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Review

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Tree Biotechnology and Environmental Concerns

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Abstract

Forestry sector has witnessed some unprecedented events in the recent past both in terms of galloping biotechnological developments and heated environmental debates over risks associated with release of transgenic trees. Improvements in the *in vitro* propagation techniques has made it possible to develop tissue culture based plant regeneration protocols just for about any tree species. And with the inclusion of every new species within the realms of tissue culture technology, it becomes a candidate for genetic improvement through recombinant DNA technology, the so called genetic engineering. Poplars and their hybrids serve as the model tree species on which most of the genetic transformation work as been carried out. A lot of work has also gone in genetic transformation of fruit trees and trees of horticultural interests. Trees have been successfully transformed for traits ranging from reduction of length of juvenile phase to alteration of tree architecture to altering wood quality by lignin and cellulose modification. Moreover trees have been genetically engineered successfully to combat various types of insect pests and pathogens causing diseases. But all these developments have ignited controversies over the possible benefits and risks associated with transgenic plantations by various environmental agencies and activists. Solutions to most of these concerns can be found out with more intensive prioritized research.

Key words: forestry, *in vitro* propagation, genetic transformation, lignin modification, tree architecture, escapes.

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Introduction

The forest trees have not been domesticated to the same extent as the agricultural crops have been and hence use of biotechnology can produce better results in forestry sector compared to the agriculture. However there are couple of inherent hurdles to genetic improvement of forest trees owing to their large size, long breeding cycles which usually includes a long juvenile period, and complex reproductive characteristics, including self-incompatibility and high degree of heterozygosity. New developments in the field of biotechnology will answer these problems in the times not far away. Initially though the pace of forestry biotechnology seemed slower compared to that in agronomic crop biotechnology, the scenario has fast changed in last couple of years. Recent advances in biotechnological approaches, such as *in vitro* propagation, somatic embryogenesis, somatic hybridization, gene transfer and marker assisted breeding, have brought the genetic improvement of forest trees at par with that of agronomic species. Genetic transformation offers an attractive alternative to breeding because it provides the potential to transfer specific traits into selected genotypes without affecting their desirable genetic background.

Development of *in vitro* techniques relevant to genetic transformation

Two tissue culture systems have been used for the regeneration of tree species: organogenesis and somatic embryogenesis. Organogenesis is the regeneration of plants by organ formation on explants or from cell masses (callus), whereas somatic embryogenesis is the formation of embryoids or embryo like structures from cells other than gametes or their fusion product.

Historically, *Agrobacterium* has been used for the transformation of angiosperm trees while gymnosperms have been

transformed using biolistics approach. Antibiotic or herbicide resistance transgenes were incorporated into the tree genome and used as selectable markers. However, in most cases, a high frequency of non-transformed regenerants (escapes) were obtained. Presence of a reporter gene was found essential for rapid identification of cluster of transformed cells and tissues. Together with the widely used beta-glucoronidase (*uidA*) reporter gene, the "Green Florescent Protein" (GFP) gene has proved to be an efficient non destructive *in vivo* marker that allow rapid elimination of escapes (Tian et al. 1999).

Since most of the tree species are recalcitrant to the regeneration through organogenesis, somatic embryogenesis has been an efficient alternative for *in vitro* production of whole plants. Although the first objective for the development of somatic embryogenesis systems for trees was mass propagation of plus tree clones to be used in various reforestation efforts, the use of somatic embryos and embryogenic tissue as source material for genetic transformation has facilitated important achievements in genetic engineering of trees. This approach has been widely used since early reports on use of somatic embryos for generation of transgenic spruce (Ellis et al. 1993) and walnut (McGranahan et al. 1998) plants. Last couple of years have seen the use of embryogenic tissues to genetically engineer a gamut of tree species ranging from *Allocasuarina* (Franche et al. 1997) to avocado (Cruz-Hernandez et al. 1998). Unfortunately, in all these cases embryogenic cultures could be produced only from zygotic embryos obtained from the seeds. Which meant that the passage from sexual stage could have result in a drastic reshuffling of genome and the performance of the transgenic seedlings would be unknown compared to that of original clonal material for the selected traits.

For the tree species that are known to regenerate *in vitro* through organogenesis- hypocotyls, cotyledons, leaf disks and stem segments have been generally used for transformation. The production of transgenic angiosperm trees has been limited to a few genera, including *Populus, Eucalyptus, Betula, Liquidamber* and *Robinia*, and transformation is routine only for certain hybrids of *Populus*. The first report of transgenic poplar was published some one and a half decade ago (Fillatti et al. 1987), and since then more has been published on genetic engineering of *Populus* than all other forest tree species put together. Recently, relatively efficient production of *Eucalyptus*, Birch and *Robinia pseudoacacia* has been reported (Igasaki et al. 2000). Regeneration of transgenic English elm, European chestnut (Gartland et al. 2000) and *Acacia mangium* (Xie and Hong, 2001) has also been reported

In fruit trees, transgenic plants have been produced through organogenesis from apple, pear, citrus (including sweet orange, sour orange, lime, lemon, and the citrus rootstocks citrange and *Poncirus trifoliata*), persimmon and *Prunus* sp., including plums, almonds and apricot (Singh and Sansavini, 1998). In most cases transformation was achieved from seed or seedling derived tissues which were the only responsive material to *in vitro* regeneration. The availability of *in vitro* micropropagation procedures of selected mature genotypes allowed transformation of *Populus* and *Eucalyptus* clones as well as apple and pear cultivars. Grafting of adult bud onto juvenile rootstock with the aim of invigorating mature tissue for transformation has been successfully employed for the production of transgenic sweet orange plants that flowered and set fruits in a record 1.5 years time, overcoming the 10 year juvenile asexual phase barrier of this species (Cervera et al. 1998).

Development of genetic transformation systems for specific novel traits

Various traits that have been targeted by genetic engineering under numerous tree improvement projects have been broadly categorized under the following heads:

1. Reduction of length of juvenile asexual phase
2. Alteration of tree architecture and performance
3. Altering wood quality by lignin and cellulose modification
4. Insect-pest resistance
5. Pathogen resistance
6. Bioremediation through transgenic trees

Each of these have been discussed individually exemplifying the major milestones reached.

Reduction of length of juvenile asexual phase

The long juvenile phase in most of the tree species is almost always a hindering factor in tree improvement programmes and also in their commercial exploitation. Several *Arabidopsis* homeotic genes that are involved in flower initiation induce early flowering when expressed ectopically in transgenic plants. Two such genes are *LEAFY* (*LFY*) and *APETALA1* (*AP1*). *LFY* has been known to function in distantly related species, including hybrid aspens (Weigel and Nilsson, 1995). The introduction of this gene resulted in production of flowers within 7 months of vegetative growth where as it normally takes 8 to 20 years for a wild type non-transformed aspen tree to flower. This is one major breakthrough and can be targeted for species of horticultural interests too. The expression of *AP1* gene has resulted in the conversion of apical and lateral shoots into floral primordia and finally flower production (Mandel and Yanofsky, 1995). *PTLF*, the homolog of *LFY* in poplar, when constitutively expressed in transgenic poplar plants, only one line flowered precociously. Alterations in development were observed in multiple lines of transgenic poplars over-expressing either *LFY* or *PTLF* genes (Rottmann et al. 2000). Conversely, constitutive

expression of either *LFY* or *AP1 Arabidopsis* genes in citrus seedlings shortened the juvenile phase and gave way to precocious flower production. Transgenic plants thus produced bore normal and fertile flowers and set normal seeded fruits. These traits were transmitted to the next generation, resulting in trees with generation time of 1 year from seed to seed. The *LFY* lines showed alterations in growth and developmental characteristics and *AP1* plants were adult and fully normal. (Pena and Seguine, 2001).

Alteration of tree architecture and performance

Modification of plant hormone levels in trees has been achieved mainly using genes from the T-DNA of *Agrobacterium tumefaciens* and *A. rhizogenes*. Over expression of *iaaM* and *iaaH* auxin-biosynthesis genes, and the *ipt* cytokinin-biosynthesis gene from *A. tumefaciens*, in poplar has resulted in alterations in growth pattern and wood properties. When over expressed in transgenic poplar, the *rolA, rolB* and *rolC* genes from *A. rizogenes* have produced alterations in growth and rooting ability. Phenotypic modifications of *rolC* over-expressing plants were associated with reduced levels of auxins, modified gibberellin biosynthesis and increased cytokinin levels (Tzfira et al. 1998). With the aim of producing dwarf rootstock, *rolA* and *rolB* genes were introduced into apple (Zhu et al. 2001), and *rolC* was incorporated in trifoliate orange and pear (Kaneyoshi and Kobayashi, 1999; Bell et al. 1999). These transgenic plants were characterized by a reduction in plant height due to shortened internodes and smaller leaves. Moreover, these transformants in general had a greater rooting ability. Expression in sense or antisense orientations of the GA20- oxidase and other genes encoding GA-catabolizing enzymes in transgenic trees has opened up a new and a novel way of modifying the tree architecture. It has been shown that constitutive over- expression of GA20-oxidase in *Arabidopsis* resembles the effect of repeated application of GA_3 to the wild-type plants (Coles et al. 1999). Moreover, the ectopic over-expression of the GA20-oxidase gene from *Arabidopsis* in hybrid poplar has resulted in trees with faster growth in height and diameter, larger leaves, more numerous and longer xylem fibers, and increased biomass, opening up ways of modifying not only tree size but also wood quality (Eriksson et al. 2000). Conversely, it can be hypothesized that expression of GA20-oxidase in antisense orientation in transgenic fruit trees could lead to plants of limited size, thus allowing higher planting density, easier management and mechanical fruit harvesting, and therefore a reduction in labor costs (Pena and Seguin, 2001).

Altering wood quality by lignin and cellulose modification

The extraction of lignin from wood fibers is an expensive and polluting process. Lignin is the most abundant organic compound (15-35%) in the biosphere after cellulose. Lignin biosynthesis phenylpropanoid pathway in trees is a very complex one and varies according to the phylogenetic group to which the tree belongs. Lignin is made up of aromatic polymers that originate from oxidative polymerization of three types of precursors. These lignin precursors give rise to hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin. The angiosperm trees typically contain S-type units, which make them a good choice for paper production because this type of lignin is comparatively easily extracted by Kraft delignification (chemical pulp extraction) process (Chiang and Funaoka, 1990). On the other hand the gymnosperms (conifers) contain more of G-type lignin which is hard to extract. By manipulating the expression of genes in the phenylpropanoid pathway, lignin quantity and composition can be effected to a great extent (Sederoff et al. 1999). Transgenic trees with easy pulping qualities have been produced by either reducing the lignin percentage or its modification. Various approaches have been employed. Antisense suppression of O-methyltransferase (OMT) in hybrid poplar have revealed the possibility of obtaining modified lignin and increased pulp yields (Jouanin et al. 2000). Recently, reduced lignin content has been obtained by down-regulation of 4-coumarate-CoA ligase (4CL) or cinnamyl alcohol dehydrogenase (CAD). In the first case, drastic reduction (upto 45%) of lignin content was observed with the increased growth rate (Hu et al. 1999). Increased (9-15%) cellulose content and alteration of hemicellulose composition was observed in these antisense 4CL transgenic poplars (Lapierre et al. 1999).

Insect-pest resistance

Defoliating insect pests play havoc to many tree species right from seedling stage to maturity. The damage can usually be seen as reduction of tree growth sometimes even threatening its very survival, as well as alteration in tree shape and fruit quality. Two approaches have been used for combating the menace caused by insects. The first one is the insertion of *cry* genes from *Bacillus thuringensis* into the tree species in question. The *cry* genes more popularly known as the *Bt*-toxin genes produce the delta-endotoxin which results in the death of feeding larvae of insects. However its use in forestry is some what limiting because of the vast areas to be covered by tree species. Effective *Bt*-toxin gene expression has been observed in apple, poplar, spruce, larch and walnut and resulted in reduced larval feeding on transgenic material (Tzfira et al. 1998; Schuler et al. 1998; Dandekar et al. 1998). The second approach

is insertion of proteinase inhibitor (PI) genes into the target tree species. The PIs are small proteins with inhibitory effects on insect digestive enzymes. Over-expression of PI genes in transgenic poplar resulted in reduced insect feeding (Klofenstein et al. 1997; DelleDonne et al. 2001).

Pathogen resistance

The introduction and expression of viral sequences in plants interfere with the life cycle of the same or closely related challenging virus. This concept of pathogen-driven resistance proves correct in transgenic *Prunus* plants in which the insertion of coat protein gene of plum pox virus (PPV) has provided high levels of resistance against the virus through the mechanism of post-transcriptional gene silencing (Scorza et al. 2001). Different strategies have been proposed to genetically engineer plants for resistance to bacterial and fungal diseases, including production of antibacterial and antifungal proteins of non-plant origin. In poplar, increased resistance to a fungal pathogen through genetic engineering has been obtained by expression of a wheat oxalate oxidase gene (Liang et al. 2001). In apple and pear, protection against fire blight causing bacterial pathogen *Erwinia amylovora* has been achieved by insertion of genes of insect origin that code for lytic proteins like cercopins and attacins (Norelli et al. 1994; Reynoird et al. 1999). Several designed and natural antimicrobial recombinant peptides have been shown to strongly inhibit *in vitro* spore germination of various forest pathogens (Jacobi et al. 2000)

Bioremediation through transgenic trees

Recovering contaminated soils through the use of microorganisms is a well known environmental technology. Most bioremediation methods are based on the use of microorganisms that are able to breakdown toxic substances. Another strategy is the use of plants as a filtering and decontaminating system. Besides cost-effectiveness, the extensive root systems of trees provide sustainable ground water pumping capabilities and their perennial nature makes ideal for 'bioremediation through trees' or 'phytoremediation'. Genetic engineering of poplar for detoxification of soil bearing chlorophenols such as TCE has been achieved by transferring the corresponding bacterial detoxifying gene (Pena and Seguin, 2001). Similarly, poplar trees engineered with a modified mercuric ion reductase gene capable of sustaining high ionic mercury concentrations have also been obtained (Rugh et al. 1998).

The environmental concerns and prevention of transgene escape

With the improvements and refinements of the gene transfer technologies and successful production of genetically engineered plants, a debate on safety of food products from genetically modified plants and their potential environmental risks has gained the momentum. Activist groups with agendas that include prohibition of environmental release of genetically engineered trees and crops have caused serious impressions on public opinion and public acceptance of transgenics. Gene flow, or the exchange of genetic information between plantations crops and wild relatives, is a naturally occurring phenomenon. The normal movement of genes via pollen dispersal provides a mechanism, however, for foreign genes to "escape" from a genetically engineered crop and spread to weedy relatives growing nearby. Gene flow becomes an environmental issue when the associated trait confers some kind of ecological advantage. This is a particular concern in the case of herbicide resistance genes, for example, where transfer of the resistance trait to weedy relatives raises the possibility of creating "super-weeds" that are more difficult to control (Daniell, 1999). James et al. (1998) have described potential impacts from escape of such trees, and has discussed certain potential ways to decrease the possibility of such escapes. They have emphasized increased field testing and monitoring. The IFURO Working Party on molecular biology of forest trees have brought out the position statement on benefits and risks of transgenic plantations. The goal of the statement is to provide a scientifically informed voice to advise public debate on the benefits and risks of transgenic plants, especially plantation tree crops. The position statement provides a perspective from society of professionals who know the scientific aspects of the area of technology controversy in depth. Its main target audiences are government regulators, scientists and professionals in biological fields, and citizens with scientific background in biology and natural resources. The position statement is available via the Internet (http://iufro.boku.ac.at/iufro/iufronet/d2/wu20406/iufro_posstatm.htm).

While transgenic traits pose some risks for plantations and associated ecosystems, many options exist to minimize their impacts. The priority research areas should address the following key aspects (Strauss et al. 1999):

1. isolation, modification and testing of additional genes and systems for gene regulation, to impart traits without undesired effects on tree development or ecosystem function;
2. studies to support resistance management programmes for use of pest-resistant trees;
3. efficient transformation methods so that genetic diversity is not unintentionally impaired as a result of inability to produce large numbers of useful transgenic genotypes; and
4. methods to modify flowering to allow reliable containment of transgenes within plantations when ecologically prudent. Field trials are crucial for all these research objectives, and

can be carried out with a high degree of environmental safety.

Since prevention of the escape of genes into wild populations has already become such an important concern, genetic containment methods should be given top priority. Containment methods include suicide genes, infertility barriers, male sterility and maternal inheritance. Male sterility is possible only in plants where the product is not a seed or fruit, requiring fertilization. Maternal inheritance of the herbicide resistance gene and prevention of escape via pollen has been successfully demonstrated (Daniell et al. 1998). Engineering foreign genes through chloroplast genomes is a practical solution to this problem. Chloroplast transformation provides containment of foreign genes because plastid transgenes are not transmitted by pollen. The escape of foreign genes via pollen is a serious environmental concern in nuclear transgenic plants because of the high rates of gene flow from crops to wild weedy relatives. Moreover, the target enzymes or proteins for most herbicides are compartmentalized within the chloroplast by this approach.

The terminator technology has also been an answer to some extent to the issue of escape transgenes to the wild. Terminator are more appropriately known as "Genetic Use Restriction Technologies", or GURTs. Two types of GURTs have been described (Hanley and Elborough, 2002). The first GURT patent outlined the concept later described as Variety-restriction GURT, or V-GURT. This type is the emotively named 'Terminator', as it causes the production of sterile seeds. The reproductive viability of the plant is under the proprietary control of the owning company, ensuring that viable seed is not available for the farmer to harvest. This is achieved via triggering a disrupter gene prior to the sale of seed, which has a delayed effect, rendering the next generation non-viable. In 'Terminator', this triggering is by commission, that is, treatment prior to seed sale with an activating stimulus. In the technology dubbed 'Traitor' by critics, this is by omission; a suppressing stimulus is withdrawn and the disrupter is then able to act. The aim is to disrupt the creation of the next generation or to render that generation incompetent to grow and survive. This is where real ingenuity is required. It is vital to arrange the genetic elements so that the disruptor has its effect in a timely manner. The essential component is the promoter, which must respond to an exogenous compound or some triggering stimulus.

The second GURT concept is the Trait-restriction GURT, or T-GURT, where it is the inheritance of the elite trait differentiating the plant from other germplasm that is under control, rather than the plant's reproductive ability. T-GURTs can be described as less crude than V-GURTs since their effects are localized to one area of the genome, and such finesse is increasingly desirable given public concerns over the process of germplasm enhancement. Control over the inheritance of a trait may occur via regulating the expression of genes conferring the trait, or ensuring the disruption or loss of these genes in the next generation. T-GURTs may be an important consideration if the plant is part of the human food chain.

Thus, biotechnology is all set to make its mark in forestry sector. But the need of the hour is to concentrate the research on developing the genetic containment technologies to prevent the creation of "superweeds" or causing gene pollution to other crops.

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