

Ecological and population genetics research imperatives for transgenic trees

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Abstract Applied research that supplies requisite, albeit incomplete, scientific knowledge is necessary if we are to address the legal, regulatory, and social/ethical issues regarding the use of transgenic trees. The technology for creating these trees has gotten far ahead of research on the ecological and population genetics impacts that may emerge. In this paper, we propose a comprehensive, interdisciplinary scientific approach that combines experimental results with model projections. We believe that much of this work must be completed before social issues can be clarified and resolved. Broad-based failure by those in the forestry-minded scientific community to carry out this interdisciplinary research could lead either to the establishment of transgenic trees with unintended consequences, or to an inability to realize the numerous advantages that this technology may offer.

Keywords Transgenic trees · Gene flow · Ecological effects

Introduction

The potential for biotechnology to substantially benefit humankind has been demonstrated clearly through its medical applications. Elsewhere, the use of transgenics for producing agronomic crops is now widespread, although not universally accepted. By 2005, 222 million acres in 21 countries had been planted to “biotech” crops (James 2005). This included 82% of all corn, 89% of all soybeans, and 81% of all upland cotton raised in the United States (NASS 2005). Nevertheless, the Swiss voted in 2005 to ban the planting of transgenic crops (Wright 2005).

Various uses for transgenic trees have been proposed (Burdon 1994; Burley 2001; Huang et al. 1993; Mathews and Campbell 2000; Raemdonck et al. 2001; Yanchuk 2001). For example, they might enable the conservation and restoration of species affected by exotic diseases and insects (e.g., American Chestnut, *Castanea americana* (Michaux) Raf.; Carraway and Merkle 1997; IFB 2002). Transgenic approaches also offer promise for the removal of toxic chemicals from soils (Bizily et al. 2000; Doty et al. 2003; Meagher 2000; Rockwood et al. 2004; Schnoor et al. 1995); new pest-management strategies based on improvements in the genetic resistance of trees and fewer applications of insecticides and fungicides (Punjar 2001; Strauss et al. 1991); and energy-efficient production of woody biomass to enhance supplies of bioenergy and biomaterials that help reduce fossil fuel consumption (Lucier et al. 2002).

Sedjo (2005a) has attempted to quantify the economics of particular applications of transgenic trees in commercial forestry. He has estimated that worldwide, the introduction of herbicide resistance could reduce wood production costs by approximately 1 billion USD per year, while the processing of lower-lignin trees at pulp mills could have an annual impact of 7.5 to 11.0 billion USD per year. Su

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et al. (2003) have suggested that if a Bt gene had been used to prevent a single outbreak of trunk insect attack in China, this action could have stemmed their losses by RMB 5 billion (about 500 million USD). Furthermore, Cabbage et al. (2006) have presented a median estimate for the elimination of fusiform rust in the southern US of 200 million USD per annum. Finally, the tree biotechnology company ArborGen, which is a leader in developing transgenic trees, is focusing on three main benefits: fast-growing loblolly pine (*Pinus taeda* L.) for the southern US, low-lignin eucalyptus for deployment in South America, and cold-hardy eucalyptus to be planted in the southernmost portions of the pine range in the US (Hinchee 2005). However, to date, actual deployment of transgenic stock has been limited to only a few million poplar trees with the Bt transgene in China (Sedjo 2005b).

Indications are that the public, at least in North America, may be favorably disposed to the adoption of transgenic trees, particularly when their application has obvious social benefits. For example, in a Canadian survey by Walker (2005), 78% of the respondents had a positive opinion regarding transgenic trees for enhancing carbon sequestration. Their support clearly was lower when those advantages would apparently accrue to forest product companies (58% favorable response for trees that could grow more quickly and be harvested earlier). However, those favorable reactions did not seem to be founded in a strong knowledge base, because 71% of participants said they were not familiar with genetically modified trees.

Certainly, there is strong worldwide opposition among some environmental groups to any deployment of transgenic trees—see, for example, the report from a press conference sponsored by the Global Justice Ecology Project (2004). While many meetings have been conducted and several books published regarding the particular ecological and population genetics effects of transgenic trees (e.g., Strauss and Bradshaw 2004; Williams 2006a), the bulk of the experimental studies appear to have been done on agronomic species (Snow et al. 2002). Certainly, forest scientists face a significant challenge compared with their agricultural colleagues. For example, trees have much longer generation intervals and crop cycles. Furthermore, the community of forestry researchers is much smaller than that of agrosience, making it more difficult to assemble multidisciplinary teams and garner the necessary project funding.

The purpose of our paper is to promote the necessity for more ecological research into the potential impacts of transgenic forest trees. We propose a specific program of scientific study that will enable society to perform risk/benefit analyses based on sound scientific data and model predictions. Our scope here is limited to scientific questions only and refrains from any discussion of policy issues such

as whether potential consequences are acceptable or not. Although we believe that scientific research will help indicate where within that wide range the actual impacts will fall, this is a social/policy decision that science will inform but not make. We begin with a brief review of the ecological influences of transgenic plants, with the emphasis being on gene flow and fitness. From there, the discussion moves to flowering control and ecological effects, particularly as they relate to information gaps and research needs.

Gene flow and fitness effects

Definition and general description of gene flow

In its simplest terms, *gene flow* is the transfer of genes between populations. Embedded within this basic definition is a host of details and complications that make it an interesting, challenging, and important subject. In this paper, we are concerned with “vertical gene flow” from transgenic crops to receptor populations of non-transgenic plants via sexual crossing. While “horizontal gene flow” (e.g., from plants to microbes) is certainly a valid area for study, we will not address it here due to space limitations.

Vertical gene flow comprises at least two components: (1) dispersal of pollen, seed, and vegetative propagules leading to gene migration; and (2) the fitness or selection differential of transgenic “immigrants” relative to non-transgenic members of the receptor population. We will discuss how the long-term effects of gene flow depend on interactions with other fundamental genetic processes (e.g., mutation rate, natural selection, and genetic drift).

Gene flow, which occurs naturally whenever wild populations cross sexually with each other, is also common between crop plants and their wild relatives. With regard to transgenic trees, much of the popular literature has focused on potential transgene flow from transgenic plantations to “wild” or “natural” stands. However, it is more likely to occur between transgenic and non-transgenic tree crops (in both directions), possibly having important consequences (Ellstrand 2002; Snow 2002). For example, the following two practical cases illustrate potential and significant legal and commercial consequences. First, a legal concern can arise if one landowner plants transgenic trees and then the seeds derived from those trees germinate into seedlings in another landowner’s plantations. In this case, the ownership of, and the right to use, the transgene becomes an issue, along with the cost to find and eliminate the transgenic trees if the owner of the land where they become established does not want them. A second commercial concern arises if the two landowners have differing management objectives. An owner whose intended product

is strong dimensional lumber could suffer financial losses if transgenic trees engineered for easy pulping resulted in weaker lumber.

Migration rate and dispersal

Migration rate is defined as the fraction of the receptor population replaced per generation by immigrants containing the transgene; denoted by “*m*” (Ellstrand 2003). Note, this is not the same as the fraction based on the total number of immigrants from the transgenic stand because not all of them will contain the transgene.¹

Multiple steps and mechanisms are involved in the processes of migration. The first to be considered here is *dispersal*, or the movement of genes, although this does not include their establishment in other populations. Certainly, pollen movement can be a significant mechanism, especially in wind-pollinated species such as pine. Schuster and Mitton (2000) have detected gene flow resulting from pollen being dispersed over 2 km, and have speculated it might even occur over hundreds of kilometers. Nevertheless, the mean pollen movement distances for pines are more likely to be 50 to 150 m (Slavov et al. 2004). Dispersal can also happen when seeds are transported by vectors such as wind, animals, and water. Williams (2006b) believes that 99% of the pollen and seeds is dispersed through “local neighborhood diffusion” and the other 1% via long-distance dispersal, although species variation can be significant around those general numbers.

The physical movement of pollen or seeds does not, in itself, constitute gene flow. Pollen can degrade quickly, and seeds may not germinate. Gene flow can be said to occur only if viable pollen reaches a receptive flower, fertilization is successful, a seed develops to maturity and subsequently germinates, and the resulting seedling survives. To achieve this, numerous conditions must be met, including the phenological synchrony of flowering, the availability of a suitable seedbed, and ample water and light. Obviously, dispersal is a highly stochastic process, and stochastic models of gene flow can show very different results from deterministic ones (Thompson et al. 2003).

¹ Transgenes are generally hemizygous and, therefore, usually segregate at a 1:1 ratio when outcrossing with non-transgenic trees. Therefore, migration rates of a particular transgene are ~50% of the immigration rate for all trees with a parent in the transgenic stand (Ellstrand 2003). However, some researchers, e.g., Snow et al. (1999), have reported that not all transgenic lines segregate at that 1:1 ratio. This could be the result of multiple inserts. Thus situation-specific studies may be needed.

Models of dispersal and migration

Both *dispersal* and *migration rate* have been reasonably well described mathematically; we will highlight two models to illustrate the diversity of approaches and capabilities. Nathan et al. (2001, 2002a,b) have developed highly mechanistic models after examining the physics of the components for movement (including horizontal and vertical wind speed, terminal velocity of the seed). Importantly, their models consider the effects of turbulent transport, which can lead to long-distance dispersal. These models are spatially explicit and stochastic, and provide a probabilistic determination of seed distribution. Sensitivity analysis with these models has shown that fluctuations in horizontal and vertical wind velocities account for 86% of the variation in dispersal (Nathan et al. 2001).

DiFazio (2002) has constructed a landscape-scale model that simulates both local and long-distance dispersals. It is based on a geographic information system (GIS) description of a large area (tens to hundreds of kilometers in dimension), with cells containing geographic, habitat, and tree information. This model, incorporating an annual time step, can consider the consequences of location for transgenic plantations and can be used to simulate time periods that are as long as necessary for the situation being modeled. Such capability is certain to be required for risk analyses of transgenic plantations in landscapes with both commercial and non-commercial ownership. This model and its use are also discussed by Slavov et al. (2004).

Fitness and selection differential

Gene flow and its effects are determined not only by the rate of transgene migration into non-transgenic stands but also by the fitness of the progeny containing the transgene relative to non-transgenic individuals in the receptor population. *Fitness* is defined as “the contribution of genes that it (an individual) makes to the next generation, or the number of its progeny represented in the next generation,” and “*relative fitness* is the fitness of an individual relative to the population mean.” (Falconer and Mackay 1996) A closely related term, *selection differential*, “*s*”, is the weighted-average, relative fitness of a population.

Crosses of transgenic and non-transgenic plants may give rise to transgenic progeny with higher, lower, or the same fitness as the non-transgenic plants with which they interact. Enhanced fitness might be manifested in better germination rates, a greater ability to compete, or improved tolerance toward biotic (e.g., pests, disease) or environmental (e.g., temperature, light, water) stresses. Similarly, negative fitness can result from the metabolic cost of a transgene’s functioning if there is no offsetting ecological advantage.

Whereas migration rate receives much attention in the literature, fitness is at least equally important. Burke and Reiseberg (2003) have stated, “Although transgenes will often escape from cultivation, their rate of spread will be mainly governed by their fitness effects not the migration rate.” (p. 1250). Likewise, Hails and Morley (2005) have asserted that “research effort should now focus on estimating any changes in the fitness of a population as a consequence of having a transgene.” (p. 245)

Because fitness is defined by the contribution of genes an individual makes to the next generation, it is an extraordinarily difficult concept to study experimentally in forestry, given the distances and time frames involved. Therefore, many researchers, even in agriculture, simplify the problem by examining the components of fitness, such as fecundity, seed germination rate, seedling survival, and seedling growth rate. Even so, the challenge of designing rigorous studies of fitness components is formidable. One must compare among the performances of plant populations identical in their genetic make-up except for the transgene. Further complicating factors include the need for multiple genotypes (to gain a wider inference space) and replicate transformation events within genotypes (to avoid the confounding of transgene effects with construct insertion-point effects). Such investigations require multiple environments and a range of stress factors, with and without pressure from the agent that the transgene is designed to mitigate. Finally, the metabolic activity of the transgene may induce a negative fitness effect. Possible approaches for determining fitness in forests are discussed later in this paper.

Many short-term studies have been conducted with transgenic agronomic crops. For example, Snow et al. (1999) evaluated the fitness effects of a transgene for resistance to the herbicide glufosinate in rapeseed (*Brassica*). In the absence of glufosinate, survival and number of seeds per plant did not differ between transgenic and non-transgenic plants. Working with data from creeping bentgrass (*Agrostis stolonifera* L.), Meagher et al. (2003) concluded that in the absence of the herbicide, the ecological consequences would probably be minimal.

Burke and Rieseberg (2003) studied fitness in sunflower (*Helianthus annuus* L.) transformed for disease resistance and found no difference in seed output between transgenic and non-transgenic lines in the absence of a pathogen challenge, thereby indicating no inherent fitness cost of the transgene. For field trials where the disease was present, the transgenic plants showed a lower frequency of infection after inoculation at two of the three sites; however, because the disease effect varied across locations, the transgene had no overall significant influence on seed output. This indicated no general increase in fitness for that transgene. These results illustrate two important points. First, the effect of a transgenic phenotype, compared with a non-

transgenic plant of the same genetic background, ordinarily can be assumed to be location-dependent. Second, fitness must be understood in terms of the whole biological cycle of the plant. That is, at the sunflower trial site where the transgene provided the most disease protection, the pathogen had no effect on seed output, whereas, at another site, where disease caused a decline in seed production, the transgene had no impact on disease incidence.

Similar results have been reported by Stewart et al. (1997), who compared a non-transgenic rapeseed (*Brassica napus*) line with one containing a *Bacillus thuringiensis* (*Bt*) toxin gene. In the presence of natural vegetation, used to simulate escape from cultivation, virtually all plants from both lines died, indicating a neutral fitness effect. By contrast, in cultivated plots where insect pressure was applied, the transgenic line had increased seed production, which demonstrated that the transgene could be fitness-positive. This result illustrates that fitness is situation-dependent and that crop-to-crop transgene flow may be as deserving of attention as crop-to-wild gene flow.

In another example, Snow et al. (2003) studied the fitness effects of a *Bt* transgene in sunflowers at two field sites and in a greenhouse. At one of the sites, the transgenic plants produced 55% more seeds than the corresponding non-transgenic control. At the other site, the effect was plus 14%, but non-significant. In the greenhouse, no transgenic effect on fecundity was identified, again indicating no fitness cost of the transgene itself (i.e., no metabolic drag due to transgene activity).

Although fitness studies are difficult to conduct in any crop, they are likely to be especially challenging in trees because of their generation interval, large size, and long juvenile periods. Nevertheless, we believe the investigation of fitness is essential to developing a sound scientific understanding of the ecological influences of transgenic trees. Fitness also plays an equally important, and perhaps even more complicated, role when considering the evolutionary effects of transgenes.

Population effects of gene flow

The outcomes influenced by population genetics, which result from the interaction of gene flow with genetic drift or natural selection, are theoretically determined by the relative magnitude of the migration rate, m , and the selection differential, s (Ellstrand 2003). Slatkin (1987) provides an excellent summary of these interactions and gives some useful generalizations regarding these effects. For simplicity, we will follow the theory of Ellstrand (2003), in which, “If the migration rate, m , is approximately equal to the mutation rate, then they will have roughly the same evolutionary significance”. Here, we will consider three cases: transgenes that impart neutral, negative, or positive

fitness. Again, we must emphasize that fitness is not an absolute characteristic, but can vary with location and environment.

Neutral-fitness gene flow As a rule of thumb, “the arrival of about one immigrant every other generation, or one inter-population mating per generation, should be sufficient” to overcome the effects of genetic drift on fitness-neutral genes (Ellstrand 2003). This amount of migration should allow a transgene to become established in a local population that is receiving gene flow. Ellstrand states that “interestingly, this rate of gene flow is independent of the size of the receiving population” because both the migration rate and the force of genetic drift are inversely proportional to the population size (Slatkin 1987). Greater rates of gene flow, which are not equal in direction between populations, could have quite significant effects.

For example, consider a small, protected natural area or riparian zone that is proximate to several transgenic plantations where the same transgene is used for multiple years after plantation establishment. The frequency of that fitness-neutral transgene would be expected to increase in the wild stand. In the extreme, the non-transgenic population could face “extinction” by genetic swamping. This does not mean the wild trees will die, but that the pure wild type could cease to exist. Snow et al. (1999) also has demonstrated the persistent impact of neutral transgene flow in a greenhouse study.

Negative-fitness gene flow With a negative-fitness transgene, gene flow interacts with natural selection. If the migration rate of a negative-fitness transgene is high enough, it can counter the effect of natural selection against that gene. In particular, if the migration rate, m , is greater than the selection differential, s , of the immigrant allele (Ellstrand 2003, p. 38) then the detrimental allele will be maintained in the population. This then causes the mean fitness of the population to decrease. Lenormand (2002) has modeled this process for local populations in different environments.

Positive-fitness gene flow Snow et al. (2003) demonstrated a positive fitness effect for sunflower in some locations, as did Stewart et al. (1997), who evaluated a transgenic line of rapeseed containing a *Bt* transgene. Similarly, we might speculate that transgenes for stress tolerance (drought or cold) would have positive fitness effects. In those cases, gene flow and natural selection would become positively reinforcing and accelerate the increase in frequency of the transgene. The same general logic applies to migration and the local selection differential, as it does with negative fitness. Therefore, if the gene has a positive selection differential equal to the rate of migration, then the rate of

change per generation is twice that of natural selection alone (Ellstrand 2003). The flow of beneficial transgenes from commercial plantations to wild stands can hasten local “extinction” by assimilation. Again, this does not mean that the receiving population dies, but that the wild genotype in the receiving population ceases to exist in its pure form. Under certain circumstances, we could speculate that this scenario would lead to an increase in weediness. In agronomic crops, buffers and refugia have been proposed as a method to mitigate these effects (EPA 2002).

Ecological considerations and research needs for transgenic trees

Now that we have covered the basic theory and components of gene flow, we will discuss special ecological considerations relevant to transgenic trees. Furthermore, we will propose a course of research that will enable the forestry research and technology communities to begin building an understanding of the effects of transgenic deployments. Relying on the scientific foundation established in the agronomic literature, we must emphasize that the fundamental biological factors separating forestry from agriculture are: (1) all tree crops are perennial, and (2) the very long periods over which these effects will occur. Here, we focus primarily on loblolly pine because it is the most important commercial tree species in the United States for which transgenic products are being developed.

Issues related to the effect of transgene flow on population genetics

Taking into account fitness and migration rates, it seems plausible that transgenic gene flow could affect tree species at the level of population genetics, especially in smaller and somewhat isolated populations. However, to get a complete picture, we must appreciate the relative time scales for deploying transgenics and the nature of the landscapes in which they will be deployed. This is best done in the context of the basic genetic processes of gene flow, genetic drift, and natural selection (Slatkin 1987; Stockwell et al. 2003).

First, consider the time scale of use for a particular transgene construct. The perennial nature of forest crops means that once they start flowering and before they are harvested, transgenic stands will probably disperse seeds and pollen every year. Suppose that plantation-grown loblolly pine trees begin producing both pollen and seed at age 16 (Williams and Davis 2005) and are harvested at age 25. Further, suppose we utilize a single transgene during 10 years of plantation establishment before replacing

it with another construct. That means that seed and pollen may be produced continuously from year 16 (first year of flowering for the first crop) until year 35 (harvest age of last crop). This amounts to 20 years of potential gene flow, *for a given transgenic construct*, from transgenic stands used in commercial applications. Likewise, if the product intended by the forest owner calls for 35-year rotations, then gene flow occurs over 30 years. While these are certainly significant time scales when considering ecological change, they are quite short in the context of traditional study of evolutionary change.

Considering the geographic structure of natural populations, Slatkin (1987) discusses the time scales necessary “to cause a substantial change in gene frequency” for both genetic drift and natural selection. Significantly, “the importance of gene flow relative to these other genetic forces is determined by the amount of gene flow *averaged over the time scale of change due to the other force*” [italics ours]. Slatkin states, “Roughly speaking, the time scale associated with natural selection is the inverse of the difference in relative fitness.” This is relevant to the flow of transgenes with negative fitness. Using the example of a fitness difference of 1%, its inverse corresponds to 100 generations. Thus, if “an average over 100 generations of roughly 1% of a population (were) replaced by immigrants”, then a significant change in frequency would likely occur.

Even though transgenes will be deployed in landscapes of very different geographic structure from natural forests, this analysis has at least two important implications. First, such a small amount of gene flow and its effects would be very difficult to detect experimentally, especially given the stochastic nature of gene flow from trees. “[This] average could be achieved by only three or four episodes of significant gene flow.” (Slatkin 1987). Scenarios of highly variable gene flow are not hard to construct, given that loblolly seed fall generally occurs during the hurricane season in the southeastern US, and that turbulent transfer is considered the primary mechanism for long-distance dispersal (Nathan et al. 2002b). Even if a construct is used for 20 years, and even if it does not persist past the next 25-year generation, the experimental period is still 45 years.

Second, the period of use of a transgene construct is approximately two orders of magnitude less than the time for a significant change when natural evolutionary time scales are considered. Thus, the focus of the impact of transgene flow should be on the transient dynamics of changes in population genetics, rather than on the long-term equilibrium effects. In other words, studies should consider changes in population genetics due to gene flow on an ecological time scale.

However, the geographic landscapes into which transgenic trees are planted will be very dissimilar from land-

scapes that are natural. In general, they will have increased fragmentation (Bacles et al. 2006), a shortened generation interval, contain domesticated populations intermixed with natural ones, and comprise areas that are quite intensively managed (Stockwell et al. 2003). These authors assert that “unprecedented rates of anthropogenic perturbations are expected to cause particularly strong selection” that could lead to observable changes, which they call “contemporary evolution,” within a few hundred years.

Thus, to understand transgenic gene flow, new models will be needed. These models will have to explicitly consider the potential interconnectedness of populations among which gene flow can occur. Sork et al. (1998) discuss three paradigms: (1) infinite island models, where gene flow can occur among all discrete, compatible populations; (2) metapopulation models, where gene flow occurs between only some populations; and (3) landscape ecology models that use spatially explicit information about the mosaic of the landscape. Manel et al. (2003) stress the importance of understanding the effects of genetic discontinuities and barriers to gene flow. Other anthropogenic factors, e.g. global warming, will likely make studies of the effects of transgene flow more complex. Bacles et al. (2006) indicate that there will be less gene flow from smaller transgenic populations in these fragmented landscapes, but say there is evidence that pollen flight distances may be greater.

Smaller natural populations, especially “conservation units” that may be necessary for evolutionary or ecological reasons, deserve special attention with respect to the effects of transgene flow (Manel et al. 2003). Stockwell et al. (2003) state that small populations “may have limited potential for adaptive evolution.” Slatkin (1987) indicates that “the time scale associated with genetic drift is approximately [*proportional to*] the number of individuals in a population.” Thus, for a smaller receiving population, the time scale over which we must average gene flow is shorter than for a larger population. Therefore, a small population could be impacted by a single incidence of high migration caused by some stochastic effect. This suggests that small, isolated biotopes or riparian areas that contain species with which outcrossing is possible, and which are near transgenic stands, might be fruitful places for research if we want to observe the effects of transgene flow.

Finally, we note the possibility of important interactions between sterility and other measures that might reduce gene flow with these genetic processes. To the extent that flowering control diminishes gene flow, ecological effects and those due to population genetics will be mitigated. If flowering control does drastically reduce gene flow, then the time frames for any changes in population genetics will be lengthened.

Understanding gene flow in trees—use of models

As stated above, considerable time is needed to observe and understand gene flow, the processes of population genetics, and their effects on ecology and evolution. Even if we wanted only to study ecological changes over a period of two rotations, we would still require a trial to run 50 years. Experiments lasting that long are very rare indeed, and surely society would want to have an answer regarding risks and benefits much sooner. Given this, the foregone conclusion is that we will have to use, and link together, simulation models of the various components of gene flow. Fortunately, this is just the direction that many forest-sector scientists are already taking (DiFazio 2002).

At the risk of over-simplifying, we envision three kinds of models that generally correspond to the processes comprising gene flow. In order of increasing time scale, they are: dispersal models that consider movement of pollen, seed, and vegetative propagules; ecological models that incorporate succession-related phenotypic changes in weediness and competitive ability that result from fitness differences introduced by the transgenes; and population genetics models to account for transgene fate once introgression has begun.

Dispersal models were reviewed earlier in this paper. Models to predict the ecological effects of phenotypic differences that arise from transgene spread will be necessary to predict short-term consequences, such as weediness, and mid-term effects on succession patterns. Consider, for example, the potential outcome of inserting a transgenic construct that increases growth by 40% into a superior clone that already grows 40% faster than trees from wild seed. The resulting phenotype may have double the growth of wild phenotypes. Therefore, we need ecological models to estimate whether this phenotype will alter succession patterns such as those witnessed in an open field, or will change the outcome of competition in mixed pine–hardwood stands. Just as with dispersal, these ecological models must consider spatially explicit landscapes (for a review, see Dunning et al. 1995; Turner et al. 2001). They will also have to be incorporated into harvest scheduling models, e.g., Habplan (van Deusen 1999, 2001), if we are to understand the ecological effects on and near large industrial forestland holdings.

The same basic equations and principles apply to population genetics for both trees and other crops (e.g., see Haygood et al. 2003; Roughgarden 1996), although these will have to be applied to spatially explicit landscapes to be valuable in understanding the impacts of transgene flows. These fundamentals have already been applied to investigate various aspects of gene flow in general and transgenic effects in particular. For example, Williams and Davis

(2005) considered long-distance dispersal and unintended fitness effects resulting from pleiotropy. Haygood et al. (2003) assessed the conditions for demographic swamping and genetic assimilation, whereas Linacre and Ades (2004) investigated the buffer sizes required for containment of transgenic eucalyptus.

Lenormand (2002) has presented several models that evaluate gene-flow effects in varying local habitats. In comparing deterministic and stochastic models for developing a risk analysis, Thompson et al. (2003) found that the “equilibrium...where hybrids completely dominate the patch, cannot be reached deterministically.... In the stochastic version of the model however...hybrids may dominate the patch after some sequence of environmental (parameter) variation.” (p. 204). It is argued that understanding the importance of infrequent events is “very difficult to achieve by subjective interrogation of empirical data” (p. 207), and that “events that are unlikely over periods of a few years become almost certain within scales of a few decades.” (p. 199). The implication is that short-term empirical studies, which may miss these events, could reach the wrong conclusions. Finally, Bullock (1999) has used a matrix modeling approach in which the entries represent demographic transitions over all life-cycle stages and, based on elasticity analysis, has identified those stages most likely to affect fitness.

These linked models must be capable of describing effects in large, complex, and heterogeneous landscapes, where commercial forestry is practiced, and in which the impact of deploying transgenic trees must be understood. In fact, commercial forest owners manage forested landscapes that comprise intensively managed plantations, areas managed less intensively for timber, and areas of high conservation value where timber production is a secondary objective or prohibited (Lucier 1994). The influence of transgenic plantations will be determined by how they are placed on this landscape as well as by how the whole “shifting mosaic” is controlled to mitigate potential deleterious effects through landscape management.

We believe that the long time frames over which population genetics and ecological responses to transgenic deployment occur require risk analyses that utilize these linked models. This in turn determines the nature of the experimental work necessary for understanding the impacts of transgenic trees. Such research should focus on estimating the parameters that drive these models and evaluating the predictions made. Furthermore, we agree with Thompson et al. (2003), who conclude that results from short-term experiments are very difficult to interpret without the benefit of these models and that, given the time frames involved, virtually all experiments will be short-term relative to the underlying biology.

Ecological context

With regard to our loblolly pine example, any ecological effects must be understood in the context of its normal ecological behavior as a pioneer species that most often becomes established on disturbed sites, such as roadsides or recently cleared land. Although commercial plantations comprise only a relatively small fraction of its natural range, this pine is the most commercially important tree in the US. As a crop in industrial plantations, it is usually managed quite intensively, using genetically improved (by classical breeding) planting stock, site preparation, pesticides, and fertilizer (Yin and Sedjo 2001).

In that ecological context, and analogous with the results of Burke and Rieseberg (2003), we can construct a hypothetical situation in which fitness is location-dependent in loblolly pine. For example, if pollen containing the transgene successfully fertilizes a wild tree in an older, mixed pine–hardwood stand with a dense overstory, and if the seed falls within that stand, it might not germinate, and, even so, would likely not survive to reproduce because of an inadequate seedbed and low-light conditions. By contrast, if a seed is formed when pollen containing the transgene fertilizes a flower in a genetically improved but non-transgenic stand, and if that seed falls on a site freshly prepared for a non-transgenic loblolly pine plantation, it has a much better chance of germination and survival. Such ingrowth of “volunteers” could be problematic in many Southern pine plantations.

The time scale over which gene flow effects are experienced is important because this phenomenon interacts with other natural genetic processes, especially genetic drift and natural selection. The outcome, then, depends on the rate of migration over the time scales of those processes. In addition, the longer the period over which gene flow takes place, the more time before its effects may become evident, and the harder it is to study. One short-term outcome, i.e., the ingrowth of transgenic seedlings into non-transgenic tree crops, should soon be observable. However, enhanced weediness along roads or in any disturbed field might not become apparent for a few years, and some species may take quite a while before becoming invasive (Hoenicka and Fladung 2006).

Rotation-length effects may result from transgene gene flow into wild stands (unmanaged, or managed for a variety of objectives). Williams and Davis (2005) apply the rule of thumb that loblolly starts flowering at about 16 years. Depending on the intended product, harvesting could be done from age 20 (primarily for fiber) to age 35 (for solid-wood materials). Successional effects also may include altered patterns of competition with hardwoods in mixed-species stands. Such impacts could result when transgenes that protect against insect or disease attack, or those that

dramatically increase growth, are inserted into clones that already develop much faster than natural, undomesticated trees. Evolutionary effects occur over much longer time scales, measured in generations. Slatkin (1987) suggests that gene flow can have an impact over 100 generations, or several millennia, for loblolly pine.

Another important aspect of the ecological context is the potential for mating with wild relatives. Unlike most transgenic agronomic crops that have been deregulated, transgenic loblolly pine in its natural range would have several wild relatives with which it could cross. In addition to frequent intra-specific crosses, there would be opportunity for hybridization with slash pine (*Pinus elliottii* Engelm.) or shortleaf pine (*P. echinata* Mill.). One must be cautious, however, when making generalizations about seed and pollen formation in plantations. Although their production levels are likely to be low in stands grown for fiber on rotations of 20 years or less, not all stands are grown this way. Stands that are managed for solid-wood products generally have fewer trees per acre, larger crowns, and longer rotations (i.e., >30 years); seed production in these stands can be prolific, especially from border trees.

Flowering control

As a means of transgene confinement, researchers worldwide are attempting to genetically engineer trees that do not produce functional flowers (Lemmetynen and Sopanen 2004). Different strategies are being tested, including cell ablation, which involves floral-specific expression of a cytotoxin gene; RNA interference (RNAi) to down-regulate the expression of an endogene necessary for normal floral development; and dominant negative mutations (DNMs) that lead to the production of dysfunctional versions of gene products, e.g., transcription factors, necessary for the expression of genes required for proper floral development (Meilan et al. 2004). Because the process by which trees are being engineered for reproductive sterility is discussed elsewhere in this volume (Brunner et al. 2007), we will not duplicate that information in this paper.

In addition to confinement, there are other reasons for engineering sterility. The first is to maintain juvenile rates of vegetative growth. Although the tradeoffs between reproduction and growth are debatable (Obeso 2002), indirect evidence indicates that vegetative growth slows in trees that have undergone maturation (Eis et al. 1965; Teich 1975), presumably because their photosynthate is diverted to the development of reproductive structures (e.g., cones, pollen, seed).

Flowering control may also have utility in regulating gene flow from otherwise non-transgenic trees so that concerns can be alleviated about the effects of monocultures on genetic diversity. For example, in poplar

plantations, large stands are often planted to a single genotype (clone). When trees in these plantations flower, they release pollen containing alleles from only two parents. If their resulting pollen cloud fertilizes trees in wild stands, the offspring will be considerably less diverse, genetically, than those produced by random, open matings in the wild. Although clonal forestry is currently not practiced widely, it is likely to be more common in the future and, perhaps, become an important comparator by the time transgenic trees are ready for commercial deployment.

Engineering trees to remain sterile throughout their lives is technically difficult because of their long life spans and the large number of meristems that may potentially become reproductive. Although there is evidence that transgene expression in trees is generally stable (Meilan et al. 2004) and reversion events are expected to be rare, the precedent exists for transgene instability that could lead to gene flow from trees designed to be sterile. Given that absolute sterility may not be a realistic, or necessary, goal, a more traditional method for reducing fertility, such as the use of aneuploids (Bradshaw and Stettler 1993), may provide a comparable level of safety. This approach is supported by predictions made using a gene-flow model (STEVE; DiFazio 2002) that relies on probability density functions to estimate propagule movement and reproductive success. Sensitivity analysis conducted using this model has revealed that complete sterility is not needed to achieve significant gene containment.

A significant ecological concern about flowering control is that it could deny forest-dwelling animals important sources of sustenance (pollen, flowers, and seeds). Continuing with our loblolly pine example, we might consider the possible effects of complete sterility on the brown-headed nuthatch (*Sitta pusilla* Latham), whose diet is 56% pine seeds (Martin et al. 1951). According to the Cornell Lab of Ornithology, this nuthatch “is restricted to the pine forests of the southeastern states” and is “decreasing throughout [its] range because of habitat degradation.” (http://www.birds.cornell.edu/AllAboutBirds/BirdGuide/Brown-headed_Nuthatch.html#conservation, accessed 28 March 2006). The current conservation status of this bird is somewhat ambiguous. It is listed as “threatened and declining” on the Partners in Flight Watch List (http://www.abcbirds.org/pif/pif_watch, accessed May 11, 2006). Audubon classifies it as a “yellow list species” in the continental U.S. and Alaska (<http://audubon2.org/webapp/watchlist/AudubonWatchList2002.pdf>, accessed 11 May 2006). By contrast, The Nature Conservancy classifies it as a G5 species, indicating that it is “common; demonstrably widespread, abundant, and secure” (<http://biology.usgs.gov/s+t/noframe/u175.htm>, accessed 11 May 2006). Nevertheless, wide-ranging deployment of completely sterile, transgenic loblolly pine stands could substantially

reduce suitable habitat and raise concerns about the viability of local bird populations. Therefore, research is needed on landscape management schemes that maintain habitat for species like the nuthatch in advance of large-scale establishment of transgenic loblolly pine with flowering control to modulate gene flow.

Male-only flowering control: a special case

It is conceivable that plants can be engineered to prevent the development of viable pollen and/or seed without significantly compromising the nutritional value of these reproductive structures. Similarly, it should be possible to develop trees that will inducibly and transiently flower, so that introduced traits can be introgressed into other genetic backgrounds. However, the ecological viability of trees with these constructs will need to be demonstrated.

Depending on the level of containment required, male sterility may provide an acceptable degree of transgene control that reduces the ecological concerns about food-chain effects while still allowing female flowering and seed production. Although male-sterile genotypes do occur spontaneously, albeit rarely in populations of wild trees, the responsible mechanisms are still under investigation (Hosoo et al. 2005; Wilson and Owens 2003).

Several transgenic approaches to engineering gender-specific sterility are currently being tested (reviewed by Lemmetyinen and Sapanen 2004), but definitive results have not yet been reported, primarily because of the long juvenile periods in trees, the dearth of early flowering genotypes, and the lack of an effective means for reliably inducing precocious flowering. Although it is possible to promote early flowering in some eucalypts and pines, these species are notoriously recalcitrant with respect to transformation and regeneration.

While researchers may be able to use populations of non-transgenic trees (e.g., pine) to explore some aspects of gene flow, certain questions may be answered only through long-term field trials with transgenic trees. These could include, for example, evaluating the durability of various sterility systems. In that case, it is logical to use *Populus*, for at least two reasons. First, poplars are adapted for growth over a wide geographical range. Second, poplar can be transformed and regenerated much more easily than other tree species, especially pine. Some proponents of recombinant DNA technology have suggested that male sterility may provide adequate levels of containment, especially in dioecious species. If that concept were endorsed by regulators and the general public, then full sterility would also likely be accepted, though that may be subject to an examination of other ecological effects such as the disruption of food chains that begin with seeds.

Occasionally, individuals within a normally dioecious species produce hermaphroditic flowers. This is certainly true of the genus *Populus* (Stanton, Greenwood Resources, personal communication.). There is also limited evidence that seeds derived from hermaphroditic poplar catkins are the result of selfing (DiFazio, unpublished data). Therefore, even if male sterility remains intact, seed production is still possible when there is a source of donor pollen, which is very likely from non-transgenic trees growing adjacent to commercial plantations. Seeds from fertilized trees can then be carried long distances by animal vectors, wind, and moving water.

Controlling vegetative spread

Vegetative reproduction by transgenic trees also contributes to the risk of transgene spread (Hoenicka and Fladung 2006). For example, some species within the genus *Populus* are propagated via broken branches and twigs, circumventing the sexual process (Rood et al. 2003). Viable propagules can be transported considerable distances in streams.

The primary concerns are suckers, which can proliferate on the lateral roots of certain species, as well as the formation of adventitious roots on branches shed from some of those same species. Given this, it may become necessary to develop methods for controlling vegetative reproduction. To reduce the number of root suckers, scions from commercially important genotypes could be grafted onto rootstocks that are much less prone to producing suckers. With regard to rooting, considerable progress has been made in our understanding of the genes regulating the development of adventitious and lateral roots in both angiosperms and gymnosperms (e.g., Casimiro et al. 2003; Ermel et al. 2000; Fu and Harberd 2003; Goldfarb et al. 2003; Lindroth et al. 2001; Xie et al. 2002). This information suggests several promising approaches for engineering solutions to vegetative spread, but their efficacy will have to be verified empirically.

Ecological effects of transgenic trees not related to gene flow—direct effects of phenotypes

Many potential effects of transgenic trees can arise directly from the particular phenotype and, thus, are unrelated to gene flow. In general, the concern is that a change in some property of the transgenic trees will disrupt an ecological process and lead to deleterious consequences.

Many examples are discussed in the literature. Insects may evolve to become tolerant of a *Bt* gene product, thereby becoming refractory pests. In contrast, effective control of insects harmful to the tree crop may disrupt a food chain, causing negative secondary effects for beneficial insects and for other animals that feed on insects. As

mentioned above, food chains that are dependent on seed or pollen may be severely disrupted by successful flowering control. Likewise, deployment of crop plants engineered for herbicide tolerance may promote natural selection for herbicide tolerance in weed species and eventually make them harder to control. Altered chemical properties, e.g., lower lignin content or changes in lignin structure that make pulping easier, also may affect soil decomposers and, in the long term, soil properties.

Many of these effects can be mitigated in a variety of ways. Refugia are required in agricultural crops transformed with the *Bt* gene. It also may be possible to engineer flowers that are sterile but still produce pollen and seeds with unchanged nutritional values. Impacts on the food chain may be alleviated through landscape management of the number, size, and location of transgenic stands relative to potentially affected resources. One excellent example of a sound scientific approach for studying the consequences of transgene use in modifying the nature and content of lignin is Halpin in this volume.

Recommended scientific program of study regarding the ecological and population genetics effects of transgenic trees

Our recommended scientific approach encompasses six components:

1. Understand the efficacy and stability of transgene expression for sterility;
2. Determine the fitness effects of various transgenes for critical aspects of forest-tree life cycles;
3. Develop and link together predictive models for:
 - a. Seed and pollen dispersal;
 - b. Ecological (e.g., successional, including weediness) effects of transgenic phenotypes;
 - c. Impacts of gene flow on genetic populations in ecological time frames within spatially explicit landscapes;
4. Conduct field experiments to estimate the parameter values for these models;
5. Directly test model predictions;
6. Investigate the direct effects of transgenic phenotypes on selected ecological processes.

Comments on some of these steps are presented below.

Efficacy and stability of transgene expression

The strategies being used to genetically engineer trees that will not produce functional flowers rely on the stable expression of a transgene. For example, one approach to flowering control depends on the expression of a mutated,

non-functional form (i.e., a DNM) of a gene product necessary for proper floral development (e.g., a transcription factor). However, based on a report by Lolle et al. (2005), these mutations may not be maintained in subsequent generations because of a postulated extra-chromosomal mechanism that repairs DNA in a template-directed manner. Cell ablation and RNAi approaches also may be problematic. For example, mutations in a cytotoxin gene, or changes in its promoter (methylation), may lead to decreased expression in vegetative tissues (Lemmetynen and Sopanen 2004).

Furthermore, an introduced gene can be silenced when the host plant is either exposed to elevated temperatures (Broer et al. 2003; Köhne et al. 1998) or has undergone dormancy (Scorza et al. 2001). Similarly, Meilan (unpublished data) has observed a case in which a transgenic tree exhibited what was thought to be somaclonal variation only after the plant had undergone dormancy in the field. Given the foregoing, more than one environmental cue might be able to trigger transgene silencing.

Although these stability studies can be conducted in isolation (i.e., where no inter-fertile wild relatives exist within the range that pollen could move), federal regulators may be reluctant to approve long-term field tests involving trees that have been engineered for flowering control because of the risk of transgene escape. Moreover, trees have long juvenile periods, such that a seeming absence of flowering is not necessarily an indication that the transgene is truly being expressed. Thus, it might be best to conduct expression stability trials only with transgenes that affect traits other than flowering. Nevertheless, if that latter character is being evaluated, it would likely need to be done after the trees had undergone maturation. Regardless of what trait is being assessed, trees must be monitored for transgene expression and phenotypic stability under field conditions, in a variety of environments, using several transgenic lines (independent events), and over many years (i.e., after several dormancy cycles). In addition to baseline and year-to-year measurements, the potential for seasonal changes in expression also should be examined. At this point, specific recommendations concerning the number of years and locations cannot be made, but determining what is minimally sufficient is an important scientific question.

Fitness effects of transgenes

Obtaining estimates of fitness, or the selection differential, in trees will be particularly difficult because of their long generation intervals. Much creativity will be required to design and conduct these fitness experiments. However, we agree with Hails and Morley (2005), who indicate that “research effort should now focus on estimating any changes in the fitness of a population as a consequence of

having a transgene.” (p. 245). These studies must account for the effects of different environments (locations or weather/climate) and the pressures that various stressors (e.g., herbivores, abiotic factors such as cold or drought) exert on the trees. Such effects are discussed in those papers on experimental determination of fitness that we have reviewed above.

To study fitness, we should take advantage of the previous commercial movement of provenances or the introduction of exotics capable of interbreeding with native species. For example, in the early 1980s, the Weyerhaeuser Company began planting loblolly pine from the lower coastal plain of North Carolina onto much of their land in Arkansas and Oklahoma. Earlier research tests had shown that this eastern provenance grew approximately 20% faster than the local loblolly pine. These new plantings extended to and beyond the original western range of the species (Lambeth et al. 1984). Some of these stands are now 25 years old, and, because they are managed on wide spacings, have large crowns and should have been producing seed for several years.

Operational plantations of the North Carolina source now exist in close proximity to plantations established from seed of the western provenance. In addition, many research studies with families from both sources planted side by side have been established. Therefore, if effective genetic markers can be developed, we have the opportunity to learn much about fitness. For example, using research trials, we could compare the percentage of trees in the next generation of the two sources with those from the originally planted mix, and also calculate the rate of inter-provenance hybrid formation between those two sources. In this way, we might determine if improved growth is a positive fitness trait, information that would be very useful in making decisions about transgenic pines containing constructs for enhanced growth.

Similar opportunities are being exploited in Canada, where natural populations are being sampled close to exotic plantations. There, hybridization rates are sufficiently high to impact the genetic composition of wild stands within a few generations (Meirmans et al. 2005). Components of fitness at critical life-cycle stages can be examined by grafting 2-year-old transgenic seedlings and non-transgenic seedlings from the same clone onto the tops of trees in established seed orchards (Bramlett and Burris 1995). In loblolly pine, these scions can flower in their second year and produce seed the next year. Controlled pollination can be accomplished in enclosed bags to prevent transgene escape, and the cones can be harvested before they open. This design would allow analyses of seed production and germination, and a determination of seedling vigor and competitive ability in various environments. Experimental plots containing these seedlings could be harvested before

flowering, in accordance with existing regulations. Done in a wide variety of environments, with and without the presence of the particular stress factor the transgene is designed to mitigate, these trials would provide considerable advancement in our scientific understanding. In general, they should not be any more difficult to complete than are fitness studies with agronomic crops, especially as top grafting is an operational practice in commercial loblolly pine seed orchards.

Models: development, estimation of parameters, and testing

Landscape models for dispersal and ecological interactions have already been developed. Research is currently underway to improve spatially explicit models for population genetics. It may be sufficient to link these models virtually, using the predictions of one set of models to inform the initial state of the next model(s). However, literal linking, as in a single computer program, is much more difficult, involving issues of spatial and temporal scaling. Publications have generally described these models and detailed the experimental methods for estimating their parameters. However, such models should not be accorded credibility until they have been independently tested; there is no reason not to subject a model to a critical test capable of falsifying it (Chalmers 1976; Hempel 1966; Popper 1959).

Because of its relatively small size, the forestry research community will be unable to conduct as many fitness assessments as are possible in agriculture. Ellstrand (2002) points out that such studies require the cooperation of “plant evolutionists, ecologists, and population geneticists”. To that, we might add the role of molecular geneticists, physiologists, and applied forest management scientists. Bringing diverse, interdisciplinary teams together will be a challenge. Williams (2006b) advocates a “public–private partnership... a technology trust.” Much could be done through the establishment of a special working group in IUFRO that brings together scientists from these disciplines on a worldwide basis. However this is done, the resources will be limited. Therefore, we advocate that the first step be to identify a small set of the most critical research questions regarding fitness (i.e., those involving likely traits to be deployed and in situations where significant effects might be expected) and then concentrate the initial work on them. Results from those studies will help demonstrate the magnitude and seriousness of the concerns regarding transgene deployment.

Conclusions

If the benefits of transgenic trees are to be realized by society, a sound scientific basis must be developed so risk/

benefit analyses can be completed that aid in evaluating their acceptance. We believe that the regulators of this technology, the scientists who work in this and related fields, the entrepreneurs who are investing considerable sums of money and who expect to earn a return on that investment, and citizens at large (whether favorably inclined or skeptical), will all gain from, and should demand, such analyses.

A substantial and growing body of peer-reviewed research is available regarding population genetics and the ecological effects of transgenic plants. Although most of the preliminary work has been done with agronomic crops, such efforts have set a precedent for what are the basics of the scientific approach. Forestry researchers who fail to measure up to the standards of those earlier endeavors do so at the risk of having their research marginalized (or worse, failing to obtain public support).

The perennial habit of forest trees, their long generation intervals, and the stochastic nature of many of the processes involved, present formidable challenges. Nevertheless, we have now identified approaches that build on existing research results; as more studies are done, they will pave the way for future advancements.

What will happen when transgenics are deployed in forested landscapes? The answer is that “it depends”, and that scientists must help sort out the potentially significant effects from those that are insignificant. The outcome depends on the landscape configuration for transgenic plantations in relation to wild stands, non-transgenic plantations, sensitive natural areas, and the holdings of other forest owners who manage for a variety of objectives. The outcome also depends of what mitigation measures are applied: sterility and its effectiveness, buffers, and refugia. It depends on what transgene constructs are used and the fitness of those transgenes in different ecological situations. Finally, it depends on numerous stochastic effects, such as whether a thunderstorm or hurricane creates conditions for a significant long-distance dispersal event.

Although studies of these phenomena are hard to perform, we believe that without them, science-based, risk–benefit analyses of transgenic tree deployment will be difficult, if not impossible, and that the potential advantages of these technologies may not be realized.

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