Carlson, J. 2005. Biological dimensions of the GMO issue. In, proc. of conf. on restoration of American chestnut to forest lands, Steiner, K.C. and J.E. Carlson (eds.).

BIOLOGICAL DIMENSIONS OF THE GMO ISSUE

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Abstract: Genetic engineering is opening new opportunities in tree improvement, including access to novel genes for disease resistance. American chestnut is one of the tree species for which genetic transformation has been reported. Thus restoration of American chestnut could benefit from the genetic engineering technology, should suitable genes for blight resistance be identified. However, questions regarding the efficacy and safety of genetic engineering with trees, and other perennial plant species, have been raised including the stability of integration and expression of foreign genes, long term effects on non-target species, transgene dispersal through seed and pollen, etc. Many of these concerns could be alleviated if reliable approaches were available for the confinement of transgenes. The National Research Council assembled a committee of twelve researchers from various disciplines to evaluate the measures for biological confinement of transgenes. This paper will summarize the committee's findings regarding the unique concerns that arise in the application of GE to trees, and the potential that exists for applying bioconfinement methods with GE trees.

Keywords: National Academies; genetic engineering; trees; biological confinement; transgenes

INTRODUCTION

The ability to apply GE to plant improvement extends beyond annual crops to woody perennial plants including forest trees (reviewed by Pena and Seguin 2001) and fruit and nut trees (reviewed by Trifonova and Atanassov 1996) such as American chestnut (Conners et al, 2002a,b). Examples of successful GE with trees are appearing with increasing frequency in the scientific literature. Applications for field trials of GE trees have been submitted in the US, Canada, and the EU over the past 10 years for a wide variety of tree species ranging from pines to persimmons, and poplar to papaya (www.isb.vt.edu/cfdocs/fieldtests1.cfm).

The Committee on the Biological Confinement of Genetically Engineered Organisms was established by the National Research Council in 2002 to evaluate the status of and potential role of biological confinement technologies for Genetically Engineered Organisms (GEOs). The study was solicited by the U.S. Department of Agriculture. The National Research Council assembled a committee of 12 experts from various disciplines. The committee prepared its report over a 15 month period, meeting several times at the facilities of the National academies if Science in Washington DC and Irvine, California. In 2004, the Committee on the Biological Confinement of Genetically Engineered Organisms published its findings (Kirk, Carlson, et al 2004). This report is available in hardback edition from the National Academies Press, or as downloadable files at the NRC web site (http://www.nap.edu/books/0309090857/html/). This paper will review the background rationale for the study, the findings of the committee regarding trees and other woody perennials, and the committee's recommendations.

The federal policy framework for the regulation of biotechnology products was created in 1986. This framework followed a vigorous discussion amongst scientists and the public over a period of several years, and has continued to be debated since. Many new GEOs have been developed since the framework was established. More recently the application of GE technology has expanded from annual crops to perennial plants and animals, including forest trees such as American chestnut. The expansion of GE to long lived organisms raised the issue amongst the regulatory community of how best to ensure confinement of transgenes over the long term (addressed in McLean and Charest 2000). Examples of failure of physical confinement protocols and the concerns raised in the public over the "escape" of transgenes into the food supply and the environment, suggested that it was time to also look at biological confinement opportunities.

The committee was charged with addressing the following questions:

1) What bioconfinement methods for GEOs are available, and how feasible, effective and costly are these methods?
2) What do we know about when and why methods fail, and what can be done to mitigate those failures?
3) When these methods are used in large-scale applications, what procedures can be used to detect and cull individuals for which the bioconfinement methods have failed?
4) What are the probable ecological consequences of large-scale use of bioconfinement methods at the population, community, and landscape levels?
5) What new data and knowledge are required for addressing these important questions?
6) What is the social acceptability of bioconfinement methods?

The committee focused on risks associated with the dispersal of a transgene or transgenic organism into a place, population, or biological community for which it was not intended. Long-lived species that disperse easily can present particular risks due to the inefficacy of physical confinement methods, and the potential for escapees to interact with wild populations. Thus the committee was asked to pay particular attention to transgenic fish, shellfish, trees, and grasses.

The concerns most often associated with risk of dispersal of transgenes include 1) The evolution of increased weediness or invasiveness; 2) Effects on nontarget populations — including humans; and 3) The potential for transgenes to disperse into the environment during field tests before being deregulated. The impact of transgene dispersal from perennial plants such as trees often involves issues of gene flow into natural populations. Gene flow issues with GEOs have been addressed in several studies, including trees (Slavov et al. 2002) from both theoretical and applied perspectives, and will not be dealt with much here.

WHEN WILL BIOCONFINEMENT BE NECESSARY FOR TREES?

GE trees are appearing with increasing frequency. Since 1989, more than 230 permits for field tests of GEO plants have been approved by the Animal and Plant Health Inspection Service in the United States, including 18 woody plant species. At least 65 permits have been granted in other countries for field trials on GE trees and other woody plants. The tree species for which field tests have been approved in include pines, persimmons, apples, walnuts, spruces, Sweetgum, aspen, plum, poplar, pear, and papaya (www.isb.vt.edu/cfdocs/fieldtests1.cfm).

Numerous traits are being engineered in trees, including lignin modification, increased growth and productivity, enhanced utilization of resources, pest and disease resistance, stress tolerance, herbicide resistance, optimization of mycorrhizal symbioses, phytoremediation of contaminated soils, and even production of anticancer drugs (Sederoff, 1999; Merkle and Dean 2000).

Specific concerns arise regarding the potential for escape of transgenes with forest trees. One concern is that long-distance gene movement occurs naturally with trees. Pollen and seeds from trees can be carried very long distances by wind, animal, and water vectors. Also, interfertile wild or feral relatives are quite common among tree species. Since hybridization can occur so readily among trees, even exotic trees used for GE may require confinement if within pollen flow distance to related tree species. Another important issue is that trees are more likely to be keystone species within their ecosystems than other plants and animals. When keystone species are impacted, invasion and non-target impact consequences can be large and spread far beyond the species itself. Finally, forests trees carry a higher level of societal importance and impact than most agricultural species. Concerns over forest health are more than economic; they extend to issues of importance of place, esthetics, recreation, nature, and ecosystem services.

BIOCONFINEMENT TECHNOLOGIES

The committee searched the literature to identify as many bioconfinement methods as possible. Each technique or potential technique was described and its strengths and weaknesses evaluated. The following bioconfinement methods were reviewed:

- Sterility
- Mortality of Vegetative Propagules
- Confining Pollen-Mediated Spread of Transgenes
- Transgenes Absent from Seeds and Pollen
- Artificially Induced Transgene Expression
- Reducing Gene Flow to Crop Relatives
- Repressible Seed Lethal Confinement
- Cross-Incompatibility
- Fitness Reduction in Transgenic Crop-Wild Progeny
- Phenotypic and Fitness Handicaps
- Reduced Exposure to Transgenic Traits

The category of sterility**,** included the use of interspecific hybrids, sterile triploids, unisexual plants lacking mates, transgenic sterility, ablation of reproductive organs, and reversible transgenic sterility (a "GURT" or Genetic Use Restriction Technology).

A GURT approach to bioconfinement of transgenes that has received a great deal of attention and research is known as "trait-genetic use restriction technology." Trait- GURTs are alternative approaches to reduce the effects caused by unwanted transgenes by activating a transgenic trait at a specific time through a specific artificial stimulus, such as a chemical spray. In this way, non-target organisms are spared long term exposure to the trait, the targeted species may be less likely to develop resistance, and GEOs that escape confinement should not have any selective advantage as the trait would not be expressed in the absence of the inducing agent in nature.

Several opportunities for confining pollen-mediated spread of transgenes were considered such as nontransgenic male sterility, transgenic male sterility, transgenes in chloroplast DNA transgenes in chloroplast DNA, and apomixis (for asexually produced seeds).

Creating GEOs in which the transgenes are absent from seeds and pollen has been proposed by use of non-transgenic scions on transgenic rootstock, and by programmed excision of transgenes before reproduction. Also, programmed cell death was evaluated as a means to induce mortality in vegetative propagules.

Approaches to reduce exposure of non-target organisms to transgenic traits through tissue-specific gene expression have also been proposed, including chloroplast-targeting of gene expression, limiting expression to roots and tubers, vascular tissue-specific gene expression, flower- and fruit-specific gene expression, pollen-specific gene expression, and seed-specific gene expression.

EXAMPLES OF BIOCONFINEMENT METHODS

Sexual reproduction of genetically engineered plants can be blocked by including a gene that renders the organism either permanently sterile (nonreversible transgenic sterility) or conditionally sterile until an appropriate trigger is applied, such as the use of a chemical spray on a plant (reversible transgenic sterility).

Engineered sterility has its strengths and weaknesses for bioconfinement of GEOs. The main strength of the approach is in its overall effectiveness of confinement. Since with trees the primary risk of transgene escape is through pollen and seed, reliable sterility could overcome much of the risk associated with GE trees (Strauss, et al. 1995). However, there were weaknesses found in the general application of sterility as well, such as concerns that engineered sterility has not been adequately tested yet to determine how effective and reliable it will be for long-lived organisms, that it may be unsuitable for farmers who save seed from specific crops for replanting the next year, and it is still uncertain how well engineered sterility will be accepted by the public. With trees, the specific concern would be those cases in which the seed or fruit crop may be a highly desirable feature, such as in the production of mast for wildlife, in which cases engineered sterility would be counterproductive, unless reversible.

Although most of the above approaches are still untested in the field, their efficacy is generally recognized as having great potential. The options for engineering the nuclear genome to effect sterility in trees that are being tested in poplar includes ablation, gene suppression, and dominant negative mutants. Ablation of floral organs involves the regulated expression of a bacterial cytotoxin using a floral promoter from the species to be engineered. This approach targets expression of the cytotoxin to the tissues of the developing flower or floral buds, killing those tissues and thus preventing flowering. Engineering floral ablation in this manner is relatively easy to accomplish. However, very few if any genes are expressed absolutely exclusively in one cell type in plants. Promoters are more likely to be floral predominant rather than floral exclusive expression, thus raising the possibility that the cytotoxin will also be expressed in non-floral tissues. Thus with the ablation approach, it will be necessary to mitigate deleterious side effects in vegetative tissues prior to use. Another approach is to create dominant negative mutations in which a gene known to be essential for floral development is mutated in vitro to produce a protein that is stable in the cell but no longer active. Over-expressing that mutant gene produces an protein that interferes with the function of the wild type protein, causing sterility.

One of the most active areas of research in plants is gene suppression by RNA-interference (RNAi). RNAi is a natural system for regulation of gene expression in which small double-stranded RNA molecules interfere and thus suppress expression of endogenous genes. This can be engineered by expressing an inverted repeat of a small fragment of DNA homologous to the target gene (De Buck et al. 2001; Klahre et al, 2002). It should be possible to create sterile trees by RNAi targeted at genes that are of essential for floral development. Much is being learned about the genes regulating reproduction in trees, and this knowledge will offer opportunities for engineering sterility by manipulating the expression of nuclear genes as described above. Dr. Steven Strauss of The Tree Genomics, Biotechnology, and Breeding Program in the Department of Forest Science at Oregon State University, is conducting research on engineering sterility in GE poplar trees using floral genes in poplar as their model system. Table 1 lists some of the genes that are known to be involved in floral development in Populus species (as of May, 2004)

Table 1. Genes controlling flower development in Populus.

Arabidopsis Gene	Function in Arabidopsis	Poplar Homolog(s)
AGAMOUS (AG) *	Stamen & carpel identity	*PTAG1* *PTAG2*
APETALA3 (AP3) *	Petal & stamen identity	*PTD*
APETALA1 (AP1) *	Flower initiation; perianth identity	*PTAP1-1* *PTAP1-2*
LEAFY (LFY)	Flower initiation	*PTLF*

*MADS-box gene, member of a large plant gene family that regulates the expression (transcription) of other genes.

Field trials will be an important step in evaluating new GEO trees and in validating bioconfinement techniques such as engineered sterility. Strauss (2003) points out that a key issue in evaluating the safety of new GEOs and also of bioconfinement techniques should be the expected fitness consequences of escapes. That is, how great a risk to the environment or to people is posed by individual escapes, when bioconfinement techniques are not 100% effective. To be a risk, in most cases a transgene must amplify a great deal to have significant environmental impact. An individual GEO tree that has escaped into a natural population of millions of trees will have little of no impact on the overall makeup of that population, unless the transgene increases in frequency quickly. Small releases of GEOs thus have a major built-in safety buffer of scale in field trials. Unless the transgene provides a great differential benefit under strong selection pressure, the key force governing spread of the transgene will be genetic drift. Under genetic drift, a selectively neutral transgene is as likely to be lost from a natural population as maintained, and if not eliminated would require many generations to reach a frequency high enough to be a stable component of a natural population. Furthermore the maximum frequency that a neutral transgene might reach in a natural population will be lower, the fewer escapes from sterility that occur in the GEO population. This indicates that there should be a relatively high tolerance for incomplete sterility in field trials of GEO trees. If so, it may not be necessary to always accomplish 100% sterility to have an effective means of bioconfinement for transgenes.

APPLICATION OF GENETIC ENGINEERING TO AMERICAN CHESTNUT

The application of GE to American chestnut would involve transgenes for resistance to the blight resistance. A major issue with the release of GEOs with transgenes for resistance to disease and other pests is whether or not that will lead to resistance in the targeted organisms, as has occurred with the use of chemical pesticides. This is a particular concern with GEO trees which need to be able to rely on their transgenes for resistance over the course of many generations of the pest population. Several factors influence the development and containment of resistance in targeted pests. These factors include 1) the genetic basis of resistance, 2) the initial frequency of resistance alleles in the target pest population, 3) the competitiveness of resistant individuals in the pest populations, and 4) the resistance management strategy employed.

To ensure the long term usefulness of transgenes in GEOs for pest and disease resistance, it is necessary to have an effective plan to manage against the development of resistance to the transgene product in the pest populations. The key factors for a successful Resistance Management program include 1) knowledge of the biology and ecology of the pest, 2) low initial frequency of resistance genes in the pest populations, 3) low survival of pests when they are heterozygotic for the resistance genes, limiting transmission and build up of resistance genes in the pest population, and 4) the establishment of nearby refugia where the GEO is not present to ensure that susceptible alleles remain in high frequency in the pest populations.

Many different designs for refugia have been tested, but it most commonly it is recommended that 20% of the total area under cultivation, or crops, be planted as a refuge with non-GEO plants. The exact position of the refugia vary with the pest and crop species in question, but refugia may be embedded as rows within in the GEO field, or as borders or Blocks in the GEO field or even as a separate neighboring field. An alternate design includes a separate 20% Refuge that is sprayed with pesticide not related to the transgene product, to control the numbers of pests entering the GEO plots.

Figure 1. American chestnut tree with cankered trunk and new sprouts from the base that are not yet showing infection.

It is important to note, that there should be much less concern about the break down of resistance in chestnut trees than would normally be the case with crop plants. The sprouts that continue to arise from the roots of old wild chestnut trees will provide a large, continuously distributed natural refugia for the production of wild type blight spores (figure 1). The widespread presence wild-type sprouts that occurs throughout the natural range of American chestnut will provide the ideal refugia for preventing mutations in the *Cryphonectria* fungal populations from getting the upper hand. Thus, blight resistance in chestnut, whether from the back-cross breeding program or from genetic engineering, should be relatively stable over time in the forest, and perhaps require less active management than disease resistance in annual crops rather than more.

RECOMMENDATIONS OF THE COMMITTEE

After an exhaustive search which included interviews with experts from academia and the biotechnology industry, the committee reported the following major findings of its study:

- Most GEOs will not require confinement.
- The need for bioconfinement should be evaluated on a case-by-case basis.
- The use of redundant bioconfinement methods will be necessary in some cases.
- Biological confinement of GEOs should be undertaken in the context of an integrated confinement system.
- The need for confinement should be considered at the beginning of the design of a GEO, and be part of the entire development process, not just at the end.

The Committee on Biological Confinement of Genetically Engineered Organisms concluded its study with the following list of recommendations for the USDA.

- The need for bioconfinement should be determined on a case-by-case basis.
- The need for bioconfinement should be considered early in development of a GEO.
- The level of confinement needed should be defined early in development of GEO.
- The stringency of the integrated confinement system should reflect the predicted risk and severity of consequences of GEO escape.
- Bioconfinement techniques should be relevant to the temporal and spatial scales of field release.
- Confinement techniques should be tested experimentally.

- The phenotypes of novel GEOs should be compared with the progenitor organisms.
- Due to the long times required, field tests of bioconfinement methods with trees should be started as soon as possible, even if tests must first be conducted with GE trees not requiring confinement, to produce data the needed for later releases.
- Redundancy of methods can be used to improve confinement in high risk cases.
- An Integrated Confinement System should be used, involving technical, organizational, and regulatory elements.
- Methods should be developed to facilitate environmental monitoring for escapes, including easily identifiable markers, and sampling strategies.
- Transparency and public participation should be important components in developing and implementing appropriate bioconfinement approaches.
- The possibility of human error should be taken into account as a factor when determining bioconfinement methods and evaluating their efficacy.
- The international effects of failures of confinement should always be considered.
- International cooperation on the confinement of GEOs should be pursued.
- More research is required on methods for biological confinement of GEOs.

The committee went into some detail on the reasons why more scientific research is required. Research is needed to characterize ecological risks and consequences and develop methods and protocols for assessing the environment effects of confinement failure. Research is needed to develop reliable, safe, and environmentally sound bioconfinement methods. Research is needed to identify and develop methods and protocols to assess the efficacy of bioconfinement. Research is needed to identify economic, legal, ethical, and social factors that might influence the application of techniques, and their regulation. Research is needed to develop a better understanding of the dispersal biology of organisms targeted for genetic engineering and release. Research is needed to develop a better understanding of how species become invasive. Finally more research on risk assessment and safety management specific to GE trees (as a follow up to Lu et al 1999) are needed.

In addition, the Committee prepared a template for risk evaluation to be used in the process of planning GEOs, including in decision making on the need for and methods of biological confinement. This risk evaluation template could be a good tool for the National Park Service to use in evaluating the use of specific GE trees in ecosystem restoration and enhancement projects.

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