.

The occurrence of a transgene repeat is often accompanied by methylation of the promoter and/or the transgene (Kumar and Fladung, 2000). However, not every transgenic line harbouring two T-DNA copies in repeat form is consequently silenced from the beginning. Two 35S::*uidA* transgenic poplar lines, characterized by the presence of T-DNA repeats, that were cultivated either under greenhouse or *in vitro* conditions, had at the time of writing revealed GUS expression over a period of seven years in plants. It remains unknown whether these lines are 'insensitive' to repeat-related transgene inactivation, or silencing has occurred but was not detected so far, or silencing of the transgene may happen sometime in the future. It is also interesting to note that transgenic radiata pine was still expressing a transgene reliably at age nine years of the trees that were originally transformed using biolistic techniques, leading to highly complex integration patterns.

Taken together, the fact that silencing is possible but may happen sometime in future is in particular important when the efficiency of strategies for biological confinement of transgenic plants is discussed, e.g. use of genes leading to male

and/or female sterility. Gene silencing of these genes would allow crossings of transgenic woody plants with their natural relatives even when a low rate of instability is assumed. The question is whether the out-crossed transgene can 'survive' in the gene pool of the natural population or will disappear (DiFazio *et al.*, 2004).

GROWTH PARAMETERS AND UNINTENDED EFFECTS

Few reports are available on the performance of transgenic trees under natural environmental conditions in long-term field trials. In a recent report, transgenic poplars carrying antisense transgenes of lignin biosynthesis key enzymes were field tested for growth indicators, interactions with insects and paper-making characteristics. It was concluded that transgene expression did not interfere with tree growth or fitness under field conditions (Pilate *et al.*, 2002).

Also during the field trial with *rolC* transgenic trees in Germany, results on growth and other parameters were obtained in different risk assessment-related scientific projects (summarized in Fladung *et al.*, 2004). Measurements of height as well as stem diameter were made every year during the field experiment. Tree height revealed higher values for the controls and the rbcS::*rolC* transgenic aspen compared with the 35S-*rolC* transgenics. Further, the dynamics of growth as well as stem diameters at 10 cm height of transgenic and control aspen trees were significantly different. In 1999 and 2000, the stem diameter in the control trees was double that of the transgenic aspen trees of equivalent maturity (Gruenwald, Ruel and Fladung, 2001). However, a higher annual increase in stem diameter was found in the 35S::*rolC* transgenic plants than in the control aspen trees, which showed constant increase. Further, the leaf size of the 35S::*rolC* transgenic aspen was much smaller than the controls (Gruenwald, Ruel and Fladung, 2001), and also the length-to-width ratio was different (Fladung, Muhs and Ahuja, 1996). The effect of the 35S::*rolC* gene construct on flushing of greenhouse-grown plants has already been described earlier (Fladung, Muhs and Ahuja, 1996; Fladung, Großmann and Ahuja, 1997). In spring, the 35S::*rolC* transgenics started to flush at least two weeks earlier than the controls and transgenic plants carrying different gene constructs. A similar effect was observed in spring of every year in the 35S::*rolC* transgenic aspen grown in the field.

The effects of the expression of the sugar beet chitinase IV gene on growth and growing habit, and the quality and leaf phenology of the chitinase transgenic silver birch lines, were monitored during three growing seasons in the Finnish field trial. The traits monitored are important for adaptation as well as for birch breeding. The attractiveness of chitinase transgenic birch to larvae of the cambium miner (*Phytobia betulae* Kang, Diptera: Agromyzidae), causing an aesthetic defect to birch wood, was also studied. Three lines out of fifteen were frequently different from the control plants in growth and leaf phenology, and these differences are suggested to be due to position effect of the transgene. The level of the transgene expression was not detected to have an influence on the growth parameters studied nor leaf phenology. In a field trial with transgenic and non-transgenic radiata pine

in New Zealand, transgenic trees were indistinguishable from controls with regard to growth characteristics (Walter, in prep).

The level of transgene expression, however, correlated with parameters related to stress status of a tree indicated by the increased amount of red colour in the leaves and lowered general condition of the transgenic trees. The stress status of the tree was described by the amount of red colour in the leaves because the ecophysiological function of foliar anthocyanins has been suggested to be related to the protection of the photosynthetic apparatus in the plants experiencing environmental stress (Hoch, Zeldin and McCown, 2001; Close and Beadle, 2003). The variation in the occurrence of *Phytobia* spp. was explained mainly by the differences in plant size, not by the level of transgene expression (Pasonen *et al.*, 2008). The expression of the sugar beet chitinase IV gene in transgenic birch *per se* did not cause significant changes in plant morphology, but was presumed to influence the stress status of the transgenic plants, which is expected to make the transgenic plants less fit than the wild-type plants.

CONCLUSIONS

Based on the authors' experience with field-released transgenic trees, support and encouragement is given to:

- the adoption of a case-by-case assessment process, with a focus on scientifically informed decision-making, with regards to deployment of genetically modified trees in plantation forestry;
- an informed and evidence-based decision-making process on GMO deployment by government authorities, ensuring that any potential risk is evaluated in the context of accepted practice;
- the continued development of environmental risk assessment technologies for genetically modified trees, in the context of currently accepted forestry practice;
- the adoption of a precautionary approach where there is either a scientifically substantiated and quantifiable risk of GMOs becoming invasive weeds, or of introducing foreign genes into native forest with potential adverse impacts on biodiversity values or plant growth characteristics;
- the active development of risk mitigation strategies, where a risk is identified. For example, where the spread of genetically modified material through pollen or seed dispersal is considered a risk, sterility techniques should be evaluated and deployed to prevent the formation of seed or pollen, or both;
- encouraging the development of GMO trees that are unable to spread genetically modified material.

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10. Social, legal and regulatory issues related to transgenic trees

R.A. Sedjo

Genetic engineering has already had a huge worldwide effect on agriculture. Genetic engineering is the use of recombinant DNA and asexual gene transfer methods to modify organisms (Strauss *et al.*, 2001) and produce so-called genetically modified organisms (GMOs) or transgenics. Although the first commercial genetically modified crops (tomatoes) were planted in 1994, 1996 was the first year in which a significant area (1.66 million hectares) of crops was planted containing genetically modified traits. Since then there has been a dramatic increase in plantings, and by 2005–06, the global planted area reached almost 87.2 million hectares. This is equal to five times the total agricultural area or nineteen times the total arable cropping area of the United Kingdom.

Almost all of the global genetically modified crop area comprises the four main crops in which genetically modified traits have been commercialized, namely soybean, maize, cotton and canola. In 2005, genetically modified traits accounted for 29% of the global plantings of these four crops: genetically modified soybeans accounted for the largest share (62%), followed by maize (22%), cotton (11%) and canola (5%). In terms of the share of total global plantings to these four crops, genetically modified traits accounted for a majority of soybean plantings (59%) in 2005. For the other three main crops, the genetically modified shares in 2005 were 13% for maize, 27% for cotton and 18% for canola (ISAAA, 2006).

Much of the biotechnology already developed for agriculture has direct applications in forestry, and many of the biotechnological innovations being introduced to forestry are being adapted directly from agriculture. Innovations such as the introduction of the herbicide-tolerant gene into tree seed stock follow directly from the success of the same herbicide-tolerant gene in agricultural crops. Research similar to that in agriculture is also being undertaken with disease- and pest-resistant genes, as well as other gene-altering modifications. It is anticipated that these innovations could result in substantially reduced wood costs through increased wood yields, the reduction of plantation establishment costs and reduced tree losses through the growing cycle. Also, biotechnological research in forestry is moving in the direction whereby genetic alteration would enhance wood quality by producing desired modifications in fibre characteristics, lignin content or limb thickness in a manner that would reduce processing costs. All of these modifications have the potential to generate financial benefits through reduced production costs and enhanced productivity. Additionally, conservation benefits could be achieved from the restoration of certain species ravaged by disease, such as the American chestnut (Bailey, 1997).

Internationally it is recognized, both through the Cartagena Biosafety Protocol and the United Nations Industrial Development Organization, that there is a need to form national regulatory systems to control the release of genetically modified organisms into the environment (Pachico, 2003; Sedjo, 2005). However, many countries have a variety of legislative and regulatory processes involving transgenics that predate the various international initiatives.

While there is a general agreement that existing procedures in some countries provide the basic process for deregulation, specific procedures and protocols may need to be worked out for trees, both industrial wood and orchard. This understanding was reflected in the meetings held in July 2003 outside Washington, DC, organized by USDA APHIS, which discussed some of the regulatory problems unique to transgenic trees. For example, there remains the question as to whether regulation ought to focus on the process of transgenics or on the character and attributes of the plant, irrespective of the process. In the United States of America, discussions continue and some regulatory changes are expected. In addition, developers are looking to devise field trials that will provide more efficient, low-cost testing procedures, including adequate testing in relatively short periods, with procedures to test a number of genes in one trial. These approaches differ from the current concept that each gene needs separate testing and that complete testing is required *de novo*. Also, there is re-consideration regarding allowing contingent deregulation, which might provide for continuing testing and monitoring for some time after partial deregulation.

Although the existing provisions are designed to provide for deregulation given that the requisite criteria are met, the paucity of transgenic tree deregulation over the past decade raises questions about how the law and regulations are being applied. The absence of successful transgenic tree deregulation appears to be having an important effect, not only in that transgenic trees are not deployed, but also upon the vigour of the science and on the scientists involved in transgenic tree research (Bradford *et al.*, 2005).

LEGAL AND REGULATORY ISSUES

The general regulatory framework for transgenic trees, which is similar in many respects to that for crops, deals with two major areas of concern: food safety, and consequences for the environment. However, since food safety is rarely a problem, the focus of transgenic tree regulation is the environment. While regulatory systems vary by country, the usual case is for transgenic trees to fall under the same general set of regulations as crops and other plants. However, deregulation protocols may be modified to recognize the longer lifespan of trees and the associated longer-term deregulation problems.

In all regulated situations in the United States of America, and most countries globally, transgenic plants are automatically regulated and therefore require a deregulatory process before they can become commercial. In some countries, however, the criterion is based on the novelty of the plant, and thus regulation can also apply to non-transgenic genetically modified plants.

The long lives of trees make monitoring for potential problems more difficult than for annual plants. Most tree improvement programmes try to identify superior trees early in the cycle. This allows utilization of the superior-performing trees quickly, although with only a limited amount of information. Such an approach requires continual refining and adaptation of the genetic stock, and may contain surprises in tree performance. There is a fair degree of support in the United States of America industry for a conditional deregulation, whereby distribution would be limited and monitoring would continue for a specified period of time or until outstanding uncertainties were resolved.

CONCERNS REGARDING TRANSGENIC TREES

As the regulatory structure suggests, the primary reason for regulation of transgenics is the concern that there may be health, safety or environmental risks. The problem areas for trees are largely environmental (e.g. see Mullin and Bertrand, 1998). The regulators must behave as if the introduction of transgenics may pose new risks of environmental damage. In the United States of America the existence of concerns about the extent to which transgenics could become weed pests is clearly reflected in the Federal Plant Pest Act. More broadly, there are concerns that damage due to gene flow could occur or that transgenics could in other ways disrupt the environment (DiFazio *et al.*, 1999). Some have likened the introduction of a transgenic into the environment as providing a similar risk to the introduction of an exotic, some of which have become invasive. However, many ecologists have argued that the risks of a transgenic are generally lower and more predictable than for an exotic, since the transgenic has only a few introduced genes and the general expression of these is known. Thus, the gene expression associated with transgenics should be more predictable than with an exotic, in which the full expression of most of the genes is unknown, and any problems arising with a transgenic would be easier to identify and manage.

In any event, the primary concern with transgenic trees continues to be environmental risks, and that remains the focus of their regulation. Trees, being perennials, differ from the annual plants common in agriculture because of their long life and delayed flowering. We should note, however, that trees are not the only long-lived plants considered for genetic engineering. Other long-lived plants importantly include many of the grasses. Delayed flowering generally makes the examination of the impacts of the introduced genes over generations more difficult, but not impossible, since certain tissue-culture approaches may be helpful in mitigating the intergenerational delays. Nevertheless, regulatory complexities are likely to persist.

Thus far, only a few trees have been deregulated. In the United States of America, papaya has been deregulated and a plum tree appears about to be deregulated. In China, a transgenic poplar appears to have been commercialized (Xu *et al.*, 2004), although the extent to which it is deregulated remains unclear.

RISK AND COVERAGE

There are at least two major issues when determining the nature of regulation. First are the types of plants that are covered. Second is the level of acceptable risk. An issue in the development of the appropriate criteria for determining whether plants, including trees, are to be regulated centres around whether the regulation should apply to the transgenic process itself or to the attributes of the plant or product, such as whether it may generate concerns about weediness or other adverse risks.

Some biologists have argued that regulation would better be applied to plants on the basis of the plant attributes, rather than simply on the basis of the process of genetic engineering. The decision would be based on the novelty of the plant independent of the process used in its development. This criterion would be applied, in principle, to all novel plants, including genetically modified plants, whether the modification occurred by traditional breeding or genetic engineering.

The argument of those suggesting novelty as the critical criterion is that the transgenic process itself does not inherently lead to more risky products. Rather, it is argued, the regulatory process should focus on the changes and the attributes, whether generated by traditional or transgenic approaches, that could provide a social or environmental risk. The risks associated with the attributes of the products ought to be regulated and hence the products themselves, regardless of the process used in their development.

RISK REGULATION IN SELECTED COUNTRIES

Countries vary in their approach to risk. The formal United States of America decision criteria are that the product has "no significant or unreasonable adverse risks". Note that some "reasonable risk" is allowed. Reasonable is sometimes equated to allowing no more risk than would be expected from plants developed through traditional breeding. As currently practised, regulation in the United States of America is applied only to transgenic plants. Using this approach, all transgenic plants and trees are automatically classified as regulated articles that must go through the deregulation process to be eligible for commercialization. Alternatively articulated, any plant that involves the insert of a gene using a non-sexual approach is defined as a transgenic and is automatically a regulated plant. The European Union's decision criteria are particularly adverse to risk and require that all genetically modified plants do not present any additional or increased risks. Thus, the European Union calls for zero-risk criteria. This is a more severe standard than that of the United States of America or Canada, which accept some risks. In general, deregulation procedures are the same for all transgenics. More generally, although most countries agree on the need for some types of risk assessments for plants, there is as yet no consensus as to the degree of potential harm that will be tolerated, that is, the degree of severity of the risk (Pachico, 2003).

The question of what to regulate is also answered differently in various countries. While most countries automatically regulate transgenics, Canada

applies the criterion of novelty for regulation of both traditional and genetically engineered GMOs. However, no tree modified by traditional sexual processes has yet been required to go through formal deregulation procedures, whereas in almost all cases transgenic plants and trees require deregulation in Canada (personal communication, Phil MacDonald, Canadian Food Inspection Agency, Quebec City, 23 October 2003). Thus, in practice, the initial selection of the transgenic process may be an initial proxy for novelty.

Another question has been whether all regulated plants should be subjected to the same procedures in order to achieve a deregulated status. Some have maintained that a different deregulation channel should be adopted depending upon an initial assessment of the level of risk of a plant. China, for example, has a risk scale running from 'no risk' to low, medium and high risk, with the stringency of the deregulation procedures reflecting the category. A preliminary appraisal gives the plants a risk rating in one of these categories. Those in the no- or low-risk range have a relatively easy deregulation process, while those given a higher initial risk rating are required to go through a more extensive deregulation protocol. Many have argued that such a system might be appropriate to the United States of America (Strauss, 2003, 2007).

However, in some countries the law and regulatory structure remain unsettled. Chile, for example, allows field testing of certain transgenics, but does not allow or have a procedure to commercialize transgenics. Brazil had a prohibition against certain transgenic crops, which has been widely violated. However, recently some of this prohibition has been lifted (www.isaaa.org/kc).

The countries discussed above are not the only countries involved in deregulation and field trials of trees. While it is estimated that in recent years about 61% of worldwide tree trials have been in the United States of America, a host of other countries are undertaking tree field trials, including Australia, Canada, Chile, France, Italy, Japan, New Zealand and South Africa.

THE UNITED STATES OF AMERICA AS A CASE STUDY

An example of the regulation of transgenic trees is found in the experience of the United States of America (see Sedjo, 2004a, b).

An overview: law and regulations

The Federal Plant Protection Act 2000 gives the Secretary of the USDA the authority to adopt regulations preventing the introduction and dissemination of plant pests. Pursuant to this authority the USDA, through APHIS, regulates "organisms and products altered or produced through genetic engineering that are plant pests or are believed to be plant pests". Such products are known as "regulated articles". It is unlawful for any person to introduce a regulated article into production without first obtaining permission from APHIS. However, any person can submit a petition to deregulate, seeking a determination that a regulated article does not present a plant-pest risk and therefore should not be regulated (Section 12.7.3).

Additionally, the National Environmental Policy Act (NEPA) "requires a federal agency such as APHIS to prepare detailed EIS [environmental impact statements] for all 'major Federal action significantly affecting the quality of the human environment'". NEPA's responsibility is to ensure that APHIS will have available detailed information concerning significant environmental impacts and will have carefully considered the information. It also guarantees that the relevant information will be made available to the public. If a proposed project will significantly affect the environment, then an EIS is required. If an EIS is not required, the agency must prepare an environmental assessment to determine whether the environmental impact is sufficient to warrant an EIS. An environmental assessment is a concise public document that briefly provides sufficient evidence and analysis for determining whether to prepare an EIS or, alternatively, a finding of no significant impact.

Agencies and responsibilities

In the United States of America, three main agencies are involved in regulating transgenics: APHIS; the Food and Drug Administration (FDA) of the USDA; and the Environmental Protection Agency (USEPA). The FDA is involved with food safety, and the USEPA with pesticides and toxic substances under various legislation and overall environmental safety (NEPA).

The legal responsibility for protecting agriculture from pests and diseases from all sources resides with APHIS, and under the Federal Plant Pest Act, which mandates monitoring of plants that offer potential pest risks. The Plant Protection Act (Title 7 U.S.C. Sections 7701 *et seq.*) provides additional legal authority to APHIS, which, drawing from these two acts, has the authority and responsibility to determine whether a genetically altered plant, crop or tree is likely to become a plant pest or provide unacceptable risks to the environment. While APHIS has considerable experience with crop plants, it has only limited experience with trees.

Products of biotechnology, however, do not always fit comfortably within the lines the law has drawn based on historic function and intended use of products. In 1986, the Coordinated Framework for the Regulation of Biotechnology was adopted by federal agencies (see 51 Fed. Reg. 23302; 26 June 1986) to provide a coordinated regulatory approach. Products of biotechnology are regulated according to their intended use, with some products being regulated under more than one agency.

Deregulation process: some details

Transgenic plants are automatically defined as a 'regulated article'. The general deregulatory process for trees is essentially the same as for crops and other plants and is designed to assess a transgenic plant to determine if it provides increased risks of harm over that of traditional breeding. If it is found not to provide an unacceptable level of risk, it can be deregulated. The regulatory approach of APHIS requires three steps: permitting, notification and petition to deregulate.

For regulated articles, a permit must be obtained for the importation, interstate movement or release of the article into the environment. Deregulation requires field testing, which provides information as to the characteristics of the regulated article. Next, the deregulation process requires that a petition for deregulation be submitted to APHIS. Upon receipt and evaluation of the petition, APHIS, utilizing a scientific committee and a public participation process, makes a determination of whether to deregulate. APHIS has three ultimate options: to deregulate fully, to reject the petition or to provide qualified deregulation, e.g. to deregulate for a specific geographic region. Once a determination of full deregulated status is made, the product and its offspring no longer require an APHIS authorization for transport, release or commercialization in the United States of America. If the regulation is qualified, the article is treated as fully deregulated within the specified region, but subject to all of the regulatory restriction outside that region. If the petition is rejected, then full regulation continues.

It should be noted that a regulated article can be commercialized without being deregulated. This is common in biopharmaceutical products where the article is utilized but never deregulated. In this case the regulation provisions on the article continue.

The implementation of the Plant Protection Act related to transgenic plants centres on assessing the safety and environmental implications of the modified plant. Field testing is one of the major sources of information and is typically undertaken by the developer and occurs under controlled conditions for most genetically engineered organisms, particularly new or genetically modified plants. Field testing is designed to ensure that new plants are as safe to use as those generated by traditional breeding. The tests are also designed to prevent controlled items from escaping into the natural environment while being tested. Thus, strong containment measures are required. The developer is authorized by APHIS to gather information through field trials as well as though laboratory tests, literature reviews and other approaches, to confirm that the product has the new intended property and to determine that it is as safe to the environment as traditional varieties.

The final step of the deregulation process requires that a petition for deregulation be submitted to APHIS that details the field test results (including the use of statistical analysis) and provides a literature review and any other relevant information and/or experience. When enough information is gathered, the developer can petition APHIS to make a "Determination of Nonregulated Status". When APHIS receives a petition, a team of agency scientists begins the review. The agency announces to the public that the petition has been received, and the completed petition is made available for public review and comment. In these reviews, the APHIS standard is that an organism must not directly or indirectly cause disease or damage to plant, plant parts or processed products of plants. Additionally, the environmental implications are examined. It is common for the scientific review committee neither to accept nor reject the petition initially, but to return it to the developers with requests for additional information.

Also, it should be stressed that the overall assessment by APHIS includes a consideration of the potential effects on the wider environment to ensure that any environmental impacts are not likely to be significant. Broader environmental considerations are mandated under the National Environmental Policy Act of 1969. Furthermore, if the plant has pesticide properties, such as the introduction of a Bt gene, USEPA becomes involved in the deregulation of such a transformation. USEPA would have responsibility since the plant would involve pesticides and/or toxic substances. In this case, two agencies would be actively involved in the deregulation process, which undoubtedly would raise the costs to the developer, perhaps substantially. Up to now, there have been few pesticide-resistant transgenic trees, and most of the current research and development in the United States of America appears to be of the type unlikely to fall directly under USEPA pesticide and toxic substance regulation.

Ultimately, APHIS has several possible responses to a petition: it can approve the petition in whole, approve in part or deny the petition. APHIS can also determine that the plant poses no significant risk in certain geographic areas, but significant risk in others, and therefore approve the petition only within a given geographical area.

Tree deregulation

There are three types of trees that APHIS might consider deregulating: orchard, ornamental and wood trees. Over the period 1987–2001, wood trees were involved in only 1.2% of the total number of field tests of genetically modified plants in agriculture and forestry. Most of those, 91%, occurred in the latest reported period (1997–2001). A total of 90 wood-tree field tests were undertaken, representing four tree genera, between 1987 and 2001, with poplar being involved in well over one-half of the trials (www.isb.vt.edu/cfdocs/fieldtests1.cfm). Although trees make up only a small portion of the plants tested and about 57% of the trees are timber trees, the number of trees tested has increased dramatically in recent years (as has the total number of plants of all types).

The general approach to the petition process in APHIS appears to be to work cooperatively with the developer. Petitions are seldom rejected outright but they are often returned as being incomplete or providing insufficient information. Despite increasing field testing in recent years, only one tree has been deregulated by APHIS: a papaya fruit tree. This tree was experiencing severe disease problems (papaya ring spot virus) in Hawaii (AgBiotech Buzz, 2002). A GMO was developed to address the disease, and the transgenic papaya was deregulated and is now in widespread use in Hawaii. Despite this success, few other trees of any type appear ready for imminent deregulation. An exception is a plum tree that suffers from plum pox virus, a viral disease of stone fruit trees such as plums, peaches and apricots. Transgenic plants expressing viral genes have been shown to exhibit varying degrees of resistance to the virus (Levy *et al.*, 2000), and recent reports suggest that a transgenic plum tree may be nearing deregulation. Thus far, however, APHIS has received no petitions

for the deregulation of a transgenic forest tree. Worldwide, there is only one documented commercially released transgenic forest tree, in China, that has been deployed (Xu *et al.*, 2004).

APHIS performance

Deregulation is based on assessment of the results of field testing, statistical analysis and literature review. APHIS reviews about 1 000 applications for field testing of transgenics each year. Only about 59 transgenics, representing 13 species, have been deregulated over a 15-year period. Examples of deregulated articles include salt- and drought-tolerant Bermuda grass, maize-expressing proteins with pharmaceutical applications, virus-resistant squash, soybean with altered oil profile, Bt maize, and herbicide-tolerant and insect-resistant cotton.

To date, however, APHIS has authorized thousands of field tests for more than 50 plant species, mostly related to agricultural crops. Many of these have achieved deregulated status. So far, however, only a relative few (124) field tests of genetically altered trees have been authorized (McLean and Charest, 2000), including transgenic spruce, pine, poplar, walnut, citrus, cherry, apple, pear, plum, papaya and persimmon.

Recent court decisions

Recent court decisions in the United States of America appear to require the regulatory authorities to apply more stringent standards than they had, in fact, been applying. Although the decisions apply to perennial grasses, the inferences suggest that similar standards will probably apply to trees. In the alfalfa seed decision in the United States of America District Court for the Northern District of California (Case 3:06-cv-01075-CRB Document 83, Filed 02/13/07), the court ruled that APHIS erred in applying an exception and not undertaking an EIS, as sometimes called for by NEPA (Geertson v. USDA 2006). An EIS requires a substantial increase in time and costs for APHIS and also imposes large additional costs on the developer. This EIS process allows opponents to raise hypothetical and conjectural negative environmental impacts for detailed scrutiny. A similar opinion came from the District of Columbia District Court (Civil action 03-00020 [HHK]) regarding the Scott Company's genetically engineered creeping bent grass (ICTA v. USDA/Scotts 2006). Both of these cases involved the introduction of pesticide-resistant genes to seed grasses, and the issues appear likely to be applicable to the transfer of certain types of genes to trees. While pesticide-resistant genes in trees are apparently not imminent, the fact that the APHIS procedures were deemed by the courts as "arbitrary", and therefore inadequate, necessitates the revision and complication of APHIS deregulatory procedures, at least for certain types of transgenic innovations.

SOCIAL ISSUES: POSITIONS OF USERS AND MARKETS

This section characterizes the attitudes of various groups towards transgenic trees and the regulatory structure. These characterizations are not based on scientific

sampling procedures but rather reflect general impressions based on documentation from various groups and conversations with some of their members.

Attitudes towards transgenic trees and regulations

Numerous groups have an interest in transgenic trees. These include tree growers, tree processors, tree developers, direct and indirect consumers of forest products, as well as environmentalists. Not surprisingly, attitudes towards transgenic trees vary substantially among these groups. Additionally, as has been shown in various surveys of attitudes towards transgenic foods, attitudes towards transgenics generally tend to vary considerably across countries.

Tree breeders and developers

Not surprisingly, among transgenic tree developers, whether in the private sector or with universities, the attitude towards transgenics is basically positive. These groups generally believe that there is a place for some type of regulation. There is common criticism, however, of the United States of America approach of requiring all transgenics to go through the same deregulation process. As noted earlier, a common view among transgenic biologists is that certain types of transgenic changes are predictable so that a formal deregulation approach is not required. However, such an approach would obviously require some preliminary assessment to determine which transgenics require a more comprehensive assessment.

Tree planters and growers

While many tree planting firms engage in tree improvement, and some are involved in research to improve the ability to clone trees, especially pine, few forest industry firms are directly engaged in tree genetic engineering research and development. The industry structure that has emerged in the past decade in North America has seen the work on transgenics being undertaken largely by universities and specialized research firms. This differs from an earlier period when individual forest firms often included work on transgenics as part of their overall tree improvement programmes. An explanation of this restructuring apparently is, at least in part, the desire of forest firms to distance themselves from the activity of genetic engineering during a period of questionable public acceptance. Additionally, there are almost surely economies of scale in concentrating research efforts in a few places rather than fragmenting the efforts.

In general, tree planters and growers are looking for opportunities to reduce costs and increase productivity. Transgenics offer both possibilities and thus, in concept, are attractive to tree growers. However, tree growers are very sensitive to actual and expected market behaviour and thus, given some of the current controversies over transgenic products, are somewhat wary.

Environmentalists

A systematic inquiry at the booths at the World Forestry Congress in Quebec City (September 2003) found the attitude of environmentalists towards transgenic

trees tends to run from extremely hostile to quite sceptical. Strongly 'green' organizations, such as Greenpeace, exhibited great hostility, with ominous predictions of how transgenic trees would damage the natural environment. The guidelines of the Forest Stewardship Council, a certifier of acceptable forestry practices, specifically prohibit the certification of transgenic trees. At the other end of the spectrum are organizations (e.g. The Nature Conservancy) that have no institutional position on transgenics. These organizations have some staff professionals who agree that transgenic trees may have a role in forestry's future. However, they point out that this issue is generally out of the mainstream of their organization's direct concerns.

Consumers

Two groups of consumers might have attitudes on transgenic wood. Consumers of wood as an input to other production, such as a pulp mill, find that transgenic trees with certain characteristics are desirable for their production needs. Trees with more fibre, less juvenile wood, and less or more easily removable lignin, for example, have characteristics that reduce processing costs and are therefore, in principle, desirable. A concern of these producers is whether such products will be acceptable to consumers.

The second group is consumers of final products (paper, lumber, panels, etc.) that are made from transgenic wood. From a product-performance perspective, there is little reason to believe that the final products from transgenic wood would be less suitable to their needs. In fact, in some cases the transgenic wood might produce a better final product. If processing costs were reduced, the lower price of the product would be a desirable feature. Also, there are generally no food safety issues involved with wood, although cellulose is sometimes used as a filler in foods. Thus, except for any philosophical concerns about transgenics, the products made from the wood ought to be acceptable to final consumers. The extent to which final consumers might actually resist transgenic wood products remains problematic. Some insights might be gained from the experience of certified wood and ecolabelled wood products. There is little evidence that consumers are willing to pay a price premium for certified wood. However, some firms may find it to their advantage to be certified, presumably because certification imparts a competitive advantage, even if not a price advantage. How these attitudes might translate to a transgenic wood market remains to be determined. It could be that consumers might prefer natural, non-transgenic wood, other things being equal. However, a modest price discount could overcome this tendency.

CONCLUSIONS

As forestry makes a transition from foraging wild forests to tree cropping, the potential of plant improvements that will contribute to general social and economic benefits increases. Innovations that can be developed along the lines of those in crops, such as herbicide and pest resistance, and innovations involving the form and fibre characteristics of trees, offer promise. Although the life cycle of tree

improvement often means long delays between innovation and the realization of financial benefits, a number of potential transgenic innovations offer possibilities of the early capture of benefits.

Although transgenics appear to offer substantial potential for increasing productivity in forestry, there are concerns about risks that might be involved, particularly environmental risks. The purpose of regulation and the deregulation process is to ensure that these transgenic innovations are safe. Nevertheless, consumers are sensitive to these situations, and these concerns could be translated into the performance of markets for wood-based products.

Thus far, no country has publicly approved the deregulation, and hence commercialization, of a transgenic forest tree. Only one tree – the papaya – has been deregulated and is now commercialized in Hawaii. A transgenic plum tree resistant to pox appears about to be deregulated. In China, a transgenic poplar has been released on a scale that is not entirely known, but it appears to be approaching deregulatory status, if it is not already there. Finally, many forest trees are currently in field trials in several countries, and it appears further deregulation is likely to occur in some countries in the relatively near future.

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11. Forest biotechnology: more than wood production

R. Kellison

The announcement in 2007 by ArborGen LLC (www.arborgen.com) of their acquisition of the seed orchards and nurseries, inclusive of the advanced breeding programmes and materials, of International Paper Co., MeadWestvaco Corp. and Rubicon Limited's Horizon2, emphasized that the joint venture would increase wood production from planted forests while alleviating the drain on native forests. The conclusion is that transgenic trees will grow faster than their non-transgenic counterparts, and that they will be more resistant to insects and diseases and more tolerant of environmental extremes such as cold and drought. The claim has validity as exemplified by the results from the earlier chapters of this book. But there is more to transgenics than just trees that grow bigger and faster, have more resistance to pests and have greater adaptability than run-of-the-mill trees or those from advanced tree breeding programmes. There are benefits from forest biotechnology aside from tree growth and plantation yield. The multitude of the 'aside' benefits will probably have greater value in the long run than growth and yield. The 'aside' benefits will include phytoremediation, species restoration, afforestation, biofuels and bioprocessing. The list does not necessarily stop there. The future products from bio-engineered trees are limited only by one's imagination.

PHYTOREMEDIATION

Toxic wastes are of two types: those that escape, either by accident or design, from their intended use, and those that are residues from an approved use. A prime example of the former type is trichloroethylene (TCE), a solvent that is used worldwide to remove clothing stains and as an industrial degreaser. The second type is exemplified by chloroform, which is the by-product of chlorine that is used for purification of drinking water.

Bioremediation, the forerunner of phytoremediation, gained attention when it was observed that the plumes from oil spills contracted in the presence of underground water. Research showed that the contraction was caused by microbes in the water that, through denitrification, turned the contaminants into CO_2. Further research showed that the denitrification process was effective against pollutants of oil, chlorinated solvents, pesticides, agricultural chemicals, creosote and gasoline (Chapelle, 1985).

The positive results from the denitrification process led to the discovery that some plants can detoxify contaminated soils. Such plants produce enzymes that can break down trichloroethylene (C_2HCl_3) into chloride ions, which is a harmless

salt that the plant sheds, and recombines the carbon and hydrogen with oxygen to produce water and carbon dioxide. One of the plant groups found effective in detoxifying contaminated soils is poplar (*Populus* spp.) (Newman *et al.*, 1997). Poplars are indigenous to the northern hemisphere, with numerous species of similar phenology that hybridize with one another under controlled conditions. The hybrids find common use in commercial forestry because of fast growth, pest resistance, adaptability, wood properties and ability to be vegetatively propagated.

The enzymes in poplars that metabolize the contaminants are from a group of cytochromes called P450, which are common to both plants and animals. In research trials, unaltered poplar plants can metabolize the TCE into salt while recombining the carbon and hydrogen with oxygen to produce water and CO_2. The limitation of this process is that it is very slow. To speed the process, a gene from P450 in mammalian livers of rabbits has been inserted into the plant. That gene causes the P450 genes of poplars to overexpress the enzymes, which causes the pollution degradation process to be speeded up manyfold in comparison with P450 of the non-engineered plant. Research is continuing by Dr Sharon Doty and colleagues (Doty *et al.*, 2000) at the University of Washington (United States of America) on the use of promoters to enhance the production of the inherent P450 in poplars to have the same effect as those with the transgene from rabbit livers (http://uwnews.org/article.asp?Search=p450&articleid=37313).

The research just described is confined to the laboratory, or to very limited and highly controlled research trials. The potential value of the technology has such tremendous application to the polluted sites around the world that it will be only a matter of time before it finds common usage.

AFFORESTATION

Afforestation is the occupancy with trees of landscapes that are barren of forest cover. Some of those landscapes have never borne forests during the modern era and others have been denuded of trees by humans for alternative uses of the land. Within the latter category, large areas of land are barren because the soils have been depleted of nutrients and moisture holding capacity, have become water saturated in low lying areas or have become subject to invasive insects and diseases. Other areas have reverted from being highly productive for agronomic cropping to wasteland because of salt intrusion. The intrusion is very often the result of inadequate irrigation where the minerals are not flushed from the rooting zone of the plants or, in other situations, displacement of fresh water by salt water from the sea or impounded waters.

With the advent of forest biotechnology, trees will be genetically engineered to occupy adverse sites, such as those with ambient temperatures too hot or too cold for normal tree growth. Other lands, whether arid or water-saturated, will be candidates for afforestation or reforestation, and still others with soil nutrients in limited supply or oversupply will, one day, be supporting thriving forests. In addition to additional wood production for anthropogenic uses, such forests will serve as windbreaks, wildlife refuges, recreational areas and, most importantly, for carbon sequestration.

Examples exist where plants other than forest trees have been genetically engineered to tolerate high levels of salinity, drought, cold and high temperatures. Working with the wine grape (*Vitis vinifera*) in northern Nevada (United States of America) tolerance has been shown for extremes in temperature and adverse soil conditions by manipulating cell length of the roots (Cramer *et al.*, 2005). The process involves the selection of mutants of *Arabidopsis thaliana* for salt tolerance, which is then genetically engineered into the grape plants. Interestingly enough, tolerance to salinity conveys added tolerance to drought, and cold and high temperatures. That technology is suitable for transfer to forest trees, the results of which will occupy some of the most adverse sites for tree growth in the world.

Care will have to be exercised to assure that the extension of forests to lands of marginal productivity does not create a problem of equal or greater intensity. An example of such a travesty would be the additional drawdown of water on which a community or municipality might be dependent. Conditions already exist in some parts of the world, such as in South Africa, where plantation forestry is restricted at the local level because of an inadequate water supply. Used judiciously, however, the benefits of forest biotechnology will help solve more problems for the human population than it creates.

SPECIES RESTORATION AND CONSERVATION

Heritage forest tree species are threatened and endangered throughout the world. The situation is exacerbated by transnational movement of goods from continent to continent. Along with those goods are hitchhikers of the insect and disease phyla. Such pests are often benign in their indigenous range, but become catastrophic when introduced to new environments in the absence of natural biological control agents. On top of that are the indigenous pests that create havoc for tree monocultures because of changes in climate. This section will deal with those two types of forest destruction.

Heritage trees

Heritage trees are those that are threatened, endangered or have high social and economic value. The epitome of that category is American chestnut (*Castanea dentata*). That species comprised about 30 percent of the overstorey forest of the Appalachian Mountain range, with extensions into the Central and Lake States (United States of America), inclusive of southern Ontario (Canada). Its nut production had tremendous importance for wildlife as well as for Native Americans and European colonizers as a food supplement, and as a bartering commodity for essential goods and services. Additional values were for wood products that were essential for buildings, conveyances, fences, furniture and myriad other uses. In addition to the wood being easy to split, saw, form and assemble, it was durable. The tannins responsible for durability also found other uses such as in leather tanning. In short, chestnut was the all-American tree (Bolgiana, 2007).

Its prominence began to wane, probably in the late 1800s, because of a disease dieback syndrome. The decline, which killed the aboveground portion of the

tree but left the root system unaffected, was identified in 1904 by the New York Botanical Garden as *Endothia parasitica*, a pathogen from the Orient. The pathogen, subsequently named *Cryphonectria parasitica*, enters through wounds to form stem galls that girdle the tree. Trees of small size (10-cm range at breast height) are quickly killed, whereas those of larger size die at a progressively slower rate. Within a 40-year period, the pathogen had made its way to the ends of the range of the once-dominant tree species.

Efforts were initiated to select and breed for blight resistance within American chestnut, but the results were inadequate to justify continued funding. Research was also initiated with hybrids from the native species and Chinese (*Castanea mollissima*) and Japanese (*Castanea crenata*) chestnuts, both of which have higher resistance to chestnut blight than does the North American species. Even though some progress was made over a 40-year period, the results were sufficiently variable that the work by public agencies to find a cure was largely abandoned. The exception to abandonment by public agencies is the work being carried on by The Connecticut Agricultural Experiment Station under the guidance of Dr Sandra Anagnostakis (Anagnostakis, 2007).

In lieu of public funding, formation of The American Chestnut Foundation (TACF) and The American Chestnut Cooperators Foundation (TACCF) in the 1980s was initiated to continue the cause for restoration of American chestnut. The emphasis of TACF was to use a backcross breeding programme with Chinese chestnut to obtain disease resistance while maintaining the tree phenotype and nut production of American chestnut. TACCF, in contrast, concentrated its efforts on finding trees with partial resistance and escapes of pure American chestnut and hybridizing those to produce progeny with added resistance.

While both programmes have made progress, TACF is nearing completion of backcross breeding that is producing a tree of 15/16th American chestnut and 1/16th Chinese chestnut (Sisco, 2004). While high achievement is expected from the backcross progeny, the theory of quantitative genetics means that the product will not be one with complete Chinese blight resistance or one with complete American phenotype. Breeding experiments have also revealed that only two or, at most, three genes are responsible for disease susceptibility or resistance. To identify those genes, a project is under way with collaboration from the universities of North Carolina State, Clemson, Penn State, Syracuse and Georgia, in addition to the USDA Forest Service, The Connecticut Agricultural Experiment Station and TACF. Good progress is being made in this endeavour, with the welcome news that the results can be used to enhance the screening process in the backcross breeding programme. In addition, it lays the groundwork for direct insertion of the resistant genes from Chinese chestnut into American chestnut to engineer a blight-resistant tree.

Application

The application of achieving disease resistance in American chestnut is fast approaching. It bodes well as the pioneer for other tree species that are threatened

or endangered by invasion of insects and diseases from abroad. Chief among the threats are sudden oak death caused by a root pathogen (*Phytophthora ramorum*), and the insect invasives of emerald-ash borer (*Arilus planipennis*) and sirex woodwasp (*Sirex noctilio*).

The common carriers of the sudden oak death pathogen are the landscaping plants of rhododendron (*Rhododendron* spp.) and azalea (*Azalea* spp.), but the oaks (*Quercus* spp.) of the Pacific Southwest are especially vulnerable (Barrett *et al.*, 2006) and, in laboratory tests, many of the oaks of the Eastern Deciduous Forest have also proven to be highly susceptible. The emerald ash borer of Asian origin has, within a decade, killed about 30 million trees of white ash (*Fraxinus alba*) in Illinois, Indiana, Ohio, Pennsylvania and Wisconsin, and has even been found in the Canadian province of Ontario (www.emeraldashborer.info). The sirex woodwasp of European origin has been a common pest in pine plantations in the southern hemisphere, specifically in Australia, New Zealand, Chile, Argentina and Brazil. Under plantation conditions, the pest can be reasonably controlled by good silvicultural practices and by biological means. In native stands of pines common to the northeast and north-central parts of the United States of America, however, control becomes extremely complex (Haugen and Hoebeke, 2005). Biological control, including both the genetic engineering of plants for resistance and biological manipulation of the insect, seems to be the only reasonable method of countering the pests.

In addition to the exotic pests are those of indigenous origin that are causing catastrophic losses, presumably as a result of global climate change. The one that is claiming international attention, especially in western Canada and southwestern United States of America, is the mountain pine beetle (*Dendroctonus ponderosae*). Within British Columbia, hundreds of square kilometres of the naturally occurring monoculture of lodgepole pine (*Pinus contorta*) have been killed, leaving a desolate landscape. On a smaller scale, in the Pacific Southwest of the United States of America, especially in Colorado, the insect has denuded the landscape of live trees of lodgepole and ponderosa (*P. ponderosa*) pines. The cause of these catastrophic events is purported to be the lack of prolonged freezing temperatures, which allows successive broods of the insect to continue unabated.

The effort to maintain tree cover and to colonize areas formerly occupied by a native tree species is becoming ever more important as plagues proliferate. American chestnut can be the pioneer species because of its appeal to a wide audience for restoration, even as a transgenic. At the same time, its recolonization of diverse sites will pave the way for dealing with other species that are beset with plagues, such as those of lodgepole and ponderosa pines.

Biofuels

Global climate change is catching the attention of nations worldwide. Global warming is thought to be a major contributor to climate change because of the elevated load of CO_2, which is presumed to be creating a greenhouse effect. CO_2 levels have increased from about 280 ppm in pre-industrial time to 381 ppm in

2007. Anyone doubting the incremental increase has only to look at the trend from 1958 through 2007 from readings made by the National Oceanic and Atmospheric Administration (NOAA) at Mauna Loa Observatory, Hawaii. During that 50-year period, every annual amount is higher than the year before.

Burning of fossil fuels is the primary cause of the increasing amount of atmospheric CO_2. In the United States of America, for example, some estimates are that more than 80% of atmospheric CO_2 levels are from the burning of fossil fuels. Allocations of that total by user segment are: electricity generation (34%), transportation (28%), industrial use (19%), commercial use (6%), residential (5%), and agricultural use, including forestry (8%). The values change somewhat on a worldwide basis, with estimates that 20% of atmospheric deposition is due to deforestation, primarily in the tropics.

In addition to the adverse effects of greenhouse warming from the burning of fossil fuels, civil strife in the areas where the petroleum reserves are found have made the long-term availability of the resource questionable. That combination of limitations has caused governments in various parts of the world to look for alternatives sources of fuel. Even though solar power is a bountiful source of energy relatively little use has been made of it because of the expensive photovoltaic cells needed to convert light to energy (Cohen, 2007). Nuclear, wind, geothermal and water forms of energy generation hold potential, but they have been relegated to low priority because of initial cost, regulatory issues and real or perceived safety concerns.

Alternative fuels. including products like ethanol and methanol that can be made from biofuels, have been hyped by some countries for the past 40 years. As a result of the petroleum crises in the mid-1970s, Brazil decreed that 20% of its gasoline usage would be replaced by ethanol, the feedstock of which would be sugarcane (*Saccharum* spp.). The technology in automobile engine manufacture in that country has advanced so that cars of today are equipped to operate efficiently on ethanol of 80-percent grade. In conjunction with the improved manufacture of ethanol from sugar cane, scientists have been active in increasing the yield of the crop per unit area. Biomass yields were increased by 3.5% annually from 1978 to 2000, and the yields had yet to plateau. In combination with increased yields, the sugar content of the plants has increased proportionally. That, along with the added area for sugar cane production, which is projected to increase from 5.7 to 11 million hectares, makes Brazil the leading country in the world for the production of biomass fuels (Orellana and Neto, 2006).

Other biomass crops that are candidates for fossil fuel replacement are maize (*Zea mays*), switchgrass (*Panicum virgatum*) and wood cellulose. Relative to the cost of gasoline in 2005, ethanol from maize, switchgrass and wood cellulose were 29, 50 and 57% more expensive, respectively (Pimentel and Patzek, 2005). Those values are slightly less onerous than they were in 2005 because of the higher price of gasoline, but in some respects they have not changed greatly because of the higher prices for the feedstock, especially maize. The price of maize has roughly doubled during that time because of competition for the limited resource, but the

costs for production inclusive of equipment, seeds, chemicals for plant nutrition and weed control, and harvesting and transportation have increased similarly. More and more emphasis is being given to plant residues and, especially, plants grown specifically for energy production are gaining in priority. Woody biomass is gaining favour over switchgrass because of its ability to be stored 'on the stump' and to be harvested as needed. The harvesting of switchgrass is done at maturity, otherwise the energy content begins to decline slowly at first and rapidly with increasing age beyond maturity.

The prognosis is that woody biomass will be genetically engineered to increase the syringal type of lignin at the expense of guauacil. The genetically engineered plants will be grown within easy haul distance of the bioenergy plant. Portions of the southern United States of America are in a favourable position for such operations because of the option to convert abandoned pulp mills to ethanol production. Such facilities are already equipped for the processing of timber for pulp and the only remaining addition is the conversion of the cellulose to sugars and the sugars to ethanol. It is estimated that such a facility could be retrofitted for about 25% of the cost of building a new converting plant.

A limitation to conversion of cellulose to ethanol in today's world is the desired enzymes. Great progress is being made in the discovery of new enzymes and the creation of additional ones by biotechnology organizations. The prognosis is that a cornucopia of enzymes fit for rapid conversion of cellulose to ethanol, together with genetically engineered plants that are rapid growing and have a high syringal to guauacil ratio, will one day be offsetting as much as 25 percent of the fossil fuels needed for industrial society. As a case in point, the United States of America is set to enact into law an energy bill that boosts ethanol use to 36 billion gallons by 2022, up from 5.5 billion gallons in 2007. Of the 2022 total, 21 billion gallons was expected to be from raw materials other than maize. The prognosis is that trees will be a major contributor to that alternative fuel.

Paper manufacture

With an expanding world population that today is at 6.5 billion people and is expected to peak at 9 to 10 billion people by 2050, the need for paper will continue to increase. The increase will come with the numbers of people while, at the same time, per capita consumption will decrease because of reliance on computer technology. Thus there will continue to be a huge market for paper and paper products.

In the same way that trees will be grown for conversion to fuel, trees will be genetically engineered for high cellulose content for the manufacture of paper and paper products. Alterations will be made in the pulping with reliance more on enzymatic action to separate the cellulose from the lignin, thus supplanting the costly steps now encountered in both chemical and mechanical pulping. Similarly, the caustic chemicals used for bleaching the pulps will be greatly reduced in favour of enzymatic bleaching. The processes of both pulping and bleaching will be greatly simplified and, as a result, greatly reduced in cost and environmental impacts.

The two methods for separation of cellulose from the lignin of woody biomass are chemical and mechanical pulping. The most common method of wood property separation is by chemical pulping, which is done in combination with causticizing chemicals and heat. The pulp yield of such an operation varies from about 45 to 55%, by weight, depending on species of the woody biomass. Mechanical pulping, on the other hand, produces pulp yields of 85 to 90%, which is accomplished by grinding at high and costly energy levels in the presence of heat. The major difference between pulps of the two methods is in the lignin removed. Mechanical pulps have limited use because of the retained lignin, which causes papers to yellow when exposed to ultraviolet light; they therefore find application in lower-grade products or in limited combination with chemical pulps.

Both types of pulp require bleaching to some degree to meet paper and paperboard specifications, but bleaching of chemical pulps is less intrusive in cost and in environmental impact than mechanical pulps. That scenario is likely to change as the result of biotechnology. With the use of fungi, such as the white-rot basidiomycete *Ceriporiopsis subvermispora*, the lignin between the cells (fibres and tracheids) as well as the lignin within the cell walls can be separated from the cellulose by mechanical means (Teeri, 2004). Such a process would limit the need for bleaching.

Even though the pulping and bleaching process with fungi is operational on an experimental scale, it has not yet achieved commercial application because of logistics and the lack of advanced-stage enzymes. The logistics deal with the inability to distribute the fungus equally through large piles of chips and to the time required for the fungus to chemically separate the lignin from the cellulose of the woody cells. The former limitation should be overcome with design alterations at pulp mills, and the latter will come about with the discovery and genetic engineering of enzymes that will speed the process with uniformity. Energy consumption alone with the envisioned process will be about 30% less than with pure mechanical pulping (Shukla, Rai and Subramanyam, 2004).

CONCLUSIONS

Mention has been made of only a few of the benefits of biotechnology in the forestry sector: bioremediation, afforestation, conservation and restoration, and biochemical processing of wood for fuels and paper and paperboard. The list can go on to include pharmaceuticals and foodstuff from trees, carbon sequestration through extension of forest plantations to marginal sites as well as to genetically engineered trees that speed the process of sequestering carbon while sequestering larger amounts in the tree parts and in the soil (Kellison, 2007).

Concerns have been raised about the negative ecological impacts that forest trees might have on ecosystems where escapes might occur. Those concerns need to be studied, which will probably result in strict guidelines being applied for commercial application. We ought not, however, to be overly conservative because of the population increases in the world coupled with a limited land base. In fact, the land base is steadily diminishing due to human development, inclusive of expansion of industry, housing and land-use alternatives.

A case in point for keeping the options open for an expanded use of biotechnology for humans, medicines, domestic animals, agronomic crops and forest trees is the situation arising in the European Union. That suite of countries has been opposed to plant biotechnology, be it agronomic crops or forest trees, and has enacted legislation that bans the use of transgenic crops for human consumption, either directly or indirectly. The situation is now arising, however, where the demand for non-transgenic farm crops is exceeding domestic or international supply. The projection is that in one or, at most, two years some of the imported grain for animal feed will be of the genetically engineered variety (Mitchel, 2007). There will be no other option because the crops for animal feeds are progressively being used for ethanol production. The question then becomes "is it equally undesirable to consume meats from farm animals that have been fed transgenic crops as it is to directly consume the transgenic crops?" This question presumably answers itself in the long run. With population increase and arable land decline in the world, the populace will have to make use of its every resource if the human race is to survive.

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12. Regulation for genetically modified forest reproductive material moving in international trade

H.-J. Muhs

When European countries started provenance research with the main indigenous forest tree species, including some exotic ones like Douglas fir, from the1880s, it became obvious that populations from different origins and provenances of the same species react differently in growth and other characters. In the further historic development of modern forestry, IUFRO played a big role, especially in provenance research (Kriebel, 1992) and acted in many cases as a forum for discussion on how to put the results into practice. So it happened in the following century, as results of provenance research have formed the basis to develop recommendations and also rules for proper use of that material in reforestation and afforestation.

Since the 1950s, the whole legal system has been modernized in many countries. In the countries that later became the European Economic Community (EEC), property and the free use of it (despite the many restrictions existing) became very important rights. Thus, the rules for use of the material in reforestation were obsolete, because the owners themselves could decide what material to use (free choice of species and provenances). Consequently, in 1966 the EEC enforced the first regional regulation for the 'marketing' of forest reproductive material, rather than the 'use' of that material: Council Directive 66/404/EEC (EEC, 1966).

Meanwhile, that Council Directive has been revised, last in 1999 as Council Directive 1999/105/EC (EC, 1999) (EC = European Communities, later becoming the EU = European Union). The philosophy behind this was to establish rules for the production and marketing of reproductive material and give the user and consumer of that material all necessary information so as to enable them to make the best choice. In this respect, the Council Directive can be seen as a regulation to boost consumer protection.

In addition, the Council Directive will also enhance the production of forest reproductive material by setting standards for production, which includes activities such as: seed collecting and processing; vegetative propagation; producing clonal material; producing new types of basic material for the production of reproductive material; raising plants in the nursery; handling of the material at all stages from beginning to delivery to the consumer; and certification (see below). Wherever breeding is involved in these activities, it is wise to adhere to the regulation

otherwise it could happen that a new breeding product does not receive approval (see below) and as a consequence will be excluded from the EU market.

REGULATIONS FOR MARKETING OF REPRODUCTIVE MATERIAL

There are two regional regulatory schemes, that of the EU (Council Directive 1999/105/EC; EC, 1999) as mentioned above, and that of the Organisation for Economic Co-operation and Development (OECD), namely the OECD Scheme for the Certification of Forest Reproductive Material Moving in International Trade (OECD, 2007). The OECD Scheme was established in 1974, thereafter amended several times and revised in 2007. The OECD member countries (of which 25 were participating in the Scheme at the time of writing) and the EU member states (currently 27 have full membership, of which ten are simultaneously participating in the OECD Scheme) agreed to harmonize their regulations.

When in 1987 the issue of genetic modification came into the picture and the first genetically modified (transgenic) poplar clone was tested in the field, it was necessary to consider whether this type of breeding product would automatically be included in the regulation. An expert group established by the OECD assembly of Designated Authorities participating in the OECD Forest Seed and Plant Scheme worked from 1993 to 1996 on a proposal to revise the Scheme. The expert group recommended a revised version, which does not regulate the procedure of genetic modification, because this was not necessary and out of its competence while ruled on the national level or on the EU level, but included some requirements for genetically modified reproductive material to easily facilitate marketing under the regulation with a view to providing full information to meet the demand for consumer protection.

The proposal was not adopted by OECD, because it did not achieve unanimous agreement, the reason being the inclusion of requirements for genetically modified reproductive material in the text, which could not be accepted by one member country.

The OECD then took another approach for revising the Scheme, because many other items still needed to be revised and harmonized with the EU regulations, which had been in conflict and hindered the trade between OECD countries and EU member states. This part of the text consists, for instance, of using the same definitions of terms, identical descriptions of the types of basic material and categories, the same specifications for the national register and the certificates and the same concepts for the selection and testing procedures. While the OECD Scheme should not contain any additional requirements for genetically modified material, those paragraphs regulating the two advanced categories “Qualified” and “Tested” were omitted, in which such requirements were incorporated. The result was the OECD Forest Seed and Plant Scheme (OECD, 2007), which includes only the first two categories “Source identified” and “Selected” instead of four, while the two advanced categories are under consideration for further extension. That is the reason why the OECD Scheme has no regulation concerning genetically modified material.

The proposal was adopted and harmonized by the EU member states. The result was Council Directive 1999/105/EC (EC, 1999), which consequently also contains the requirements for marketing of genetically modified material. In the following discussion, the Council Directive will be the only reference for regulations for marketing of genetically modified forest reproductive material at regional level.

For the release of genetically modified organisms into the environment in general, another Council Directive has competence. Therefore the genetically modified forest reproductive material needs to meet the requirements of two directives: Council Directive 90/220/EEC on the deliberate release into the environment of the genetically modified organisms, and Council Directive 1999/105/EC (EC, 1999) on the marketing of forest reproductive material. The following sections deal with requirements that genetically modified material must fulfil to get permission for release into the environment and at the same time to obtain approval to produce reproductive material for marketing.

METHOD OF OPERATION OF THE REGULATIONS

Both sets of regulations operate according to the same principles. The government will designate the Authority to implement the Scheme or Directive in the country and to control all necessary operations. Where a country already has a national regulation, it would be advantageous to combine the authorities of the national and the international regulatory schemes. In case of the members of the EU, this is already practised.

The regulation comprises definitions and rules under which the forest reproductive material shall be certified. The procedure can briefly be described as follows: the main principles are approval and certification. The basic material will be approved, after that it can serve for the production of reproductive material. The basic material can consist of a seed source, stand, seed orchard, parents of family(ies), a clone or clonal mixture, of which all, except the seed source and stand, may be derived from genetically modified material. The procedure for approval starts with the declaration of what shall be approved (type of basic material), the exact location and delineation of the basic material so as to clearly identify it (unit of approval), and after having approved the basic material according to the rules (see below) each unit of approval shall be identified by a unique register reference.

The register reference will be listed in the National Register of approved basic material (see below). Each unit of approval is related to a category. There are four categories recognized in the Directive, namely “Source identified”, “Selected”, “Qualified” and “Tested”. Reproductive material derived from approved basic material will be certified according to its nature (either derived from seed or clonal material) and status (category). The Certificate of Identity will reproduce all relevant information on the basic material from the National Register and add the information related to the actual lot of reproductive material. Each certificate has a number and a member state code. All lots of forest reproductive material will be

accompanied by a label containing the certificate number and code together with other information relevant for the actual lot.

RULES FOR GENETICALLY MODIFIED FOREST MATERIAL

Safety requirements

The procedure for basic material that is genetically modified has to satisfy the requirements of the two regulations mentioned above. The regulations are implemented and controlled by two different Authorities in the member states of the EU. Council Directive 90/220/EEC regulates the procedure of genetic modification and sets up requirements for the material to be released into the environment. It is not the place here to outline Council Directive 90/220/EEC and the philosophy behind it. Here only the requirements to be satisfied for the release of reproductive material will be summarized, which are explicitly demanded in Council Directive 1999/105/EC (EC, 1999).

If the basic material consists of a genetically modified organism within the meaning of Directive 90/220/EEC, such material shall only be accepted if it is safe for human health and the environment (Art 5,1 of Directive 1999/105/EC). This is the fundamental requirement, which all genetically modified organisms have to fulfil. For forest basic material as well as for crops, an environmental risk assessment as laid down in Directive 90/220/EEC shall be carried out additionally. If all these requirements are met, the genetically modified basic material will be accepted for inclusion in the National Register (see below) after having been authorized in accordance with the Directive (Art 5,2b).

The meaning of the last sentence may not be clear for those who are not familiar with Directive 1999/105/EC. It actually means that the basic material, which has satisfied the requirements above, is not free for immediate commercialization. But the basic material is accepted for inclusion in the National Register of basic material. To get a full inclusion for the basic material, the other requirements set up in the Directive 1999/105/EC have also to be satisfied, which are necessary to get approval and thus permission to produce for commercialization reproductive material from the basic material.

Approval

The unit of approval is the basic material, for instance a clone as noted above. A single gene construct cannot be approved, as it exists only in an organism and can only be expressed in an organism. Consequently, each clone of a group of clones, of which all are transformed by the same gene construct, must be tested separately. It is obvious that each transformation is unique, because the position in the genome and the composition of the flanking regions of the position are different. Further, each clone contains another genetic background and therefore transgene expression may vary.

What are the special requirements for genetically modified basic material set up in the Directive 1999/105/EC? Genetically modified material can only be marketed under the category Tested (Art. 6d and Annex V). After authorization

by the Authority responsible for release into the environment has been granted, the basic material must be tested in the field, because field testing is compulsory. (Early tests, which may be accepted for approval under certain conditions, are not feasible in the case of genetically modified material.) The basic material can be tested in two ways, either by genetic evaluation of its components or by comparative testing. If genetic evaluation is preferred, the identity, origin and pedigree of the evaluated components of the basic material, together with the crossing design used to produce the reproductive material, must be documented. Pedigree involves not only information about parents and their characteristics, but also the origin of a gene construct and other genes used for transformation that have been incorporated into the genome of that component. The evaluation must satisfy certain well described requirements and must be superior to standards. Test duration is not laid down in the rules, but it is understood from the philosophy of the regulation that half of the rotation age may be accepted. In certain cases the full rotation age may be necessary to judge whether results satisfy the requirements.

As the genetically modified material must also be field tested according to Directive 90/220/EEC for deliberate release, a question could be in which order the test should be put. Usually the field testing according to Directive 1999/105/EC has to be done after the material has fulfilled the requirements and received authorization for release. The reverse order is inefficient. Another question concerning whether the subsequent genetic modification of approved basic material, which is already on the market, is possible without field testing once again, can be negated. The genetic modification leads to a severe change of the target trait and possibly also of non-target traits. Thus the testing is necessary.

Registration, the National Register, and separation of lots

After approval, the basic material enters the National Register, with each unit of approved basic material having a unique registration reference (Art. 4,2b). Full details of each unit of approval shall be recorded, together with its unique registration reference, in the National Register (Art.10,1). The following information shall be provided as applicable: Botanical name, Category, Purpose (to be stated if use for forestry functions other than timber production is foreseen), Type of basic material, Register reference, Location (for the category Tested: a short title and the exact geographical position where the basic material is maintained), Altitude, Area (size), Origin and an indication "in case of material of the category Tested, whether it is genetically modified".

The registration reference will accompany the material during all stages of production and processing of the reproductive material derived from that basic material, up to the final step of certification. The rules state clearly that lots containing genetically modified reproductive material have to be kept separate at all steps (Art. 13,1k); mixing is not permitted. Mixing of lots of other than genetically modified reproductive material may be allowed under certain conditions.

Certificates and labels

In the case of forest reproductive material derived from basic material consisting of a genetically modified organism, any label or document, official or otherwise, for the lot shall clearly indicate that fact (Art. 14,7). An official document is the certificate. There are three models for certificates, two of which cover reproductive material that may contain genetically modified material: Certificate of Identity for reproductive material derived from seed orchards or parents of family(ies) and Certificate of Identity for reproductive material derived from clones and clonal mixtures. Among the 21 or 17 items, respectively, to be filled in on the certificates, one is related to genetically modified material and must be answered: "Has genetic modification been used in the production of the basic material: Yes or No?" The same applies for the labels.

Note that 'clonal mixture' does not mean a random mixture of anything vegetatively propagated, but is a well defined term. A clonal mixture is a "mixture of identified clones in defined proportions". And a clone is defined as a "group of individuals (ramets) derived originally from a single individual (ortet) by vegetative propagation, for example by cuttings, micropropagation, grafts, layers or divisions" (Art. 2c). Therefore the clones marketed singly or the clones in a mixture marketed as clonal mixture are identified and remain identifiable during all stages of the production of that material. Also material must be declared as genetically modified that consists only partly of genetically modified organisms, for instance only a few clones in a clonal mixture.

More extended definitions of a clone are given by Ahuja and Libby (1993). Rules for clonal propagules either derived by different methods of *in vitro* propagation or micropropagation including by genetic engineering were presented and compared as long as 15 years ago (Muhs, 1993). An extra category for this material was under discussion at that time, but the development took a slightly different route, which can be seen from the rules above. The genetically modified material is fully integrated in the regulation for marketing of forest productive material.

ACCEPTANCE

Transparency is an essential part of acceptance. This has been considered in the regulation. To promote transparency the requirement has been adopted that the methodology used for the test and the detailed results obtained shall be made freely available (Annex V, 1e). This requirement is very important for the user and consumer, because they can make up their mind about the suitability of the material for reforestation purposes on the sites in question. If genetically modified material is involved, full information about the pedigree (see above) must be given also. The public can also deal with the matter and raise awareness, which may help to increase the acceptance of the issue of genetic modification of forest trees. But this seems to be a long way off, and to be dependent on many factors, such as the objectives, the methods used, the effects on the environment, and the policy of the breeder or their agency.

The public will gain even more importance as it has the power to influence official policy (in European countries much more than in many other countries). For instance, a bad policy on the part of the forest owner or company interested in growing genetically modified forest trees, by publishing wrong information or concealing information, can lead to strong public reactions against the project, although all necessary permissions according to the Directives have been granted. So far, no genetically modified forest tree has been planted in the EU member states on a commercial scale. This may change in future and those interested in growing genetically modified forest trees should involve the public at an early stage to avoid unacceptable behaviour. The reaction of the public regarding the cultivation of genetically modified crops in the past provides an example, because information from the company was scarce or had been concealed. The result was a reaction rejecting everything connected with genetic modification. It is hoped – and there are promising signs – that transparency and clear declaration and information will reap their rewards.

OUTLOOK

It was far-sighted to establish rules for genetically modified forest trees before breeders start producing such material for commercialization, because they have guidelines on how to proceed. They know that it could take some years to go through all the tests laid down in the rules. That is one of the reasons for their hesitation. In future, methods for the transformation of gene constructs into a genome will improve and methods to address proper positions in advance of where to insert it could be developed. Also, the search for suitable genes, which have a more specific effect, may be successful in future. Thus, after substantial improvement in methods and gene availability, genetic modification may also in future have a chance with forest trees.

Before then, some missing elements of the rules should be developed, in particular the environmental risk assessment with special reference to forest trees. As forest trees are long-lived organisms, experiments with genetically modified trees should be examined over a long period and monitored thereafter up to the end of the rotation. Criteria to be examined and monitored should be developed specifically for forest trees in addition to the general ones set out in Directive 90/220/EEC.

Tests should be extended by regular checks at given intervals for the stability of the gene constructs incorporated in a host genome and their expression. It has been found in many cases that transgene silencing, as well as transgene repeat formation and transgene integration, are sources for unstable expression (Kumar and Matthuis, 2004). Thus, as these factors also show great influence on the expression, additionally the expressivity, which may be defined as the function of the degree of expression in relation to the growth development and seasonal conditions over years, may be analysed. It is not helpful, for instance, if the stability of a sterility gene is lost or the gene will not longer be expressed, before the trees reach the age when they start flowering. It is not even acceptable that expressivity at that age is

reduced to a level that does not fully prevent the formation of fertile flowers. This example can also be applied to many other gene constructs and traits.

Concerns of the public may increase in future regarding the protecting or patenting of cultivars and varieties such as clones or parents of family. The public has experienced some examples in varieties of crops that have been developed by a company and used worldwide, and these varieties have replaced the local, and in many cases well adapted, ones. The company used doubtful methods to urge the farmers to buy its improved seed. As a result, the local farmer and breeder will lose income and the diversity of varieties available will decrease. It is time to think about the future development of the technique of genetic modification and its consequences. The public has great concerns that should be taken seriously.

Another example of unwanted side-effects of poorly framed policy is a case in the United States of America in which a farmer's canola crop was contaminated, without his awareness, by the pollen of a genetically modified variety that his neighbours were growing. The company that had developed the genetically modified variety claimed that the farmer was growing this variety illegally. The unusual court judgement urged the farmer not to grow his own canola variety any longer, but rather to buy the genetically modified canola variety from the company in future. Although the situation is complex, it shows the complications of poor policy. The farmer, Percy Schmeiser, continued to fight for his right to grow his choice of canola, and was recently awarded the Alternative Nobel prize for his efforts to preserve the local and well adapted varieties of various crops (not only canola) bred by farmers around the world.

SUMMARY

The issue of genetically modified forest trees first arose in 1987, when the first transgenic poplar was produced. In 1999, Council Directive 1999/105/EC (EC, 1999) of the EU was enforced as the first regional regulation, and included rules for the marketing of genetically modified forest material moving in international trade. The OECD Scheme as the second regulatory scheme (OECD, 2007) contains no special rules for genetically modified material, although the countries participating in the Scheme have been working actively towards establishing such rules. Their acceptance has been blocked by a lack of unanimous agreement.

The rules for genetically modified material have been discussed in detail above. Requirements to be fulfilled appear in the two EU Directives. After the safety requirements have been satisfied and authorization for release has been granted, the reproductive material has to undergo tests, because it can only be approved in the category "tested". After successful testing the genetically modified basic material will be approved, each unit will be registered individually and listed in the National Register. Certificates and labels will contain a clear indication that the reproductive material has been derived from genetically modified basic material. The regulation supports transparency by obliging the breeder to make freely available details of the methodology used in the test and the detailed results obtained.

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