## REVIEW

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# Biosafety in *Populus* spp. and other forest trees: from non-native species to taxa derived from traditional breeding and genetic engineering

**Abstract** Forest trees are fundamental components of our environment, mainly due to their long lifetime and important role in forest ecology. In the past, some non-native tree species and taxa from traditional breeding have induced severe environmental impacts such as biological invasion, changes in the 'gene pool', and spread of diseases in forestry. Genetically modified trees obtained in different research groups worldwide are particularly confronted with increased concerns regarding biosafety issues. In the light of current biosafety research worldwide, various threats facing forests and natural tree populations are evaluated in this review: biological invasions, horizontal gene transfer, vertical gene transfer and effects on other organisms. Results available from groups working in biosafety research and risk avoidance using forest trees, with emphasis on transgenic trees, are reviewed. Independent biosafety research as well as the establishment of biosafety research programs for forest trees financed by national and international authorities is now more important than ever before. Biosafety problems detected in the past clearly show the importance of a prior case-by-case evaluation of non-native species, new taxa and also genetically modified trees according to the precautionary principle before their release to avoid risks to the environment and human health.

**Keywords** Biosafety · Vertical gene transfer · Horizontal gene transfer · Non-native species · *Populus* · GM trees · Biological invasion · Gene flow · Transgenic trees

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## Introduction

Human beings and their activities have increased the changes occurring in the environment. Until recently the arrival of new organisms into geographically isolated environments was a rare event. Modern means of transportation have broken old geographic boundaries. Non-native organisms, when introduced to a new area, can out-compete native species, cause disease to, or destabilise and occasionally ultimately wipe out native species. Invasive species constitute a threat for biodiversity at the local and regional level, since the spread of newcomers can alter the richness and abundance of the flora and fauna of the original ecosystem. As a result, native species are abruptly faced with new species and new environments with which they have had no evolutionary history.

Some species have developed the capacity to adapt to new sites and to displace the original populations. This phenomenon has been widely recorded and it is known as 'biological invasion' (Kowarik 2003a, b). A biological invasion is a process which starts after the introduction of organisms which have evolved in other areas or under the impact of humans (Kowarik 1999). Many non-native trees and crop plants have allowed increasing productivity and became a fundamental part of human economy whereas others have developed into serious ecological problems. However, besides (a) non-native plants, potentially invasive plants comprise two additional groups: (b) taxa resulting from traditional breeding, and (c) genetic modified plants (GMPs).

Biological invasion is widely recognized as the second leading cause—after habitat destruction—of species endangerment and extinction (Vitousek et al. 1997; Wilcove et al. 1998); invasions can also cause massive ecological and economic damage. According to Wilcove et al. (1998) exotics are responsible for extinction of 57% of the endangered species in the USA. Other important threats to biological diversity are represented by interbreeding between non-native/bred plants and native relatives, which alters the 'gene pool', and the introduction of non-native animals and microorganisms.

The Cartagena Protocol on Biosafety (29 January 2000) has been one of the most important attempts to regulate the exchange of genetically modified organisms over country borders (CBD 2003). This protocol, based on the precautionary principle, aims at ensuring an adequate level of protection of native ecosystems through the safe transfer, handling and use of organisms resulting from modern biotechnology. Unfortunately, the term 'biosafety' has been often reduced to potential risks only due to GMPs. As a consequence biosafety problems derived from non-native and bred trees species have been neglected in many countries.

In this review we present the current biosafety status of forest tree species, defining the term 'biosafety' as the prevention of large-scale loss of biological diversity and integrity due to human activities. Therefore, we present and compare the risks derived from non-native and traditional bred trees with potential risks of GM trees. Results from initial biosafety research on genetic modified trees from the genus *Populus* are also reviewed.

## Threats to biosafety in forests

Forest trees have some special characteristics, compared to other plants. The most important difference regarding biosafety issues is their longer lifetime. Trees must cope with very different environmental conditions for many years, even hundred of years. This is a basic factor for assessing biosafety risks in the forestry. The time-lag between introduction of non-native species or taxa into the forest and the onset of a biosafety problem is a very important factor to consider regarding tree biosafety research. For some successful invasive species, a series of events after colonization is more important than intrinsic 'colonizing ability'. In fact, two enigmatic phenomena associated with successful invasives suggest that many species are not preadapted to become successful invasives and that the right circumstances must conspire for invasiveness to occur and perhaps evolve (Ellstrand et al. 2000). Kowarik (1992, 1995) studied the spreading dynamic of American non-native tree species, which have been growing for many years in a region near to Berlin/Germany. The lag-time between introduction of a non-native tree and the beginning of a biological invasion, was very variable between different species. Only 6% of non-native tree species became invasive during the first 50 years, 25% of species showed lag-times of up to 100 years, 51% until 200 years, and 18% of species begun a invasion even later (Table 1) (Kowarik 1992, 1995). The lag-time factor has very important implications for the evaluation of risk factors related to forest trees.

Biological invasions

*Invasion of non-native tree species*

A low proportion of plants introduced into a new environment becomes invasive. It has been found that about 62% of the crops that had come under APHIS (Animal and Plant

**Table 1** Time-lags between the first introduction of non-native trees to Brandenburg/Germany and the beginning of an invasion process (Kowarik 1992; Kowarik 2003b)

| Tree species | Time-lag (years) |
| --- | --- |
| *Prunus persica* | 415 |
| *Juglans regia* | 374 |
| *Thuja occidentalis* | 324 |
| *Fraxinus ornus* | 246 |
| *Corylus colurna* | 222 |
| *Laburnum anagyroides* | 198 |
| *Acer negundo* | 183 |
| *Celtis occidentalis* | 172 |
| *Robinia pseudoacacia* | 152 |
| *Populus × canadiensis* | 165 |
| *Aesculus hippocastanum* | 124 |
| *Ailanthus altissima* | 122 |
| *Pinus strobus* | 117 |
| *Quercus rubra* | 114 |
| *Sorbus intermedia* | 112 |
| *Pseudotsuga menziesii* | 112 |
| *Prunus mahaleb* | 54 |
| *Prunus serotina* | 29 |

Health Inspection Service, US Department of Agriculture) oversight were not persistent in native environments and thus could be considered non-invasive (Hancock et al. 1996; Hancock 2003). Another 21% persist for a few generations in native environments but eventually disappear. These have slightly higher native fitness than the non-persistent crops, but they can still be considered non-invasive, because they do not spread outside the agro-ecosystem. About 17% fall into the persistent category; these can be ranked as invasive, because they readily reproduce outside the agro-ecosystem and spread (Hancock et al. 1996; Hancock 2003).

Williamson (1993) proposed the 'tens rule'. According to this rule approximately 10% of introduced non-native species disperse, 10% of them are able to persist in the new environment, and 10% of persistent non-native species become a 'pest species'. The proportion of invasive species is higher in tropical regions and especially in tropical islands (Usher 1988); in Hawaii, for example 20% of introduced organisms are considered invasive (Loope and Müller-Dumbois 1989). Baker (1965, 1974) listed traits associated with the most successful weeds: broad germination requirements, seed dispersal over short and long distances, discontinuous germination, vigorous vegetative reproduction, rapid growth to flowering, brittle propagules, continuous seed production, vigorous competitors, self-pollination, polyploidy, unspecialised pollinators, long lived seeds, very high seed output, and plastic seed production.

Binggeli's (1996) reported 653 invasive woody plants species world-wide. He classified 184 from this as highly invasive and 134 of the invasive species have been spread by forestry for years (Table 2). A selection of some highly invasive trees is presented in the following:

*Prunus serotina*: This plant species was one of the first North American tree species introduced in Europe

**Table 2** Problematic exotic trees species (wild or hybrid trees) subject to control in Germany (adapted from Kowarik 2003a)

| Trees species (wild/hybrid) | Conflicts with | | | Initial introduction | | Pathways of secondary releases | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Agriculture | Nature conservation | Silviculture | Deliberate | New taxa | Garden ornamental | Plant for hedges/ shelterbelts | Silvicultural crops | Soil improvement | Erosion control | Beekeeper's plant | Game shelter/ forage |
| Acer negundo | | □ | | □ | | ■ | ■ | | | | | |
| *Pinus nigra* | | □ | | □ | | ■ | | ■ | | | | |
| *Pinus strobus* | | □ | | □ | | ■ | | ■ | | | | |
| *Populus* × *euroamericana* | | □ | | | □ | ■ | ■ | ■ | | | | |
| *Prunus serotina* | | □ | □ | □ | | ■ | ■ | ◊ | ◊ | | | |
| *Quercus rubra* | | □ | | □ | | ■ | | ■ | ■ | ■ | ■ | ■ |
| *Robinia pseudoacacia* | □ | □ | | □ | | ■ | ■ | ■ | | | | |
| *Pseudotsuga menziesii* | | □ | | □ | | ■ | | ■ | | | | |

Additional information: The relevance of pathways has been estimated for Germany as (■) recently relevant, (◊) relevant only before 1950

(Starfinger et al. 2003). During its long history as an introduced species, the way it has been perceived and used has changed radically. The motivation for the first introduction and plantings of *P. serotina* may have been its ornamental qualities. Today, it is widely distributed in Germany and figures prominently on lists of problematic invaders (Kowarik 1999) and successful invaders (Starfinger 1998) in central Europe. More than 30 years of intensive fighting with all means of control have achieved no substantial success. *P. serotina* has become and will remain a naturalized component of the central European flora (Starfinger et al. 2003).

*Melaleuca quinquenervia*: This Australian tree species is invading the Florida Everglades. It was originally introduced into the region as an ornamental plant, in the hope of turning the everglades into timber-producing forest. After a fire, however, this tree rains millions of seeds onto the burnt land and thus spreads very fast (Myers 1983).

Invasive forest trees have been studied for many years (Binggeli 1996; Cock 2003; Huenneke and Vitousek 1990; Kowarik 2003a, b; Reichert and Hamilton 1997; Richardson and Higgins 1998; Rouget et al. 2002; Simberloff et al. 2002; Starfinger et al. 2003). The investigation program SCOPE (Scientific Committee on Problems of the Environment 1982) studied the ecology of biological invasions worldwide for 10 years. SCOPE results indicate that invasiveness of a plant species into a new ecological system is a very complex and often barely predictable phenomenon (Kowarik 2003b; Williamson and Brown 1986).

*Invasion of pathogens related to forest trees*

The ecological impact of introducing non-native insects and microorganisms has been dramatic for some tree species (Cock 2003; Coyle et al. 2005; Dwinell 1997; Kowarik 2003b; Liebold et al. 1995; McNabb 1971; Witt 2002)

(Table 3). For example, the Asian chestnut blight fungus (*Cryphonectria parasitica*) arrived in New York on nursery stock in the late nineteenth century and spread over 100 million ha of eastern North America in less than 50 years, killing almost all mature chestnuts *Castanea dentata* (Anderson 1974). Chestnut was a dominant tree species in many forests, and though it is not extinct, large individuals are extremely rare. Thus, this species is not able to fulfil its previous ecosystem functions (Simberloff 2003).

The Dutch Elm Disease, *Ophiostoma* (=*Ceratocystis*) *ulmi* (Buisman) Nannf, from east Asia, was found in the Netherlands and northern France in 1918 and in North America in 1930. The illness was transported from Asia to Europe and North America with imported elm wood. Most European elms have been victims of this disease, in Southern England for example, 70% of 22 million elms died between 1971 and 1978 (McNabb 1971).

Gene transfer between non-native/bred and native tree species

Gene flow between organisms that are sexually compatible is called 'Vertical Gene Transfer' (VGT). The introduction of a small number of *Populus* × *euramericana* clones and *P. nigra* varieties, which can intercross with wild *P. nigra* trees, has generated concerns related to the integrity of the *P. nigra* 'gene pool' in Europe (Cagelli and Lefèvre 1995; Lefèvre et al. 1998). However, it has been suggested that VGT may not be a major threat for *P. nigra* (Tabbener and Cottrell 2002). Two factors may contribute to VGT in poplars. Firstly, most *Populus* species are dioecious, and thus obligatory out-crossers. And secondly, in addition to being wind-pollinated, the long white, silky hairs attached to the short stalks of the seeds promote wind dispersal over great distances, resulting in high rates of migration (Schreiner 1974).

**Table 3** Some important exotic pest and diseases (Dwinell 1997; McNabb 1971; Kowarik 2003b; Simberloff 2003)

| Illness/plague | Origin | Secondary expansion | Affected plants |
|---|---|---|---|
| Insects | | | |
| *Eriosoma lanigerum* | North America | Europe (since 18th century) | Apple |
| *Corythucha ciliata* | North America | Germany (1983) | Plane |
| Fungi | | | |
| *Ophistoma (=Ceratocystis) ulmi* | East Asia | Europe (20th century) North America (1930) | Elm |
| *Cryphonectria parasitica* | Asia | North America (19th century) | Chestnut |
| Bacters | | | |
| *Erwinia amylovora* | North America | Germany (1971) | Fruit-trees |
| Virus | | | |
| PlumPoxVirus (PPV) | Balkans | Germany (1961) | Plum, Apricot, Peach |
| Nematodes | | | |
| *Bursaphelechus xylophilus* | North America | Asia, Europe | Pines |

**Table 4** Natural and introduced *Populus* hybrids in the environment (modified from OECD 2000)

| Parentage | Hybrid designation [Synonym] (Common name) |
|---|---|
| *P. alba* × *P. grandidentata* | *P.* × *roulwauiana* Boivin |
| *P. alba* × *P. Adenopoda* | *P.* × *tomentosa* Carr. (Chinese white poplar) |
| *P. alba* × *P. Tremula* | *P.* × *canescens* Ait. Sm. (grey poplar) |
| *P. alba* × *P. Tremuloides* | *P.* × *heimburgeri* Boivin |
| *P. angustifolia* × *P. deltoides* | *P.* × *acuminata* Rydb.[syn. *P.* × andrewsii Sarg.] (Lanceleaf cottonwood) |
| *P. angustifolia* × *P. balsamifera* | *P.* × *brayshawii* Boivin (Brayshaw's poplar) |
| *P. angustifolia* × *P. tremuloides* | *P.* × *sennii* Boivin |
| *P. balsamifera* × *P. deltoides* | *P.* × *jackii* Sarg. (Jack's poplar) |
| *P. balsamifera* × *P. tremuloides* | *P.* × *dutillyi* Lepage |
| *P. deltoides* × *P. Nigra* | *P.* × *canadensis* Moench cv. Eugenei [syn. *P.* × *euramericana* Dode Guinier] (Carolina poplar, Canada poplar, Euramerican poplars) |
| *P. deltoides* × *P. tremuloides* | *P.* × *bernardii* Boivin (Bernard poplars) |
| *P. deltoides* × *P. Trichocarpa* | *P.* × *generosa* Henry [syn. *P.* × *interamericana* Brockh.] (Interamerican poplars) |
| *P. fremontii* × *P. trichocarpa* | *P.* × *parryi* Sarg. (Parry cottonwood) |
| *P. grandidentata* × *P. Tremuloides* | *P.* × *smithii* Boivin |
| *P. laurifolia* × *P. Nigra* | *P.* × *berolinensis* Dippel [syn. *P.* × *rasumowskyana* Schr. and *P.* × *petrowskyana* Schr.] (Berlin poplars, Russian poplars) |
| *P. deltoides* × *P. balsamifera* × *P. angustifolia* (natural trihybrid) | Unnamed |

Natural hybridisation has been reported between almost all sympatric poplar species, and between introduced and native poplars, both in North America and Europe (Table 4) (Schreiner 1974; Demeritt 1990). Hybridisation generally occurs between species in the same section (Brayshaw 1965; Eckenwalder 1977), although intersectional hybrids also occur. Species in different sections, though broadly sympatric, are ecologically isolated from one another, so that hybridisation occurs over large geographic areas but within a relatively narrow ecological range of overlap (Eckenwalder 1984a, b, c). Complicated natural hybrid populations may also form where three or more species are sympatric (Rood et al. 1986). Hybrids between members of the same section are produced easily and are often more vigorous than their parents, e.g. the hybrid *P. deltoides* × *P. nigra* (*P. euramericana* = *P. canadiensis*) (Kowarik 2003b).

Hybridisation is a frequent and important component of plant evolution and speciation (Riesenberg and Ellstrand 1993). More than 70% of plant species may be descended from hybrids (Grant 1981). However, hybridisation can, through one or more mechanisms, catalyse the evolution of invasiveness (Ellstrand et al. 2000). Concerns regarding uncontrolled VGT, due to the introduction of non-native and bred species, have promoted the development of genetic conservation programs in many countries (Wilson 1990; Rogers 2002; EUFORGEN 2003).

## Genetically modified trees (GM trees)

Introduced non-native tree species or new taxa resulting from traditional breeding have never been as important for the public opinion as genetically modified plants. Since the first reports on transgenic crops in the 1980s (Bevan 1984), the debate surrounding the generation of transgenic plants and the political, ecological and economic ramifications of their development have seldom been far from the public eye (Valentine 2003).

GM trees are still very rarely found in nature. Since 1989 there have been more than 100 confirmed GM tree field trials worldwide (BioSicherheit 2003). Most releases have occurred in the USA, but there are also reports in the European Union, New Zealand and China. Commercial plantations of GM trees have only been reported in China (Lida et al. 2003).

The promising prospects offered by genetic modification, especially for tree breeding, has promoted efforts to develop molecular breeding methods for woody plants. The genetic transformation of trees has some advantages with respect to conventional breeding: (a) genes from virtually any organism can be used, (b) individual genotypes can be improved for one or a small number of well defined traits while preserving the rest of the genome intact, and (c) genetic engineering is the only method available which could allow accelerated breeding of forest tree species at rates comparable to that achieved with crop plants. This latter advantage is particularly important as breeding of forest trees has been hampered by the long time they require to enter into the reproductive phase (juvenility). Many of the commercially important tree species don't flower until they are at least 15–20 years old (Hackett 1985).

The main aims of genetic transformation in forest tree breeding are (1) wood modification (Chiang 2002; Pilate et al. 2002; Campbell et al. 2003); increase in quantity and quality of wood, for example through lignin reduction in wood, (2) reduction of the prolonged juvenile phase (Meilan et al. 2001), (3) induction of sterility in order to avoid gene flow into the wild relatives (Strauss et al. 1995) and biological invasions, (4) resistance to viral, fungal and bacterial pathogens (Strauss et al. 2001; Campbell et al. 2003), (5) herbicide resistance (Strauss et al. 2001; Campbell et al. 2003), (6) cold, heat, drought tolerance (Strauss et al. 1997; Mullin and Bertrand 1998) and (7) development of plants for phytoremediation of contaminated soils (Gullner et al. 2001).

Benefits of GM trees can arise from the transfer of traits that are not readily available either in the breeding population or the genetic resource. The conservation of genetic resources threatened by the anthropogenic introduction of pest and diseases, e.g. genetic modification of elms to save them from Dutch Elm Disease, can be improved through transfer of Anti-fungal genes. Genetically modified English elm (*Ulmus procera*) trees have been already produced following transformation with different anti-fungal protein genes (Gartland et al. 2003).

Plantation forestry is displacing the harvesting of the natural forests. Fast growing, short-rotation GM tree crops would relieve pressure on natural forests (Gartland et al. 2002). The presence of transgenic sequences in plantation trees would be an additional protection to natural forests, as transgenes would allow to differentiate plantation wood from illegal wood obtained from natural forests. Pressures on that forests could be reduced with this new certification strategy.

However, despite of all promising aspects of genetic modification, this technology has been the subject of considerable controversy, with concerns raised mainly from ecological and ethical arguments. This controversy has slowed the broad use of this technique in agriculture and forestry. Amongst the potential biosafety threats argued against GM trees are:

Invasion of transgenic trees

Fitness of a tree species might be changed in an unpredictable way by genetic modification. It is important to determine whether newly introduced traits make GM trees more likely to be invasive in natural habitats. Luby and McNichol (1995) and Baker (1974) stated that weediness arises from many different characters. The invasive potential of the plant species plays a more important role than isolated genes used for genetic transformation (Luby and McNichol 1995). Both Fitter et al. (1990) and Williamson et al. (1990) oppose this statement and suggest that small genetic changes can cause large ecological alterations. Hancock (2003) has proposed the possibility of determining the potential impact of individual transgenes by evaluating their phenotypic effect (Table 5). 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 (Bizili et al. 2000), should be considered detrimental because they reduce plant fitness in the absence of heavy metal contamination. 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 are e.g. Bt toxin genes for insect resistance (Genissel et al. 2000) or those conferring tolerance against drought, salinity or high temperature (Wang et al. 2004).

Spread by vegetative means, through root suckers, is also a very important risk assessment factor for both transgenic and non-transgenic poplars (Fladung et al. 2003). Root

**Table 5** Relative fitness impact of transgenes (from Hancock 2003)

| | | |
|---|---|---|
| T-A | Neutral in the native environment | Marker genes |
| T-B | Detrimental in the native environment | Male sterility, altered fiber quality, altered fruit ripening, and storage |
| T-C | Variable, depending on invasiveness of tree or native relative | Herbicide resistance |
| T-D | Variable, depending on invasiveness of crop or native relative | Viral, fungal and pest resistance |
| T-E | Potentially advantageous in the native environment | Cold, drought, and heavy metal tolerance; improved nutrient uptake; altered development |

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 should be also considered for containment of trees.

Vertical gene transfer

Gene flow in poplar plantations and its implications for transgenic risk assessment have been studied by DiFazio (2002). 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 × Populus 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 surrounding landscape characteristics surrounding plantations also had a strong influence on the rate of flow. The development of sterility strategies may be a solution to avoid gene flow between native species and non-native species/bred taxa. If the production of pollen and seeds is reduced, gene flow can be minimised or even prevented (DiFazio 2002).

The incorporation of sterility genes into transgenic lines of trees has therefore been proposed to reduce or even avoid gene flow of transgenes into non-transgenic relatives (Strauss et al. 1995). An additional advantage of sterile trees would be the reduction of energetic costs necessary for development of reproductive structures (Brunner et al. 1998; Mouradov et al. 1998). Many sterility gene constructs have successfully been tested in crop plants, e.g. by expression of deleterious genes, like *barnase* (Mariani et al. 1990), s*tilbene synthase* (Fisher et al. 1997), the gene for ribosome inactivating protein (Palmiter et al. 1987), use of dominant negative mutations (Mitzukami et al. 1996), gene suppression strategies like antisense suppression, cosuppression, 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 trees. There are some reports in *Populus* (Meilan et al. 2001; Fladung and Hoenicka 2004; Hoenicka and Fladung 2003; Skinner et al. 2003), but the induction of sterility in transgenic trees has not really been demonstrated adequately shown so far.

The first poplars transformed with sterility genes showed a lower performance as control plants (Meilan et al. 2001). Heterologous promoters used seem to direct activity of cytotoxic gene expression in non-target, vegetative tissues ('leaky' expression) (Meilan et al. 2001). Use of floral promoters from forest trees (Skinner et al. 2003) or use of other genes may allow avoidance of 'leaky' expression.

Horizontal gene transfer

Gene transfer between organisms that are sexually non-compatible is called 'Horizontal Gene Transfer' (HGT). The possibility of transgene transfer from transgenic plants into other organisms (mainly bacteria, fungi and viruses) has become an important argument against GMPs (Stirn 2000; Peerenboom 2000). HGT between eukaryotes and other kingdoms is regarded as unusual (Brown 2003). *Agrobacterium* species, one of the best-characterised examples of HGT, are capable of inserting a defined fragment of its DNA into the genome of dicotyledonous plants (Chilton et al. 1977; Schell et al. 1979; Gelvin 2003; Valentine 2003). Ng*rol* genes (Ng*rolB*, Ng*rolC*, Ng*ORF13*, and Ng*ORF14*), 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 transformation seems to have occurred very early in the evolution of the genus *Nicotiana* (Aoki and Syono 1999).

That HGT is possible in the inverse direction, from plants into bacteria, has been concluded from sequence homologies between plant genes and the respective genes in bacteria, e.g. the glucose-6-phosphate-isomerase gene in *Clarkia ungulata* and in *E. coli* (Schlüter and Potrykus 1996). Natural HGT has been detected between the endosymbiotic bacterium *Wolbachia* and its host insect *Callosobruchus* (Kondo et al. 2002), between plants (Won and Renner 2003; Mower et al. 2004), and other bacteria and plants (Brown 2003). The mechanisms of HGT between prokaryotes, such as transduction and conjugation, are relatively well understood (Ochman et al. 2000). In contrast, mechanisms underlying prokaryote-eukaryote gene transfer, excluding that between *Agrobacterium* and angiosperms, as well as conditions by which HGT takes place are widely unknown (Kondo et al. 2002; Won and Renner 2003).

For trees a possible HGT to mycorrhizal fungi might be in particular important. Mycorrhizas are highly evolved, mutualistic associations between soil fungi and plant roots. Many forest tree species are largely dependent on ectomycorrhizal (EM) fungi for the uptake of mineral nutrients (Smith and Read 1997). The tight connection between fungi and trees, and the exchange of substances between them, might favour the occurrence of HGT. In nature, *Populus* is able to form two types of mycorrhizal symbiosis: arbuscular mycorrhiza (AM) and ectomycorrhiza (EM) (Harley and Harley 1987). So far, no report is available showing clearly a HGT event from a tree to mycorrhiza fungi (Nehls et al. personal communication). The presence of phytosymbiotic bacteria in *Populus* species (Van Aken et al. 2004) and other tree spacies represents also an important object of investigation in relation to HGT.

HGT is a very important challenge for biosafety research in the future. The list of potentially naturally occurring HGT vectors include: transposable elements, plasmids, viruses, bacteria, protozoans, nematodes, fungi and insects. Furthermore, free DNA of high molecular weight is able to persist in soil, sediments or microcosms (Lorenz and Wackernagel 1994), even though DNases are widely

distributed. DNA survival of several months or years (Lorenz and Wackernagel 1994) and over thousands of years (Pääbo et al. 1988) has been reported. To gain a perspective when assessing the potential environmental impact of DNA from GMPs, it is important to consider the amount of DNA in the environment from non-GM origins. Dale et al. (2002) concluded that the impact of free DNA of transgenic origin is negligible when compared with the total amount of free DNA available in the environment. Pollen, leaves and fruit alone result in thousands of tonnes of DNA per year being released into the environment (Dörfler and Schubbert 1998), in addition to the contribution from decaying plant and animal matter and release from microorganisms. DNA survival can be expected to depend on a variety of factors including nucleases, reducing agents, metal ion concentrations, and the presence of binding agents (Van den Eede et al. 2004). Throughout the history of evolution, all organisms have been receiving foreign DNA. Mice fed with phage M13 DNA degraded 95% of this DNA but some of it was later detectable in peripheral leukocytes, spleen and liver (Schubbert et al. 1997). DNA uptake mechanisms by the intestinal wall epithelia, and the defence mechanisms against foreign DNA are still not clear. It is also unclear if foreign DNA represents a serious safety risk factor. DNA sequences from plants, animals, bacteria and viruses have been present in human and animal feed through history (Van den Eede et al. 2004). Therefore, most sequences to be found in GMPs will have entered the mammalian gut before present time.

Transgene instability

Many papers on annual crops have shown that expression of transgenes is less stable than had originally been thought. Most of these events reported fall into the class of homology-dependent gene silencing, which involves mechanisms that function at the level of transgene transcription or post-transcriptionally (reviewed in Paszkowski 1994; Meyer 1995). Gene silencing has been reported in transgenic trees transformed with the *rolC* gene (Fladung 1999; Fladung and Kumar 2002; Kumar and Fladung 2001; Fladung et al. 2004). The stability of transgene expression has a decisive influence on the efficiency of strategies for biological confinement of transgenic plants. Gene silencing of sterility genes would allow crossings of transgenic woody plants with their natural relatives. Unstable transgenes represent an even higher risk factor in transgenic woody plants than in annual plants, because of their prolonged lifetime. However, gene instability is not an exclusive phenomenon of transgenic plants. Adverse abiotic and biotic stresses have been shown to induce genome instability in non-transgenic plants (Lebel et al. 1993; Lucht et al. 2002; Puchta et al. 1995; Ries 2000; Kovalchuk 2003; Filkowsky et al. 2004). Infection of tobacco plants with tobacco mosaic virus and oilseed rape mosaic virus was shown to induce a threefold increase in homologous DNA recombination in non-infected tissues (Dong 2004). 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 et al. 1997). Hawkins et al. (2003) evaluated the transgene expression in a hybrid poplar (*Populus tremula* × *P. alba*) clone transformed with constructs carrying a reporter gene (*uidA*) under the control of either a constitutive or a vascular-specific promoter. Analyses of transgene expression by GUS fluorometry and histochemistry were performed on several hundred trees, originating from different transgenic lines, grown under in vitro, greenhouse and field conditions. While important variations in expression levels occurred, the transgene appeared to be stably expressed throughout a 6-year period. A similar result was 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).

Silencing in 35S::*uidA* transgenic poplar was detected only for lines, which were probably silenced from the beginning shortly after the transformation process (Hawkins et al. 2003; 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 (2000a) 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 published gene silencing events in trees were observed in lines containing an elevated number of transgene copies. Silencing was attributed to different transgene copies organised either at two or more integration loci or as transgene repeat at one locus (Fladung 1999; Hawkins et al. 2003; Kumar and Fladung 2001, 2002; Fladung and Kumar 2002), or to position effect variation including flanking genomic sequences (Kumar and Fladung 2001, 2002), or to both (Fladung and Kumar 2002). Occurrence of a transgene repeat is often accompanied by methylation of the promoter and/or the transgene (Kumar and Fladung 2000a). 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 produced in our laboratory, characterized by the presence of T-DNA repeats, revealed GUS expression over a period of 7 years in plants so far cultivated either under greenhouse or in vitro conditions. It remains unknown so far 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 some time in the future. Nevertheless, transgenic lines containing

more than one T-DNA copy can easily be detected by simple molecular methods (Kumar and Fladung 2000a) and subsequently discarded from the breeding process.

Impact on non-target organisms

All tree species are part of the ecological food chain, and thus many non-target species have an opportunity to be exposed to a transgene and its products (Mullin and Bertrand 1998). GM trees transformed with intent to convey greater resistance to pathogens have been of particular concern. Ecotoxic effects on other organisms like insects or soil organisms were assumed (Myhr and Traavik 2002). Transgenic pest-protection strategies generally depend on the transfer and expression of novel or natural defensive plant genes. 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 et al. 2002). The expression of broad-spectrum antimicrobial components by GM plants may not only suppress target pathogens, but may affect plant symbionts such as mycorrhizae and rhizobia, as well as other micro-organisms involved in decomposition and nutrient cycling (Morra 1994; Glandorf et al. 1997).

It is 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 may represent a similar or probably higher risk than GM plants (Brimner and Boland 2003; Boland and Brimner 2004). However, they have not been questioned as Bt toxin carrying transgenic plants (Bt trees), are freely available in many countries and have being used in forest protection against lepidopteran defoliators for many years (Bauce et al. 2004; Kouassi et al. 2001; Cadogan and Scharbach 2003).

Allelopathic effects of non-native trees on other organisms seems to be altering the ecological balance in many ecological systems (see review by Inderjit and Duke 2003). GM trees may represent a similar ecological threat. The evaluation of possible environmental damage, e.g. 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 alone as a result of pesticide use (Dale et al. 2002). Advantages and disadvantages of Bt trees should be carefully considered.

So far, no report is available showing clearly a HGT event to mycorrhiza fungi. Experiments with transgenic trees, that carry an antibiotic resistance gene under the control of a fungal-specific promoter carried out in containment and under field test conditions, to detect HGT are ongoing (BioSicherheit 2003).

The ectomycorrhizal (EM) status of the roots was analysed in transgenic and non-transgenic trees (Kaldorf et al. 2002). For the first 2 years under field evaluation no or minor differences were found in ectomycorrhizal status be-

tween control and transgenic trees. However, long-term experiments for up to 4 years revealed significant differences in the EM community composition between 35S::*rolC* and untransformed trees (Kaldorf et al. unpublished). It is not clear whether the differences might be caused by the constitutive *rolC* expression itself, or might be a secondary effect of the dwarf-like *rolC* phenotype.

Effects on non-targeted characteristics

Transgenic trees altered in characteristics such as reduced lignin (Hu et al. 1999) or longer cellulose fibres (Eriksson et al. 2000) may also reveal characteristics which are induced by the action of the gene but are not targeted. For example, lignin is important for the stabilisation of the plant structure acting also as a barrier for pathogen entrance (Fink 1999). Thus possibly, low lignin-trees might be more prone to breaks or to pathogen attack. Pilate et al. (2002) did not detect a disturbed fitness of lignin-reduced transgenic poplar trees.

The susceptibility to pathogens can be increased by genetic transformation. As already shown for 35S::*rolC* transgenic potato plants (Fladung and Gieffers 1993) the 35S::*rolC* transgenic trees had a higher degree of susceptibility to phytopathogenic fungi *Melampsora* and *Venturia* than the untransformed plant. This susceptibility can be explained at least in part by the altered hormonal status and carbohydrate composition found in 35S::*rolC* transgenic leaves (Fladung et al. 1997; Fladung and Gieffers 1993, 2003; Kaldorf et al. 2002).

Unpredictable pleiotropic effects due to lack of gene targeting

Secondary effects following transgene introduction may arise from the expression products, or the insertion of one or more transgene(s), which can divert the gene expression patterns of the recipient plant. Kumar and Fladung (2001) demonstrated for transgenic lines characterized by an 'altered' phenotype that the transgene has integrated near a gene coding for a putative methyl transferase gene.

– Transformation constructs

(i) *Promoters*: The 35S promoter of the Cauliflower Mosaic Virus (CaMV) is currently widely used in transformation experiments including trees. A study supports that there is a 'recombination hotspot' in this promoter (Kohli et al. 1999). The CaMV 35S promoter seems to be prone to recombination during the biolistic transformation process. Some non-governmental organizations (NGOs) that are against biotechnology argue that the CaMV 35S might be also prone to recombine with other DNA sequences in the host genome, including dormant viral DNA, as well as with other viruses in the host cell. This speculation has been criticized because pararetroviral sequences, such as the CaMV 35S promoter, are not exotic to plant genomes (Hull et al. 2000; Matzke

et al. 2000; Tepfer 2002). Many plants contain already integrated pararetroviral sequences (Gregor et al. 2004; Richert-Poggeler and Shepherd 1997) and related retrotransposons (Jakowitsch et al. 1999; Kumar and Bennetzen 1999; Matzke et al. 2000). The 35S promoter has been proposed to represent a lower biosafety risk factor than viruses, endogene retrovirus/pararetrovirus and retrotransposons present in all plants (Hull et al. 2000; Matzke et al. 2000; Tepfer 2002). Indeed, in some plants such elements constitute up to 90 % of the genome (SanMiguel et al. 1996; Kazazian 2004). There is no evidence that the 35S promoter is mobile, in the way that a transposon can be (Tepfer 2002).

(ii) *Antibiotic resistance*: Due to the concerns from some environmental groups, scientists and NGOs, antibiotic resistance genes in transgenic plants came to the focus of politicians and the public because antibiotic resistance genes might be transmitted to bacteria in nature (Stirn 2000). These genes are important during the transformation process as plant selectable markers to keep alive few transgenic cells in the large pool of cells not taking-up the foreign gene(s).

The FDA (Food and Drug Administration, USA) suggested ranking the antibiotic resistance genes in transgenic plants with the kanamycin resistance on one end as the most acceptable, and the vancomycin resistance gene on the other (FDA 1998). The *nptII* gene, which confers kanamycin, neomycin and gentamycin B resistance, has a 13-year history of safe use in food crops and resistance to this group of antibiotics (Group I) is widespread in naturally occurring microbes and is seldom used in human medicine, because more potential aminoglucosides are available (EFSA 2004; Smalla et al. 1993). The European Food Safety Authority (EFSA) concluded that the use of *nptII* as a selection marker did not pose a risk to the environment nor to human and animal health (EFSA 2004). There is no rationale for inhibiting or restricting the use of *nptII* genes either for field experimentation or for the purpose of placing GM plant products on the market. A second group of antibiotic resistance gene (Group II), common in nature and useful for human medicine, which includes resistance to chloramphenicol, ampicillin, streptomycin and spectinomycin, should be restricted to field trial purposes and should not be present in GM plants to be placed on the market. Genes conferring resistance to antibiotics important in human medicine (Group III), like amikacin and tetracyclines, should not be present in GM plants at all (EFSA 2004).

## Regulation of biosafety risks

There are many national and international policies to prevent biosafety risks (Golz 1999; Shine et al. 2000). Many countries have signed national and international agreements for the avoidance of biological invasions, e.g. Bern Convention (1979) and European Community council directive 92/43/EWG (1992), and risks derived from modern biotechnology, e.g. the Cartagena Protocol (2000) and the European Community council directive 90/220/EEC (2001). Australia and New Zealand belong to the countries with the most rigorous and consistent biosafety politics in the world covering all biosafety risk factors, from biological invasions to genetically modified organisms (Fisahn and Winter 1999). New Zealand's biosafety laws classify genetic modified and non-native organisms as 'new organisms' and treat them equally. In Germany, biosafety standards are also very restrictive, though in the practice only GMPs but not other biosafety risk factors have been subject to rigorous regulations (Doyle 2002; Fisahn and Winter 1999). The high rejection of GMPs and GM trees in Germany contrasts with the open attitude towards non-native plants, which in fact can be quite freely cultivated. Furthermore, several biosafety projects have been forbidden in public German institutes (Vogel 2005) and there are plans to ban use of GM trees in the German forests by a new forest law (BMVEL 2004).

## Concluding remarks

The complicated process of invasion ecology and the high number of invasive non-native organisms integrated into our ecosystems are certainly the most important reasons why these processes have been ignored in most countries, when compared with GMPs. Some invasive forest trees have been spread by the forest industry worldwide (Binggeli 1996). Tree plagues and diseases are spreading rapidly at the present time. The proliferation of international transportation and tourism are breaking down biogeographical boundaries. Every day, about two million people cross an international border (Bright 1999). People spread, voluntary or involuntary, weed seeds, fungal spores and many other organisms from one place to another. The effective collapse of world ecological barriers is a phenomenon, as far as we know, without precedent in the entire history of life (Bright 1999).

The introduction of non-native and traditional bred trees is changing the 'gene pool' of many important tree species, such as poplars. Geographical isolation had avoided hybridisation for thousands of years in many of these species. The example provided by the poplar hybrid *P. euramericana* shows that such 'gene pool' change can also have a strong influence on the ecological balance.

GMPs, the new biosafety risk group, are convulsing public opinion more than any other biosafety risk before. Genetic engineering of trees is a very new discipline and little has been done regarding their potential for environmental impact. Most plant studies on environmental releases have targeted crops but much of this information cannot be directly transposed to trees.

Important concerns regarding GM trees, like horizontal and vertical gene transfer, invasive potential, gene instability and impact on other organisms, are not an exclusive problem of this group of plants. Novel risks introduced by new technologies are presumed greater then established ones, even if the later are less well characterized. Perhaps

the most difficult hurdle facing the advance of genetic modification is not a technical but a psychological one. The public has witnessed the consequences of deficient risk assessments in the past, e.g. waste disposals, air pollution, mad cow disease and many others (EEA 2004). The experience has resulted in a lack of confidence in, and mistrust of political institutions, corporations, and scientists as sources of reliable information (Aldhouse 2000).

The application of genetic modification to forest trees could make an important contribution to tree breeding, to the conservation of native forests and endangered tree species, to the avoidance of biological invasions, and to the reduction of environmental pollution derived from pesticides and industry. On the other hand, the application of any new technology, not only genetic modification, should occur after a meticulous safety assessment. Biosafety aspects of trees need careful consideration because of the long generation time of trees, their important roles in ecosystem functioning and the potential for long-distance dispersal of pollen and seeds. Biosafety problems detected in the past, show clearly the importance of a prior case-by-case evaluation of non-native species, new taxa and also genetically modified trees according to the precautionary principle before their release for avoidance of risks to the environment and human health.

The importance of an independent and objective biosafety research has been pointed out (DeAngelis 2000; Myhr and Traavik 2002). Biosafety research from private companies and the increasing co-operation between transnational corporations and public research institutions has raised ethical questions related to the integrity of research and the objectivity of scientists (DeAngelis 2000). Restrictions applied to public sector biosafety research by some governments (Vogel 2005) are an additional burden for an independent biosafety research.

Scientific uncertainties and the relative lack of baseline information about forest ecosystems will pose challenges to the existing methods of analysing risks and benefits. The development of efficient biosafety standards may reduce impacts caused by human beings in the environment in the future, but it will not completely eliminate potential risks. Biosafety represents a profound and global potential challenge to our economic system, to our technical conservation skills, and to our ethics.

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