Institute of Forest Biotechnology
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Genetically Engineered Forest Trees

Identifying Priorities for Ecological Risk Assessment

A publication by the Institute of Forest Biotechnology

Dear Colleague;

This publication is a compilation of research presentations, identified knowledge gaps, and ideas for next steps in answering ecological risk questions about genetically engineered forest trees. This material was generated during the second meeting from the Institute of Forest Biotechnology (IFB) that focused on ecological issues. We would like to thank all of our invited experts and our Initiative Sponsors for making this publication possible.

We hope that you find it informative and provocative. Feel free to print this PDF and use it for your personal use. If you would like a bound book of this report, please purchase it online.

We invite you to explore the Institute's other initiatives on our website, forestbiotech.org. Biotech trees hold the promise of enhancing our environment and providing sustainable resources for society if used responsibly. The IFB is dedicated to discovering the social and environmental effects of using forest biotechnologies. Responsible Use: Forest Biotechnology Principles is the next step in the IFB's focus on science, dialogue, and stewardship. All material about this initiative is online at responsibleuse.org.

Thank you for your interest in protecting the future of forestry. Please contact us or join our news group online.

Best regards,
Susan McCord

Executive Director - Institute of Forest Biotechnology

PDF Location: http://forestbiotech.org/pdf/GE_Trees_Ecorisk_online_v1.pdf
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Genetically Engineered Forest Trees

Identifying Priorities for Ecological Risk Assessment

SUMMARY OF A MULTISTAKEHOLDER WORKSHOP

Institute of Forest Biotechnology
Science • Dialogue • Stewardship

Genetically Engineered Forest Trees

Identifying Priorities for Ecological Risk Assessment

SPONSORED BY

The Pew Initiative on Food and Biotechnology

Weyerhaeuser Company Foundation

The Animal and Plant Health Inspection Service
of the U.S. Department of Agriculture

Genetically Engineered Forest Trees

Identifying Priorities for Ecological Risk Assessment

SUMMARY OF A MULTISTAKEHOLDER WORKSHOP

May 3-4, 2007 | Raleigh, North Carolina | Convened by The Institute of Forest Biotechnology

Institute of Forest Biotechnology

Science • Dialogue • Stewardship

The Institute of Forest Biotechnology
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Preface

This report summarizes a multistakeholder workshop held in May 2007 at which research scientists, federal regulators, and representatives of the forest products industry and environmental community discussed priorities for scientific research on ecological risks relating to genetically engineered (GE) forest trees. The workshop grew out of a March 2006 conference organized by the Institute of Forest Biotechnology (IFB) called "Growing Trees and Stemming Risks," held in Vancouver, Canada. A paper presented at that conference and authored by Drs. Peter Farnum, Alan Lucier, and Richard Meilan provided the impetus for this workshop.

We were struck by the idea addressed in that paper—and discussed further in the introduction to this summary—that the technology associated with GE forest trees has perhaps outpaced scientific research regarding the potential ecological risks of such trees. By identifying key research questions and encouraging scientists to collaborate to answer them, unintended negative consequences associated with the planting of GE trees may be avoided, and the potential benefits of such trees may be more readily realized. The May 2007 workshop was organized to address these issues.

We at the IFB are very grateful for the generous funding support provided by our three partners in this workshop effort—the Pew Initiative on Food and Biotechnology, the Weyerhaeuser Company Foundation, and the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture. We hope this document will illuminate the key research needs relating to genetically engineered forest trees and serve as a springboard to collaborative work on the necessary research by workshop participants and other scientists. We look forward to learning about the results of those research efforts.

—Susan McCord, Institute of Forest Biotechnology

Introduction

Forest products are a $230 billion industry in the United States, making the U.S. the largest producer of forest products in the world. The output of this industry touches every aspect of our lives. It includes both paper pulp (used for everything from newspapers to books to packaging materials) and finished wood (for lumber, plywood, and furniture, among other products) (AFPA 2006). At the same time, forests provide essential habitat for wildlife and may be used for recreational activities such as hiking, camping, hunting, and fishing (NWF 2006). In the future, Americans will likely also look to forest trees as a biofuel source, as a way to combat the twin challenges of energy security and climate change.

At present, a significant amount of wood pulp in the U.S. is derived from intensively managed stands of planted pines. Trees for biofuel would undoubtedly also be grown in such plantations (in the South and elsewhere), as they present the most efficient and cost-effective way to produce pulp (NWF 2006).

Southern pine plantations have grown tremendously in size and efficiency over the past 50 years. In the 1950s, fewer than 2 million acres of pine plantations existed in the South, and most of those produced less than 90 cubic feet per acre of pulpwood. By 2000, the total acreage of Southern pine plantations had grown to approximately 32 million acres and the productivity to more than 400 cubic feet per acre. The remarkable increases in productivity have been driven largely by improvements in site-specific silvicultural regimes (i.e., site preparation, fertilization, and weed control) as well as advancements in selective breeding (Fox, Jokela, and Allen 2004).

Many scientists and industry experts agree that the most promising future innovations in plantation forestry lie in the realm of biotechnology (Fox, Jokela, and Allen 2004).[1] Unlike traditional breeding, genetic engineering allows the control of a particular trait of interest (e.g., fast growth or disease resistance) via the manipulation of a specific gene or genes. Agricultural commodities have already seen significantly improved yields and management efficiencies with the commercialization of genetically engineered (GE)[2] varieties—though not without public controversy, particularly in Europe. In the U.S. at present, more than three-quarters of the soybeans and cotton grown, and more than half of the corn, are GE varieties.[3] Other less-widely grown GE crops include papaya, canola, flax, and squash.

GE trees are currently being studied in university and private laboratories around the world. They include varieties that are disease and pest resistant, faster growing, able to remove toxic chemicals from the soil, and/or particularly suited to biofuel production (Farnum, Lucier, and Meilan 2007). No GE trees have yet been approved for use in commercial forestry operations in the United States, however. The only GE forest trees known to be deployed on substantial scale are GE poplars in China (Farnum, Lucier, and Meilan 2007). The private company ArborGen is reportedly focusing on the development of three GE varieties: fast-growing loblolly pine for Southern pine plantations, low-lignin eucalyptus for use in South America, and cold-hardy eucalyptus for the Southern U.S. *(Farnum, Lucier, and Meilan 2007).*

Genetically engineered trees pose some different—and difficult—challenges compared to GE food

1 See also *http://www.forestbiotech.org/pdffiles/forestryevolution.pdf.*

2 Note that the terms *genetically engineered* (GE), *genetically modified* (GM), and *transgenic* plants, along with *genetically modified organisms* (GMOs), were used interchangeably in this workshop and so are used interchangeably in this summary as well.

3 See *www.ers.usda.gov/Data/BiotechCrops/.*

crops, and they may generate more concern among the public and the environmental community. For example, while most GE food crops in the U.S. do not have wild relatives with which they can cross-breed, plantation-grown trees in the U.S. are generally surrounded by natural forest stands made up of the same or similar species. And scientists do not fully understand what ecological effects might result from the escape and introgression of a transgene into a wild tree population. Ecological effects are of particular concern since forests—even intensively managed plantations—support vastly more plant and animal species than do farms, and they supply important ecological "services" such as clean water, clean air, and erosion control. Also, as discussed in the second presentation in this report, forest managers are generally held to a higher standard of environmental stewardship by the public than growers of row crops.

Research on GE trees also poses unique challenges. Trees are much longer lived and have much longer generation intervals than annual plants, making research a more lengthy and difficult process. In many cases, models must be used to achieve answers to research questions in a reasonable amount of time. In addition, the pool of forestry researchers and forestry research dollars is much smaller than that for agricultural crops (Farnum, Lucier, and Meilan 2007).

Most observers agree that significant additional research on GE trees is sorely needed. Such research could help scientists better understand the potential of such trees for solving important societal problems such as energy security, and help them discover ways to avoid or mitigate detrimental environmental and ecological impacts. A recent article stated the need this way:

> The technology for creating [GE] trees has gotten far ahead of research on the ecological and population genetics impacts that may emerge.... 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 *(Farnum, Lucier, and Meilan 2007)*.

With this need for research in mind, the Institute of Forest Biotechnology[4] and the Pew Initiative on Food and Biotechnology[5] sought to organize a workshop among diverse experts to discuss priorities for ecological risk assessment of GE forest trees. The two-day workshop that resulted enabled discussion among approximately 35 research scientists, federal regulators, and representatives of the forest industry and the environmental community on these important topics. The workshop was held in Raleigh, North Carolina, in May 2007.

The stated purpose of the workshop was to establish research priorities with a clear scope and broad-based buy-in to address the most urgent ecological risk questions for genetically engineered trees. Parameters to accomplish this included: what baseline information exists for assessing the environmental impact of GE trees; what comparators are appropriate; what consti-

4 The Institute of Forest Biotechnology is a nonprofit organization that works for societal, ecological, and economic benefits from appropriate uses of biotechnology in forests worldwide. See *www.forestbiotech.org*.

5 The Pew Initiative on Food and Biotechnology (PIFB) served as an independent and objective source of information on agricultural biotechnology for the public, the media, and policymakers. This workshop marked the final event sponsored by the Pew Initiative, which officially dissolved in April 2007. The PIFB website, *www.pewagbiotech.org*, will remain up indefinitely as a continuing resource for interested parties.

tutes a negative or positive effect; and what is an appropriate research timeline. Meeting organizers hoped that the meeting would:

- Define the terms and concepts for determining ecological risk.
- Identify the baseline context and experimental comparators appropriate for genetically engineered forest trees.
- Identify ecological risk issues unique to genetically engineered forest trees.
- Identify the most important scientific knowledge gaps for ecological research that are relevant and informative to the U.S. policy framework.
- Identify research priorities with a clear scope and broad-based support for the approach.
- Outline a funding strategy to support cross-disciplinary research on the priority issues.

This document, prepared by Larmer Consulting,[6] summarizes the presentations and discussions that took place at the workshop. The workshop was facilitated by Abby Dilley of RESOLVE, a nonprofit public policy and dispute resolution organization based in Washington, DC.[7] A complete list of participants and staff can be found in Appendix B.

Day One of the workshop consisted of 12 presentations, most of which were organized into three panels—one each on ecological effects, gene flow and hybridization/introgression, and fitness. Full-group discussion followed each panel, and the day concluded with a panel that helped to synthesize what had been heard during the presentations.

Day Two included several hours of small work-group conversations, followed by report-outs and full-group discussion. The workshop closed with a discussion of next steps.

6 See *www.larmerconsulting.com*.

7 See *www.resolv.org*.

Day One

On Day One of the workshop the organizers and sponsors provided opening remarks, and then 12 invited experts gave presentations on key topics relating to genetically engineered trees. Ten of the presentations were organized into three panels—on ecological effects, gene flow and hybridization/introgression, and fitness. Each presentation and each panel were followed by short full-group discussions. The day ended with a panel-led discussion recapping the identified risk issues and beginning to frame research questions.

At the outset, Adam Costanza of the Institute of Forest Biotechnology (IFB) welcomed participants and thanked the steering committee for planning the workshop and creating a robust agenda. He noted that the IFB has three platforms—science, dialogue, and stewardship—and that the workshop would address all three. Costanza also acknowledged and thanked the workshop's sponsors: the Pew Initiative on Food and Biotechnology, Weyerhaeuser Company Foundation, and the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA). Finally, Costanza thanked IFB Executive Director Susan McCord for organizing and convening the workshop.

Keith Pitts of the Pew Initiative also thanked the steering committee for their work. He said the idea for the workshop arose from a scientific paper on research imperatives for GE trees written by three workshop participants—Peter Farnum, Al Lucier, and Rick Meilan (2007). He noted that issues relating to GE trees were becoming increasingly critical given that trees—including GE trees—could potentially provide solutions to the pressing national challenges of energy security, food security, and climate change, but that unanswered questions about possible ecological risks posed by GE trees challenge their commercial viability. He said this workshop should be very useful to setting a widely acceptable ecological risk assessment research agenda for GE trees and to the broader public dialogue on these issues.

Facilitator Abby Dilley then briefly reviewed the meeting agenda and ground rules and asked participants to introduce themselves in turn around the room. The goal of the workshop, she said, was to have participants develop a prioritized research agenda for ecological assessment. She noted that the focus was to be on scientific research, not regulatory policy, and that ideally participants would be inspired to collaborate on that research going forward.

Context/Background Presentations

Two invited experts—Allison Snow of Ohio State University and Peter Farnum, formerly with Weyerhaeuser—gave talks at the outset that helped to set the stage for the remainder of the workshop. Snow focused on what can be learned from experience with GE crops, while Farnum discussed the context surrounding technology issues in forestry.

What Can We Learn from Experience with Other GE Crops?

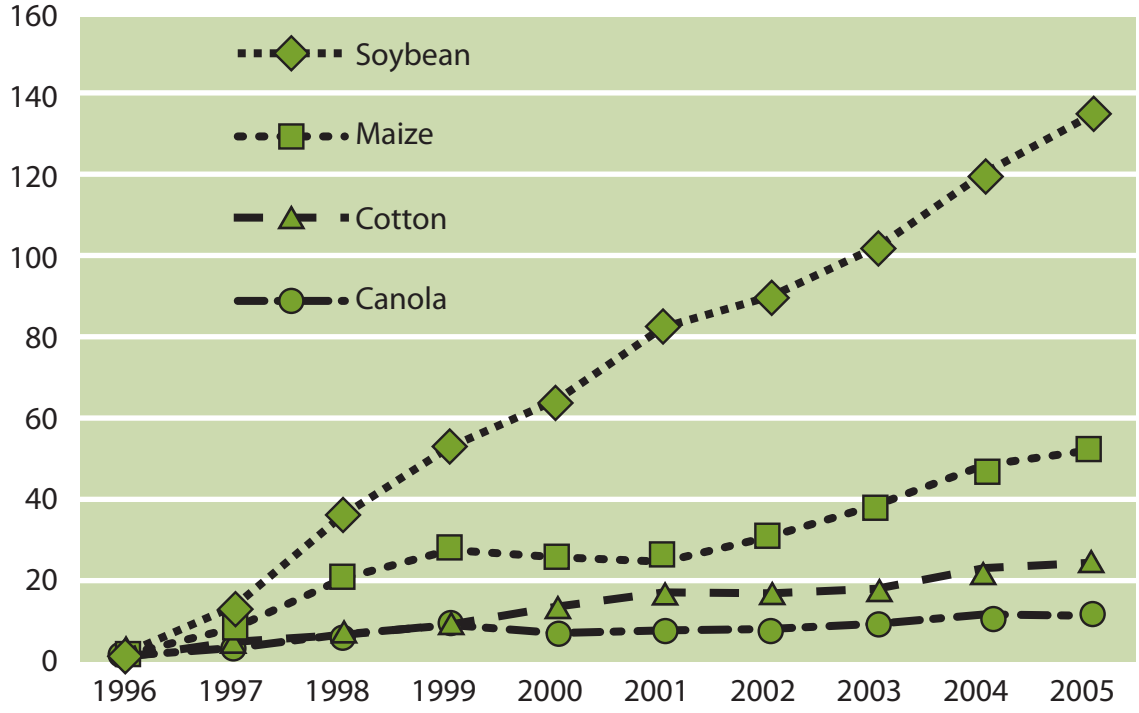
Presentation by Allison Snow, Ph.D., Ohio State University

Allison Snow is a Professor in the Department of Evolution, Ecology, and Organismal Biology at Ohio State University. She began by saying she was pleased that the workshop had brought together scientists from diverse disciplines, because collaboration among such disciplines is essential for research on GE trees.

Snow said her talk would address what is known about existing GE crops; the identification of ecological risks; expectations about transgenes; baseline knowledge needed about host plants; and the ecological and evolutionary effects of GE crops. She noted that research into GE crops has been going on for 15 years within programs funded by the USDA and other government bodies.

Only a few types of GE crops containing a few transgenic traits are currently grown around the world, Snow said. The crops most commonly

Figure 1: Global Area (Million Acres) of Biotech Crops, 1996 to 2005: by Crop



Source: Clive James, 2005

used are soybean, maize, cotton, and canola; less widely planted are squash, papaya, and a few others. The most common GE traits, Snow said, include resistance to herbicides, insects, and diseases. Examples of widely grown GE crops include Roundup Ready® soybeans, which are resistant to the herbicide Roundup® (glyphosate); *Bt* cotton and *Bt* corn, which have a built-in pesticide for *Lepidopteran* pests; and GE Hawaiian papayas, which are virus resistant.

Though the number of GE traits and crops remains small, Snow said, the number of acres of GE crops being grown has increased rapidly in recent years. (See Figure 1.) Also, many more traits and crops are currently being studied in field trials. For example, Snow said, USDA-funded research is being conducted on the ecological and evolutionary effects of new GE species and new GE traits.

Of the commonly grown GE crops, Snow said, only canola and squash have wild or weedy relatives. Thus, scientists and growers do not have much experience observing the consequences of transgenes moving into wild relatives.

In looking at ecological effects, Snow said, it is important to understand the difference between *risk* and *hazard*. Common definitions of risk, she said, include "the possibility of harm" and "unwanted outcome." In formal risk assessments, however, risk means the probability of harm times the probability of exposure. A hazard, she said, is an action, substance, or phenomenon with the potential to cause harm.

In conducting risk assessments for GE plants, Snow said, the USDA uses conventional agriculture as the baseline. That is, they seek to identify environmental impacts above and beyond those caused by conventional agriculture. Snow noted that some observers argue for using a different baseline, such as organic agriculture.

Snow said she and her colleagues, in a recent paper, identified the following possible ecological risks of GE organisms:

- Creating new or more vigorous pests and pathogens
- Exacerbating the effects of existing pests through hybridization with related species
- Harming nontarget species (e.g., soil organisms, nonpest insects, amphibians, birds)
- Disrupting biotic communities, including agroecosystems
- Causing irreparable loss of species diversity or genetic diversity within species *(Snow, et al. 2005)*

Related concerns in agroecosystems, Snow said, include the following:

- Direct or indirect harm to beneficial insects and soil organisms
- Harm to culturally important species (e.g., native butterflies, skylarks, wild relatives of crop plants)
- Requiring more (and/or more toxic) herbicides or insecticides, potentially harming flora, fauna, and human health

The general expectations for transgenes, Snow said, based on examples in published papers, are as follows:

- They confer dominant traits with Mendelian inheritance.
- They have strong expression, often with a constitutive promoter.
- The trait in the crop is also expressed in

wild relatives with the transgene.

- Those that are commercialized are unlikely to decrease plant fitness, as technology developers work to select against any such decrease.
- They may be associated with unintended phenotypic effects due to pleiotropy,[1] epistasis,[2] position effects, and other processes. However, companies generally screen to minimize unintended phenotypes before release.
- Two or more transgenes may be tightly linked and inherited together.

Snow said the primary baseline knowledge that is needed regarding the host plant—i.e., the cultivated GE plant and/or the weed or wild plant with which it may hybridize—is the extent of gene flow or exposure. Such gene flow, she said, is influenced by:

- how much outcrossing or selfing it can do
- its ability to hybridize (i.e., the potential for the transgene to introgress[3] and become established in other populations)
- the extent of seed dispersal
- the ability of the plant to spread vegetatively
- the ability of the crop plant to become feral

Figure 2

Compatible weedy relatives in USA?

Potential for outcrossing	NO	YES
LOW	Soybean	Rice Sorghum Wheat
HIGH	Corn Cotton	Brassicas Carrot Poplar Radish Squash Sunflower

- inferences from basic population genetics, including migration rates among populations and the distribution of genetic variation within and among populations

Crops with compatible wild relatives in North America, Snow said, include maize (corn), canola, sunflower, wheat, rice, sorghum, carrot, squash, strawberry, poplar, oat, clover, Bermuda grass, and forage species. Snow then shared a matrix (Figure 2) that grouped common crop plants according to their relative potential for outcrossing. Most of the GE crops planted used widely in the U.S., she said, have either no compatible weedy relatives and a low potential for outcrossing (e.g., soybean), or no compatible weedy relatives and a high potential for outcrossing (e.g., cotton and corn). The plants in the matrix that cause the most concern, Snow said, are those with compatible weedy relatives and a high potential for outcrossing, including sunflower, the *Brassicas*,[4] carrot, squash, poplar, and radish.

Snow then shared several examples of GE traits introgressing into wild and/or weedy relatives.

1 *Pleiotropy* refers to the ability of one gene to influence multiple phenotypic traits.

2 *Epistasis* refers to interaction among genes.

3 *Introgression* is the infiltration of the genes of one species into the gene pool of another through repeated backcrossing of an interspecific hybrid with one of its parents.

4 Plants belonging to the genus *Brassica* include cabbage, kale, broccoli, cauliflower, turnip, and mustard.

Cultivated squash, she said, which has single-sex flowers, is related to wild gourds. GE disease-resistant squash hybridize with their wild relatives, she continued, and the transgene conveying disease resistance may be present in the hybrid plants. The hybrids are variable; some grow quite vigorously. Likewise, herbicide-resistant GE traits in cultivated rice could find their way into weedy rice growing nearby, Snow said, even though in that case the cultivated plants are largely self-pollinating and the rate of outcrossing is low.

Snow also talked about the example of sunflower. She noted a study from 1994 showing that cultivated/wild hybrid sunflowers always occur in greater frequency closer to the cultivated crop. In fact, she said, most are found within 1,000 meters of the crop (Arias and Rieseberg 1994).

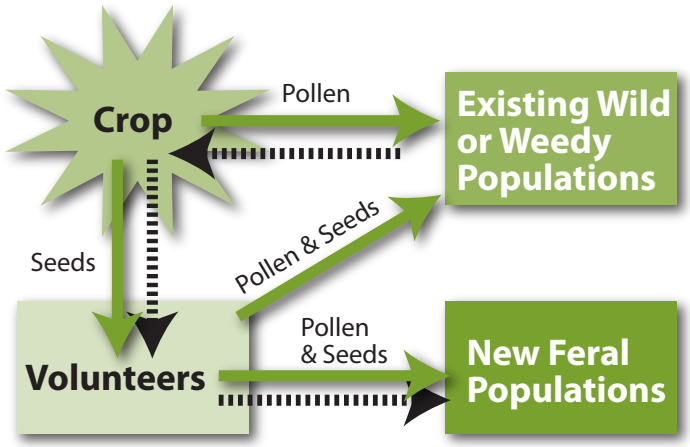
Creeping bentgrass, Snow continued, was found in a 2004 study to have long pollen dispersal (Watrud *et al.* 2004). In this case, a GE (Roundup Ready) creeping bentgrass, which can cross with nontransgenic creeping bentgrass and other related grass species, was being grown in field trials in Oregon. It had not yet been approved for commercial use. To determine the distances the pollen could travel, the researchers placed nontransgenic wild "sentinel plants" at various distances from the field trial. Their study revealed fertilization of the wild grass by the transgenic variety up to 20 km away. They also looked for hybrids among resident creeping bentgrass and found hybridization between 5 and 10 km away. Finally, they showed that the GE species also hybridized with a different but related grass species between 10 and 15 km away. It was a surprise how far the GE pollen traveled, Snow said.

Gene flow can also take place via seeds, Snow said. Seeds can be dispersed via farm vehicles, human activity, wind, and so forth, and can remain ungerminated in fields and later spring up as volunteers. This occurred with GE ProdiGene corn, which contained pharmaceutical compounds. After a field trial of ProdiGene was conducted, Snow said, all of the seed-producing plants were removed. But the next year volunteer corn plants containing the pharmaceutical compound came up among a soybean crop that had been planted in that field (Marvier and Van Acker 2005).

Genes can also spread if a crop becomes naturalized or feral. Queen Anne's Lace is an example, Snow said; this "wildflower" is actually a wild, naturalized carrot that differs from edible carrots by only a few genes.

Snow then shared a slide (Figure 3) that summarized the possible crop-wild plant interactions. Pollen from a GE crop can move into existing wild or weedy populations, she said, and likewise, pollen from the wild or weedy populations can move into the GE crop. Also, seeds from the crop can become volunteers, and the pollen and seeds of those volunteers can move into existing wild or weedy populations and/or become new feral populations.

Figure 3



What is needed, Snow said, is information about the distribution and habitats of the GE crop and its wild relatives. Sometimes that information is well known, as for many crops in the U.S. For example, she said, John Burke (a workshop attendee) did a study showing that the ranges and flowering times of wild and cultivated sunflowers overlap, so there is a high probability of gene flow from the crops to the wild relatives (Burke et al. 2002). In many regions of the world, by contrast, such baseline data has not been documented.

The types of baseline knowledge needed about host plants, Snow said, include the following:

- Lifespan (i.e., whether annual or perennial, and what the longevity is; also, seed dormancy and longevity)
- Ability to propagate and disperse vegetatively
- Extent of seed dispersal
- Ability to become feral
- Invasive ability and factors that limit current abundance
- Ecological interactions with other species (e.g., mutualisms, competition, herbivory, etc.)

Additional important knowledge includes the ecological and evolutionary effects of GE traits being put into crops, Snow said, and what those traits will do once fully transferred into wild relatives. Fitness effects (relating to survival and fecundity) are relevant to transgene frequencies and weediness, she explained. In some studies, she said, no fitness costs have been seen in controlled experiments. These include experiments relating to *Bt* wild sunflower (carrying the *cry1Ac* gene for *Lepidoptera*), fungus-resistant wild sunflower (containing the OxOx gene), herbicide-resistant *Brassica rapa*, and virus-resistant wild squash. Snow said she has found a few papers showing small fitness costs in the cultivated crop. These include studies of cultivated rice with *Bt*/CpTI, sugar beet with virus resistance, and clover with the sunflower albumin gene (see references in Chen et al. 2006).

Snow then discussed the relative fitness effects of transgenes. If there is a large fitness cost to the transgene (say a *Bt* transgene), Snow explained, and a lot of insect pressure, then the fecundity of the transgenic plant may increase (in comparison to the control), but it may not get to be much higher than the plant's nontransgenic counterpart. Whereas if there is no fitness cost to the transgene, there would be a fitness benefit to the plant. So if there's no cost, she said, the more herbivores that are around, the more protected the plant is from those herbivores (due to the transgene) compared to the control.

Snow mentioned several fitness effects of transgenic traits in wild relatives. She said fitness benefits have been detected in *Bt* wild sunflower with natural herbivory, in *Bt* canola with imposed herbivory, in virus-resistant wild squash with imposed infection, and in herbicide-resistant weeds with herbicide application. No fitness benefit has been detected in fungus-resistant wild sunflower with imposed infection, she said (Burke and Reiseberg 2003).

In controlled field experiments, the fitness benefit of the *Bt* transgene in wild sunflowers has been proven, Snow said. The *Bt* was very effective in preventing insect herbivory, including against *Lepidopterans* (*suleima*, *plagiomimicus*, and *isophrictis* (Snow *et al.* 2003).

A transgene that shows a net fitness benefit at one or more study sites, Snow said, should raise a red flag. That transgene will likely become more frequent over time and may persist indefinitely, she explained, and mitigation to remove it will not be

effective. Also, the host plant could become more abundant and more of a weed problem, depending on factors that limit population growth and metapopulation dynamics.

Snow then mentioned some ecological and evolutionary effects of novel traits on nontarget species. For example, pollen from *Bt* corn was thought to harm monarch and swallowtail butterflies. Laboratory studies showed harm, she said, but there is little actual exposure in the field. Also, *Bt* proteins in soil may persist and affect invertebrates. And, concerns exist about the farm- and field-scale effects of GE crops. Studies in the UK regarding herbicide-tolerant crops, and other studies of the effects of *Bt* on invertebrate abundance, show that some insects are negatively affected *(e.g., Andow and Zwahlen, 2006)*.

GE crops also have the potential to affect the evolution of resistance, Snow said. Many studies have been done of resistance management options in *Bt* crops, she said, and after more than eight years of *Bt* cotton, the target insect is still susceptible to the *Bt* transgene (Tabashnik et al. 2006). However, Snow added, herbicide-tolerant crops have been shown to lead to selection for resistant weeds and gene flow to weedy relatives. The more the herbicides are used, the more the resistance evolves. Snow noted, for example, that weedy volunteer canola in Canada is resistant to three herbicides (Hall *et al.* 2000).

Snow noted several challenges to predicting large-scale and long-term ecological and evolutionary effects of GE crops. For example:

- Certain combinations of genotype and environment could trigger unwanted consequences not seen in smaller studies.
- Modeling can be useful for exploring, but not predicting, consequences that are ecological and evolutionary.
- Monitoring for unanticipated or predicted effects after release is challenging and expensive.
- A lot of uncertainty exists about the extent to which researchers can ensure safety, and that needs to be acknowledged, especially if transgenes will persist indefinitely in free-living plants.

In summary, Snow reiterated that significant baseline knowledge about host plants is needed, and that the ecological and evolutionary effects of GE crops depend on the specific crop, the transgenic traits, and the receiving environment, and can be challenging to predict.

A brief question-and-answer period followed Snow's talk. One participant questioned why scientists and breeders must worry about transgenic fitness-enhancing genes, but not conventional fitness-enhancing genes. He argued for a "flatter" regulatory landscape that did not distinguish the process used. Another participant noted that with genetic engineering, genes from an entirely different class of organism can be transferred. Participants were reminded, too, that the discussion should stay focused on science, not policy.

Another participant asked Snow how ecologists decide whether a new transgene will have significant effects, given the context of high environmental variability. Snow said that, in general, scientists need to determine how a transgene affects the population dynamics of its wild relatives (e.g., whether there are large increases in population growth rates or dispersal to new sites). This must be done by conducting small studies in controlled systems and then extrapolating via modeling, she said.

Technology Issues in Forestry

Presentation by Peter Farnum, Ph.D., Weyerhaeuser Company (retired)

Peter Farnum was previously the Vice President of Technology and Timberlands for Weyerhaeuser Company. His talk covered issues relating to the context, approaches, and outcomes for addressing technology issues in forestry. As with Snow's talk, Farnum's presentation helped to set the stage for the full-group discussions later in the workshop.

Farnum first talked about public attitudes and standards for forests. He used several examples to illustrate that the public values forests highly and, like it or not, forest managers—even those operating on private land—must take public attitudes into account in conducting forest management activities. These values and attitudes, of course, have implications for the development and commercialization of GE trees.

Farnum noted, for example, a talk-radio program he heard recently in Seattle. The question being considered was, "What legal and ethical rights do trees and forests have?" The discussion was not about whether trees and forests *had* rights, Farnum said, but rather, given that they do have rights, what are those rights? He noted that the subject of agricultural plants came up, and the host of the show said, "Well, we eat agricultural plants, so it's obvious they don't have rights."

Farnum also pointed to the 1972 Supreme Court case of *Sierra Club v. Morton* as an indication of public attitudes and standards about forests. This case helped to establish who has the right to sue in environmental cases. The question was do trees have legal standing? The court decided they do not, but Farnum noted the following quote from the dissenting opinion by Justice William O. Douglas:

> The ordinary corporation is "person" for purposes of the adjudicatory processes…. So it should be as respects valleys, alpine meadows, rivers, lakes, estuaries, beaches, ridges, groves of trees, swampland, or even air that feels the destructive pressures of modern technology and modern life. The river, for example, is the living symbol of all the life it sustains or nourishes—fish, aquatic insects, water ouzels, otter, fisher, deer, elk, bear, and all other animals…. The river as plaintiff speaks for the ecological unit of life that is part of it.

Many of the resources Douglas named, Farnum said, are part of the forest ecosystem. It is clear, he said, that many people have special attitudes about forests that must be taken into account.

Even though the Sierra Club lost that suit, Farnum continued, and so could not sue on behalf of the trees specifically, the court said they could sue on behalf of people who had suffered an injury. Thus if a person's values are violated, that violation can constitute a "harm" and give an entrée to the legal system. And that, Farnum explained, is one example of how the social context is important.

Farnum then shared statistical data that helped to provide additional context. In North Carolina in 1993, he said, Weyerhaeuser conducted a survey in which 83% of respondents said they believed streams and water were a public resource, even when those resources were found on private company land. Likewise, 74% felt non-game wildlife on private forestland was a public resource. These results are not unexpected since various statutes

give governmental entities some authority with respect to these resources. However it is very surprising that 62% felt that the trees themselves, growing in industrial nurseries and planted at private company expense, were a public resource.

The point is, Farnum said, people have strong values about forests because of the public resources they see as existing in those forests, and this raises expectations for forest managers. People who manage forests have an implicit public license to operate, he said, and if they ignore that license, they do so at considerable risk.

The public's attitudes about forests and the resources within those forests, Farnum continued, also help to determine their attitudes about forest technology. The public has historically challenged applications of forest management, including clear-cutting, fertilization, effects on non-game wildlife, and so forth. These challenges play out in the context of established laws and in court. Public challenges to forest management technology are in fact the rule, Farnum said, not the exception.

Genetic engineering may be a new technology, Farnum said, but it is going to be applied in the same existing context. Forest managers will have the same objectives with GE trees as they have had with other forest management technologies and practices—faster growth, improved forest health, and more valuable raw materials. Currently, Farnum continued, scientists are documenting the potentially greater benefits, but need to provide more hard data on potential risks. There is every reason to expect that the public will challenge the use of genetic engineering in forest management, he concluded.

Farnum's suggested approach to handling these challenges included four parts:

1. Don't be surprised
2. Get involved in foundational research
3. Be transparent and inclusive, and publish
4. Separate science and policy

He talked briefly about each part of the approach in turn. First, don't be surprised. Every issue relating to forest technology is telegraphed years, if not a decade, in advance, Farnum said. For example, Weyerhaeuser had plenty of warning on the spotted owl issue, he said. The owl was listed as an endangered species in 1990, but trends in habitat loss were known for more than a decade before that.

Second, get involved in foundational research. This seems obvious, Farnum said. If the issue is important and science is going to be important to the outcome, why not get involved? With the spotted owl, he said, Weyerhaeuser could have gotten involved in research with the U.S. Forest Service in the early 1980s on habitat requirements, but they focused on other priorities instead. Though it is unclear exactly what would have happened had they gotten involved, Farnum said, the nature of the serious crisis that occurred would likely have been different. Weyerhaeuser had a better experience with regard to water quality on the Neuse River in North Carolina, Farnum said. In that case, they were prepared when significant fish kills took place, because they had participated in co-operative research into water quality that had started ten years earlier.

Farnum's third point was to be transparent and inclusive, and to publish. Research on the environmental effects of forest management should not be secret or proprietary, he said; it should be transparent. It will also be more credible if it is multi-stakeholder and multi-institutional. And, it should be published in peer-reviewed journals.

Fourth, separate science and policy. Let scientists and policymakers play their different roles, Farnum said, because science and policy require different skills. Policy skills include persuasion, negotiation, and compromise, he explained, which aren't necessarily the skills it takes to be a good scientist. At Weyerhaeuser, this separation was strictly enforced. This was beneficial, Farnum said, because the scientists were not particularly adept at policy, and the policy people did not influence scientific results.

Farnum illustrated the distinction between science and policy this way: Scientists can quantify the rate of transgene migration and the fitness of plants with transgenes, and they can describe the ecological implications. Policymakers can then decide whether those implications are significant in a legal and social sense.

There is interaction between policy and science, however, Farnum said. Policymakers may decide that "harm" is done to people who use a certain area for recreation if X percent of a certain species carries an escaped transgene. Such a decision informs scientists of the precision required in their experiments. Farnum noted too that this workshop was organized to focus on science, not policy.

Success for this four-part approach, Farnum said, is measured not by win or lose, but in terms of results that are not owned by one interest group. Rather, he said, there must be a commitment to being guided by a scientific outcome.

As an example of such a success, Farnum returned to the story of the fish kills on the Neuse River. Weyerhaeuser started collecting data on Neuse River water quality in 1980, in cooperation with a number of partners. Researchers at North Carolina State University, for example, analyzed and interpreted the data. More than 100 publications resulted from the University's efforts. When the fish kills took place in the early 1990s, he said, the state's Department of Environmental Quality could refer to those publications and call on a whole cadre of neutral experts. As a result, Farnum said, policymakers were able to make better decisions.

Farnum summarized his four-part approach with the following long sentence: Transparent multi-stakeholder research that is started early in the issue-development process, that focuses on scientific results and not social policies, and that is published in peer-reviewed journals, enhances the scientific basis for decision making and leads to better public understanding and policy/regulatory determinations.

Farnum then moved to a discussion of outcomes. The outcomes are clearly tied to the approach, he explained. He said the workshop's organizers were asking participants to identify specific scientific questions of public and regulatory concern, and then engage in foundational research to answer those questions. The research should be transparent and inclusive, he said, and should be published in peer-reviewed journals. Priorities must be set based on multi-stakeholder interest, and multidisciplinary teams must be formed. And finally, science and policy must be kept separate. (Though he added that regulatory barriers to conducting the necessary research remain within the scope of discussion.)

Farnum noted two special issues with regard to research on GE trees. First, he said, results are needed quickly, due to the late start of ecological research relative to technology development and issue identification, and also due to the time it takes to do research with trees. Second, regulatory barriers to conducting critical research regarding GE trees need to be addressed. Explaining further, Farnum said he is concerned

that the time required to complete ecological studies in forestry will delay the implementation of GE technology. He said he is also concerned that regulatory restrictions on field studies will make progress in understanding some ecological problems difficult, if not impossible. For example, the restriction on having GE trees flower in the field will create barriers to understanding gene flow. So, he said, the challenge is to develop innovative experimental approaches that shorten the experiment time and that can be done within the current regulatory structure.

As an example, Farnum said that it might be possible to conduct miniature-scale studies to estimate fitness. This could be done by creating the nursery equivalent of field plots by planting seedlings at closer densities, then observing them growing up and undergoing mortality. This would allow studies to be done more quickly, he explained. If trees were planted at a 1:12 spatial scaling, he said, time would be compressed by a factor of six. So a study that would take 18-24 years with regular spacing would theoretically take 3-4 years with dense spacing.

Farnum said certainly there are uncertainties in this approach, but a critical test could be conducted to try to falsify this hypothesis. Assuming the method was not falsified, then the relevant data could be gathered quickly, and the method could be used for studying transgenic plants, non-transgenics, and mixtures. Most important, he said, studies could be completed in 3-4 years and would result in some first-order hypotheses for the fitness effects of fast growth.

In conclusion, Farnum said, we need to focus on what's possible, not on what will not work. And we definitely need to think creatively and develop creative solutions. He said he hoped the outcomes of this workshop would include multi-stakeholder research priorities and plans, as well as necessary action steps to develop funding sources, assemble cross-disciplinary research teams, and identify barriers and ways to address them.

In the brief question-and-answer period following Farnum's presentation, one participant pointed out that pre-existing data can also be helpful in answering research questions. That is, scientists can learn from past experience regarding the movement of genes of non-transgenic trees, if they know the history of introductions in an area. Another participant argued that scientists do often need to be involved in policy, because if they do not, policy will be made by nonscientists. Policy must be continually informed by science, he said. Finally, a participant said she agreed with Farnum that foundational research is needed, but she said that often such research has gotten short shrift from federal agencies. She said agreement on the need for foundational research for GE trees would represent a significant step forward.

Panel One: Ecological Effects

Panel 1 consisted of four speakers who discussed issues relating to ecological effects. Sarah Reichard from the University of Washington addressed the issue of invasive plants; Jeffry Mitton from the University of Colorado talked about site-specific adaptation in conifers; Jeff Wolt of Iowa State University spoke about nontarget organism exposure and risk assessment; and Alan Gelfand of Duke University discussed multi-level stochastic modeling for risk assessment.

Invasive Plants: What Are They and Why Are They Relevant to You?

Presentation by Sarah Reichard, Ph.D., University of Washington

Sarah Reichard is Associate Professor in the College of Forest Resources at the University of Washington. She talked about invasive plants and the implications of invasiveness with regard to genetically engineered trees.

Reichard first talked about what invasive species do. In general, she said, they compete for resources that are in limited supply, such as water, light, and nutrients. They also compete for pollinators and seed dispersers, which are sometimes limited. For example, Reichard said, studies have shown that purple loosestrife, which is a higher nectar producer than native loosestrife, can crowd out the native species in part because honeybees differentially pollinate the invading species (Brown et al. 2002).

Invasions can have cascade effects as well, Reichard said. For example, nutrient flows change when species invade. Scientists studying invasive "nitrogen fixers" (plants that transfer nitrogen from the air to the soil) have documented that the change in nitrogen nutrient flows is persistent for a year *after* the invasive species are removed (Dougherty and Reichard 2004). So, changes to soil chemistry persist over a long period, Reichard said, and are not resolved by simply removing the invasive species.

Reichard used the example of knotweeds to illustrate the significant cascade effects that may result from an invasion. A few species of knotweed, she said, are invasive in the Northwest and can replace forest trees that grow along streambanks, including willows, cottonwoods, and some alders. Knotweeds are more efficient than the native trees at transferring nitrogen into their roots and rhizomes. (They have a 75% efficiency rate, Reichard said, whereas alders have a 5% efficiency rate and willows 33% (Urgenson 2006).) Because knotweeds store nitrogen in their roots and not their leaves, knotweed leaves have less nutrient value than native tree leaves. Thus when knotweed leaves fall in the autumn, they are a much lower-nutrient food resource for macroinvertebrates than native leaves. And macroinvertebrates are eaten by fish, so fewer macroinvertebrates mean less food for fish. Reichard said the entire aquatic food web is thereby being altered in some locations, and scientists believe this may be one reason why salmon have become endangered. The knotweed thus provides a good example of the serious, long-term effects that can result from invasive species, she said.

Reichard then turned to discussing why species become invasive. The conventional wisdom, she said, is that species become invasive when they are separated from the predators that keep them in check in their native range. An entire

field of biological control is based on this premise, Reichard said. But she explained that there is debate about how well the "enemy-release hypothesis"—which argues for releasing native herbivores[1] to suppress invasive species—really holds. A meta-analysis of 63 field studies, including more than 100 non-native plant species, found that native herbivores (especially vertebrates) did suppress non-native plants (Parker, et al. 2006). So, native generalist herbivores do provide biotic resistance, Reichard said. But species do not become invasive just because they are released from native herbivore pressure, she said; there are many other contributing factors.

Why do some species become invasive and others do not? It comes down to population dynamics, Reichard said. If a plant is a successful reproducer and stress tolerator, it is going to be a more successful invader. Thus it's helpful, she said, to look at the traits that increase the number of births (via sexual or vegetative reproduction) and those that decrease deaths (i.e., that enable stress resistance). Reichard then shared a slide (Figure 4) showing the basic model of population dynamics. In this equation, B and D represent births and deaths, while I and E refer to immigration into and emigration out of a population. If B + I is greater than D + E, Reichard said, population size will increase.

Figure 4: Basic model of Population Dynamics

$$N_{t+1} = N_t + B - D + I - E$$

$$B + I > D + E = \text{increases in population size}$$

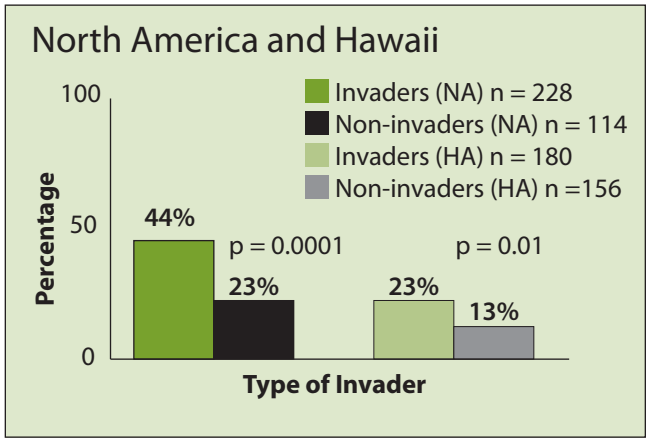
1 *Native herbivores* refers to herbivores that are native to the region from whence the invaders came.

Reichard then described a meta-analysis she conducted that looked at introduced woody species, some of which became invasive and some did not (Reichard and Hamilton 1997). She noted that the study is relevant to this workshop because some GE trees may, as a side effect, have increased invasive ability. In the meta-analysis, Reichard and her colleagues looked at 235 invasive species in North America and 181 in Hawaii that were identified in the literature and verified by experts and herbarium specimens. In addition, they looked at 114 species not known to invade but available in catalogs prior to 1930 in North America, plus 156 non-invasive species listed as grown in Hawaii before 1950 by Hawaii Garden Flora.

One of the traits analyzed, Reichard said, was vegetative reproduction capability. Vegetative reproduction is asexual reproduction that takes place through rhizomes or stolons. This ability increases the number of individuals and increases stress tolerance, thus decreasing deaths. If there is a catastrophic event, like a flood, fragments of a plant that can reproduce vegetatively may survive. Not surprisingly, then, the study found that vegetative reproduction was positively correlated with invasiveness. (See Figure 5.) It also found in the Hawaii samples that coppicing—the ability of aboveground biomass to regenerate and reproduce if it is removed from the belowground biomass—is associated with invasive ability as well.

The study also looked at the duration of juvenile periods, Reichard said. Woody plants, of course, tend to have longer juvenile periods before becoming productive than nonwoody plants. The analysis found that woody plants with shorter juvenile periods are more likely to become invasive than those with longer periods. This may relate to fast growth, Reichard said, because there is a strong correlation between early onset of reproductive capacity and rapid growth through

Figure 5: Vegetative Reproduction



the juvenile phase. Fast growth may be something that developers of GE trees will be purposely selecting for. The earlier a tree attains reproductive status, Reichard said, the more the propagules, and the trait, will spread (depending when it's harvested as a crop). This raises the question, she added, of whether it's possible to engineer for both fast growth and sterility.

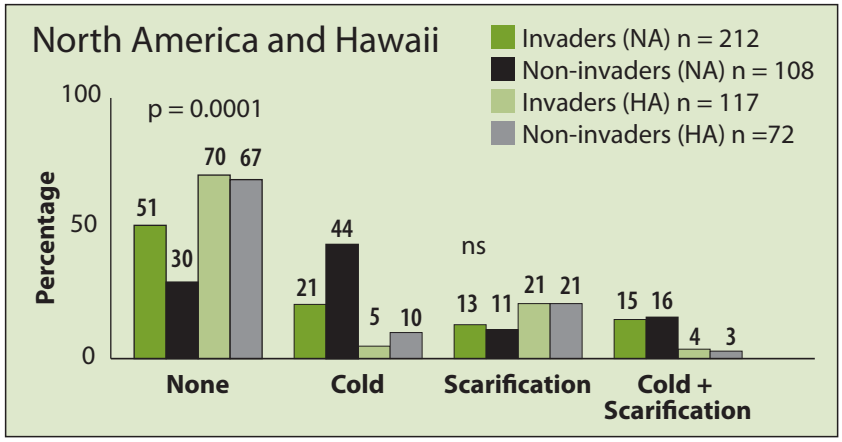
Ability to fix nitrogen is also correlated with invasive ability, Reichard said, because it's an advantage for moving into poor soils. In addition, germination requirements are correlated with invasive ability. If a seed does not have exacting conditions under which it will germinate (for example, through cold or scarification), then it is more likely to be invasive. (See Figure 6.)

Reichard next talked about some of the physiological traits of invasive plants. Purple loosestrife (*Lythrum salicaria*), for instance, is efficient at assimilating carbon and growing fast. It has been found to assimilate 208% more carbon per unit energy in leaf biomass than two natives (Nagel and Griffin 2004). Its higher growth rate may enable it to be more competitive and to rebound from stress, she said.

Other physiological traits associated with invasive plants, Reichard said, include high photosynthetic capacity and ability to photosynthesize over a long period of time. This was shown in a study comparing two invasive and two native *Rubus* species, she said (McDowell 2004). In a similar vein, evergreenness or semi-evergreenness can also be strongly associated with invasive ability—though this depends upon location. Deciduous plants tend to be less invasive on the West Coast than the East, Reichard explained. That's because in the West there's a period of summer drought, so when the plants are photosynthetically active, water is limited. But plants that are evergreen on the West Coast will have an advantage and may be more invasive.

So, what makes a species have an impact? That's not easy to determine, Reichard said, and it is very context-dependent. The traits relating to impact include: Being a good competitor (e.g., having aggressive root growth, abundant nectar, pulpy

Figure 6: Germination Requirements



fruits); nitrogen fixation (but only in situations that are nitrogen-limited); geomorphic changes; an increase in fire intensity and frequency; an ability to hybridize with natives; and structural differences. In assessing the risk of invasiveness, Reichard said, it's important to consider the context and where the plants are going to be used.

Propagule pressure is another key issue to consider, Reichard said. If a plant starts out with a small population and then has a population crash, it may not be able to rebound. But if it starts with a large population and there's a drop, it may be able to rebound. Reichard then shared two studies that have shown this to be the case. First, she said, Mulvaney (2001) studied the invasive ability of 5,000 nonnative ornamental species planted in Canberra after it became capital of Australia in 1909; Mulvaney found that the number of times a species was planted was correlated to its invasive ability. Second, Dehnen-Schmutz, et al., (2007) analyzed English nursery catalogs from the 1850s and 1860s and today. They found that 46% of the non-escaping species were still in catalogs, while 79% of the escaping species were still in the catalogs. So, Reichard said, the strongest predictor of invasive ability in England was that the species continues to be sold.

In summary, Reichard said, many factors affect invasive ability. Some biological traits that affect reproductive output and stress tolerance are correlated with invasion probability. And the number of times a species is planted may increase the probability of invasion.

Reichard said she would like to see new research address the following questions:

- Can gene flow between GE plants and natives or cultivated plants increase fitness and invasive ability?
- Can engineering for faster growth and other traits increase fitness and invasive ability in otherwise noninvasive species?
- How do we detect or monitor these species early so we can control them early?

After the presentation, one workshop participant asked Reichard if she would consider eucalyptus and poplar to be invasive. Reichard said she would, since both species have some of the traits that lead to invasiveness, such as high reproductive output and good stress tolerance.

Another participant asked if plants that can vegetatively reproduce but that are seed sterile are problematic. Reichard said she has found only two species that are invasive that *only* produce vegetatively. But, she said, some of the plants people consider to be pests are much more likely to reproduce vegetatively. In short, she said, it's not necessary for a plant to be a vegetative reproducer to be invasive, but a plant can be more invasive if it reproduces by both methods.

Finally, a participant asked if the ability to be an invasive species is inherited, such that if you change one of the traits that may lead to invasiveness a plant will still be invasive. He said he believes invasiveness to be an "eco-physiological syndrome," and that the use of invasive species as a physiological model for the effects of transgenic species on the environment is actually very coarse. Reichard commented that what's important is how traits are combined. She said her main message is that traits relating to high reproductive output and stress tolerance are the ones she is interested in as a biologist studying invasive ability, and they are also the traits GE plant developers are interested in in terms of increasing fitness.

Exquisite Site-Specific Adaptation in Conifers: Assembled by Selection, Eroded by Gene Flow

Presentation by Jeffry Mitton, Ph.D., University of Colorado

Jeffry Mitton is Professor and Chair of the Department of Ecology and Evolutionary Biology at the University of Colorado. He delivered a talk about site-specific adaptation in conifers that he prepared with Yan Linhart, who is also a Professor in the Department of Ecology and Evolutionary Biology at the University of Colorado. Mitton said the first half of the talk would focus on how site-specific adaptation is assembled by natural selection, while the second half would address how it is eroded by gene flow.

Genetic variation in conifers is very high, Mitton said, no matter how you measure it. In fact, conifers are one of the most variable groups ever studied. The genetic variation is structured, Mitton explained, such that it varies among environments. Foresters have learned that they cannot move conifer seeds around with impunity—for example, across mountain ranges or between mountains and valleys—because trees (and their seeds) are well adapted to growing right where they are. Mitton shared a map of Washington showing the large number of "seed zones" in the state. (See Figure 7.) This map shows, Mitton said, that each type of seed is well adapted to grow only in small, specific areas within the state. Mitton

Figure 7: State of Washington Tree Seed Zone Map

Source: *http://www.dnr.wa.gov/Publications/lm_wfn_seedzone_book.pdf*

said that Jerry Rehfeldt—a scientist who has studied ponderosa pine, lodgepole pine, Engleman spruce, and western hemlock—found that if you move seeds more than 300 meters up or down a slope, the resulting seedlings will not grow well. So, Mitton concluded, trees are very well adapted to where they are at present and cannot easily be moved. Mitton said he calls this phenomenon "exquisite site-specific adaptation."

Mitton then talked about "M site," a forested area in Colorado that Yan Linhart has studied for more than 30 years. Every tree on this site is known for 12 allozyme polymorphisms, Mitton said, plus mitochondrial DNA, chloroplast DNA, carbon isotope discrimination, resin pressure, whether deer eat them, whether woolly aphids are present, and the trees' age and growth rate. What Linhart has discovered, Mitton said, is the following: The seeds on the site have little inbreeding—only about 5% are inbred to some degree. And as the trees grow up, ongoing site-specific selection takes place, the inbred genotypes disappear, and more and more of the trees are heterozygous. So, the trees on this site are more heterozygous (and a smaller proportion of genotypes remain) than you would expect from natural selection over a period of 50-250 years.

Mitton said they looked at ten allozyme polymorphisms and also made comparisons with other sites. For example, they compared this south-facing site with a north-facing site across the road. And they used peroxidase polymorphisms to compare a series of seven canyons—south-facing and north-facing slopes. Very reliably, Mitton said, their results revealed allelic frequencies and genotypic differences between the hillsides. Natural selection discriminates between north- and south-facing slopes within a generation, they found. And that was true even though there was gene flow (via pollen) between the sites (Mitton et al. 1977).

Mitton then shared another example showing exquisite site-specific selection. He said it is well known that when white settlers came to Boulder, Colorado, they changed the environment. By putting out fires and tethering livestock, the early settlers allowed trees to move down from the steep mountain slopes above Boulder onto the surrounding grasslands. Mitton and his colleagues have studied the trees in the plains and determined that they are not a random sample of the trees on the slopes above them. They found, rather, that the peroxidase heterozygotes, which are more common on south-facing slopes, were the first to colonize the grasslands (Beckman and Mitton 1984). Then when the forest filled in and the canopy closed, the advantage shifted from peroxidase heterozygotes to the common homozygote, which is most common on north-facing slopes. They also found that the density of the trees changed over a few hundred meters, and the genotypic frequencies changed with tree density and the nature of the canopy.

Mitton then moved to describing research that has been conducted by him, Tom Whitham, and Neil Cobb at the Pinyon Ecology Research Group at Northern Arizona University in Flagstaff, Arizona, relating to glyserate dehydrogenase in pinion pine. In this case the researchers looked at variation on and off cinder soils, he said, and they found one locus that responded fairly regularly. There were two common alleles—2s and 3s—which combined into three genotypes (2-2, 2-3, and 3-3). The genotype that was more common on lava soils was the 3-3, Mitton said (Cobb et al. 1994). But lava soils and normal soils are such a dramatic comparison, he said, that he wondered if the differences would show up in a pinion pine stand that does not have that remarkable soil variation.

Thus he assessed pines in Owl Canyon, near Boulder. At this site, he said, he could visually see

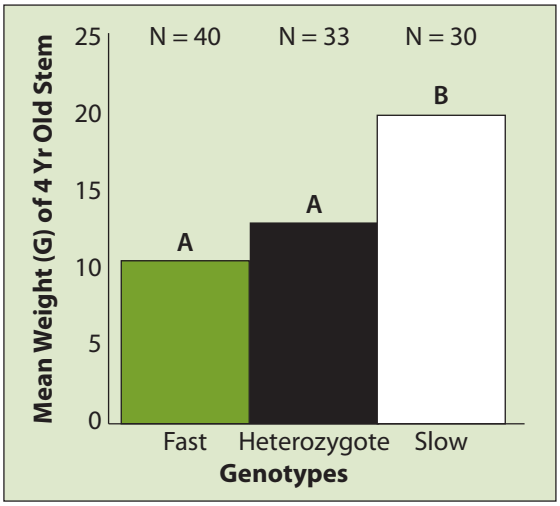
Figure 8 : Gly Genotype Frequencies Owl Canyon

Genotype	22	23	33
Wet	42	35	4
Dry	32	53	15

the differences between wet and dry sites. In the dry areas the trees were small and widely spread, with yucca and other plants growing between them. One hundred meters away were wetter sites where the trees were larger and closer together, and no yucca grew between them. When he characterized the genotypes on those two types of sites, Mitton said, he saw the same pattern as they found on lava and non-lava soils. The homozygotes most common on lava soils turned out to be the same as those growing in the dry sites near the yucca (Mitton et al. 1998). Thus it's clear that trees respond to fairly subtle variations in environment, Mitton said. He shared a slide (Figure 8) that showed the number of each genotype in the wet and dry sites.

Mitton and his colleagues also looked at growth rate and viability on lava soils, and they discovered some surprising results. Mitton shared a slide (Figure 9) showing the mean weights of a 4-year-old twig, and how they differ for fast-growing genotypes, slow-growing genotypes, and heterozygotes. What this shows, Mitton said, is that there is a 2:1 growth rate difference in trees intermixed in an area as big as a large room. If we assign a fitness of 1 to the fast-growing genotype, he said, the fitness is about .6 for the heterozygote and .4 for the homozygote (Cobb et al. 1994). Growth rate tells you viability, he explained. And he noted that in both cases, the selection coefficient against the slow-growing homozygous genotype would be greater than .5.

Figure 9 : Biomass Production



The hypothetical example in Peter Farnum's paper, Mitton continued, which was handed out to participants prior to this workshop, used selection coefficients of .01 (Farnum, Lucier, and Meilan 2007). The actual selection coefficient for this genotype is greater than .5. It's interesting to go back and read that paper again, he said, imagining the effects with an even larger coefficient such as .5.

So, how did these huge differences came about? To look at this, Mitton studied the size of the stomates in trees intermixed in a small area. What they found was that the shapes and sizes of the stomates do not overlap—some are long and narrow, some are rounder. These are trees in one site with genotypes separated by one locus (Mitton et al. 1998). And these differences can scale up, Mitton said. When they looked for range-wide variation, they found that it corresponds with summer rainfall. In dry places the 3 allele is common, while in relatively wet places, it's rare (Mitton et al. 1998).

Regarding gene flow in conifers, Mitton said there is little gene flow of mitochondrial DNA in conifers, because it is moved by seeds. But pollen can and does go far. Mitton and his colleagues did a

paternity analysis in a population of limber pine far out on the Eastern plains of Colorado. They genotyped all 177 trees at this site for ten allozyme loci. They then collected seeds from those trees and separated the endosperm from the embryo, so they could determine maternity and paternity for all of the trees on the site. This in turn helped to determine pollen flow.

Mitton shared a chart showing the results (Figure 10). It shows that quite a bit of pollen had flowed between 0 and 50 meters, but some came from beyond 550 meters. Specifically, Mitton said, 7-15% of the pollen came from outside the population (Schuster and Mitton 2000). Where did it come from? There is one tiny cluster of trees several miles a way, Mitton said, but those trees are downwind. Mitton said it is thus more likely the pollen came from populations in the mountains, 70-100 miles away.

Mitton shared one final example that showed a similar result. He explained first that, a thousand years ago, the ponderosa pine that had grown in California during glacial times started moving east, and it met and mixed with the ponderosa pine that had been growing in Arizona and New Mexico and had migrated north. Thus there is a transition zone in which the two species have been hybridizing for a thousand years. Mitton said he and Bob Latta analyzed that transition zone with mitochondria DNA (mtDNA) and chloroplast DNA (cpDNA). MtDNA is moved only by seeds, he said, and the mtDNA from those two zones is almost perfect. In contrast, the cpDNA varies more gradually over space, for wind has blown pollen in both directions. As expected, gene flow via pollen far exceeds gene flow via seeds (Latta and Mitton 1999). (See Figure 11.)

In summary, Mitton said that when he looks at a stand of trees, he sees the few that remain after stochastic accidents and natural selection have whittled down an enormous pool of seeds to a fewer number of seedlings and an even smaller pool of adults. Those that remain are perfectly adapted to their sites, he said. And those trees are standing in "the rain"—the rain of pollen carrying genes from other sites. The question is, he said, it is a downpour, or a fine mist? That's what scientists still need to figure out. But it is clear that

Figure 10: Pollen Flow

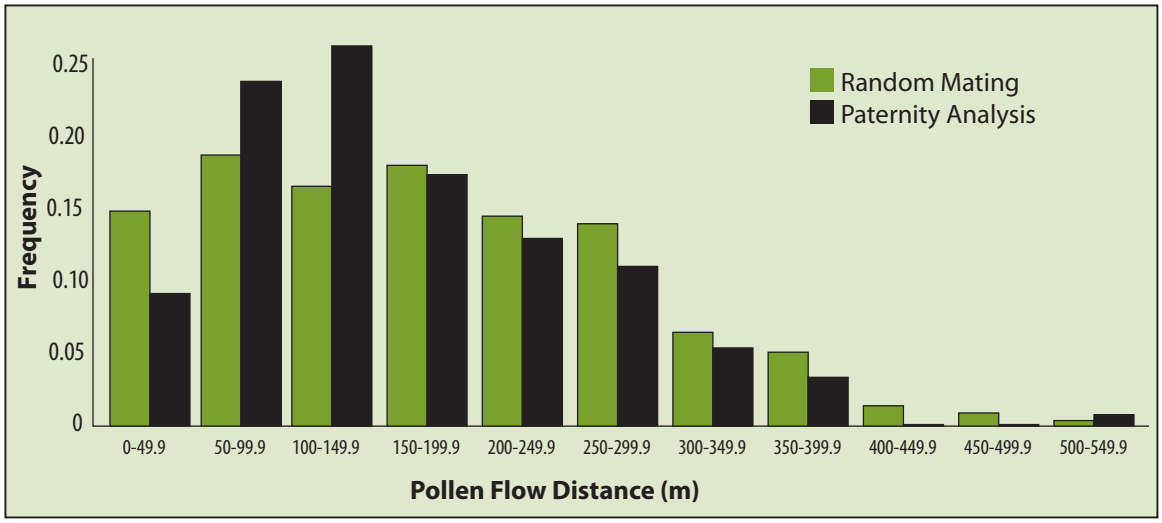
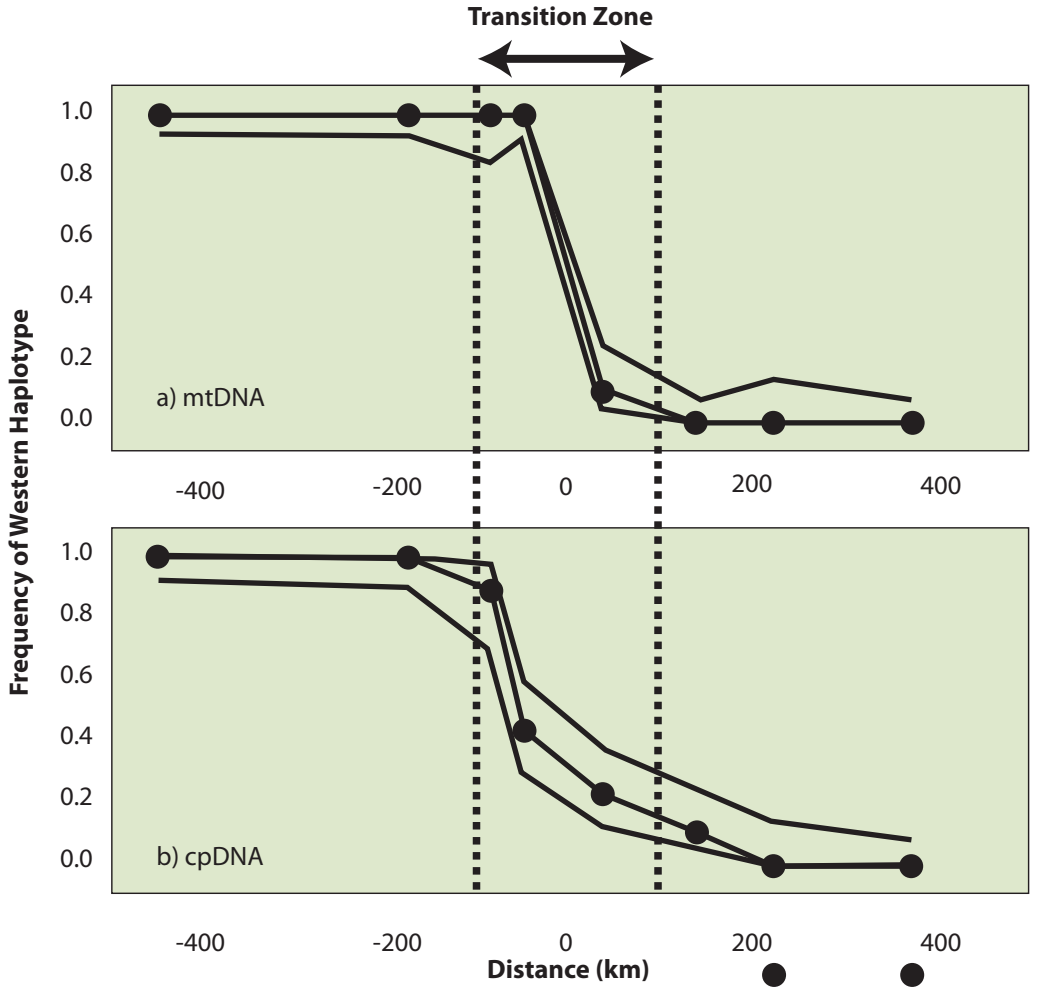


Figure 11: Gene Flow via Pollen



pollen is coming in from sites 100 meters to 20 kilometers away, at least.

After the talk, one workshop participant asked Mitton how he reconciled his results with studies of loblolly pine that have shown there to be more variation within than between seed zones. Mitton said conifers are so variable that it's easy to get that kind of result, particularly if the researchers were looking at just one trait, such as wood strength or growth rate. Mitton's interest has been in total adaptation to a site and all of the traits that relate to that.

Nontarget Organism Exposure and Risk Assessment

Presentation by Jeff Wolt, Ph.D., Iowa State University

Jeff Wolt is Professor of Agronomy at Iowa State University. He spoke about the environmental risk assessment process (also called ecological risk assessment) being used to assess the impacts of GE crops on nontarget organisms.

The formal definition of environmental risk assessment (ERA), Wolt said, is as follows: "The process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors [or actions]" (EPA 1992). This definition has been used by the Environmental Protection Agency (EPA) for 25 years for a variety of technologies, he said. "Stressors" in the case of GE plants and trees, he added, are the novel products expressed in the transformed plants.

The goals of the ERA framework, Wolt said, are to:

- Develop a unified conceptual approach to environmental assessment
- Facilitate cooperation and collaboration between assessment-related disciplines
- Increase the transparency of risk assessments to users (risk managers)
- Provide standardized tools and techniques
- Dispel the perception that ecological risk assessment is impossible (Barnthouse 2006)

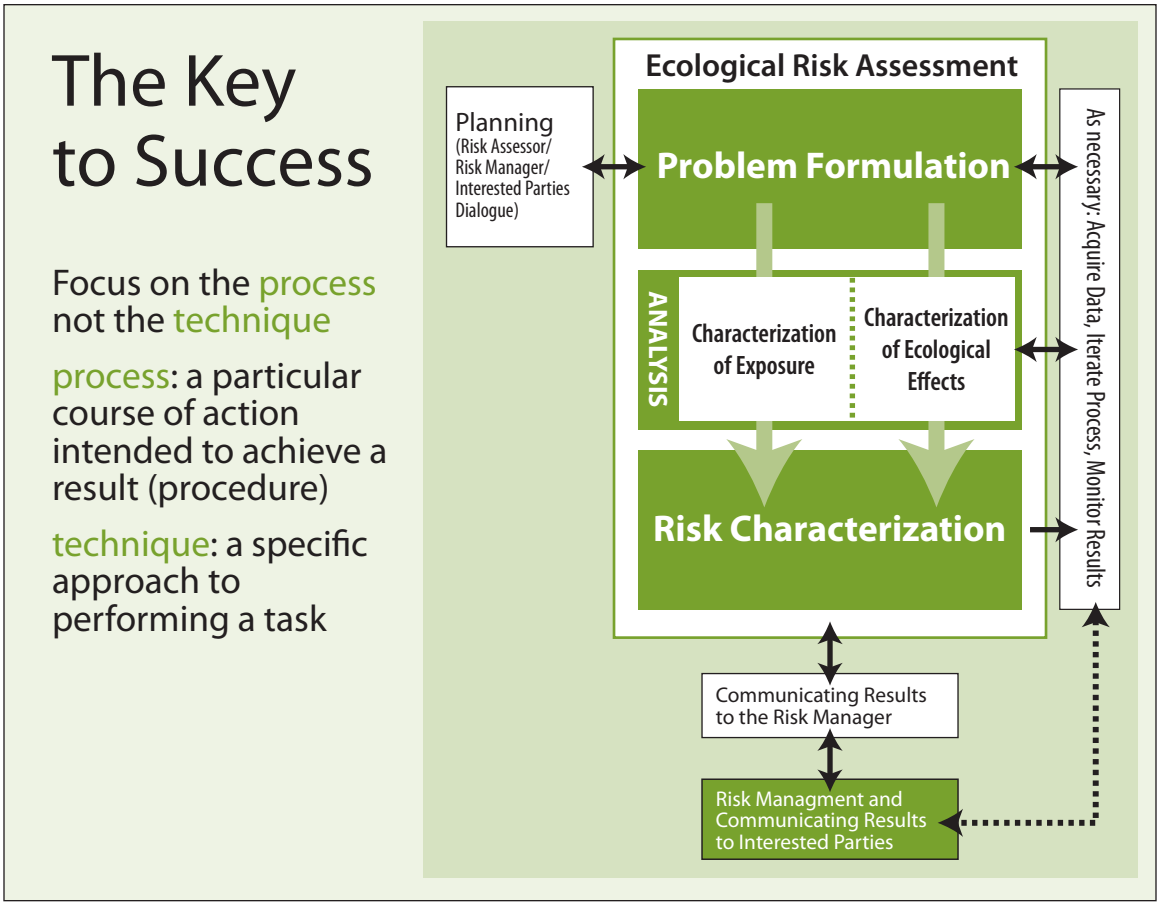
In short, Wolt said, ERA is a tool whereby the scientific community can communicate their understanding of science to policymakers and the public. It is a framework or roadmap—a logical process by which scientists can organize and integrate complex information. And in general, he added, it is a hypothetical description of a complex process.

The key to success with ERAs, Wolt said, is to focus on process, not technique. A process, he explained, is a particular course of action intended to achieve a result. A technique is a specific approach to performing a task. Wolt then shared a graphic from the EPA's framework document (Figure 12) showing the process for an ERA (EPA 1992). It shows stakeholder input at the front end and stakeholder communication at the back end, bookending the basic steps of problem formulation, analysis, and risk characterization.

Wolt's next slide showed the process in more detail (Figure 13). It begins with problem formulation, Wolt said, which includes two phases: Effects characterization (itself made up of hazard identification and measures of effects) and exposure characterization. Those two factors, Wolt said, are integrated into a risk characterization, which is delivered as a risk conclusion. The risk conclusion ideally says, "Based upon the problem we formulated, this is our state of knowledge (in probabilistic terms) and this is our state of uncertainty." So, Wolt said, the risk assessment outlines what is known and unknown.

Wolt then reviewed some definitions. *Risk*, he said, is the likelihood of harm to be manifested under relevant conditions of exposure (i.e., the probability of harm given exposure). Ideally risk describes the joint probability of exposure and effect (risk = f(exposure, effect)). Also, it is ideally fully quantitative, though Wolt said in reality with GE plants it may not be. We thus need to be open to weight-of-evidence analysis, he said, in looking at the risk from a GE plant.

Figure 12: The key to success with ERAs

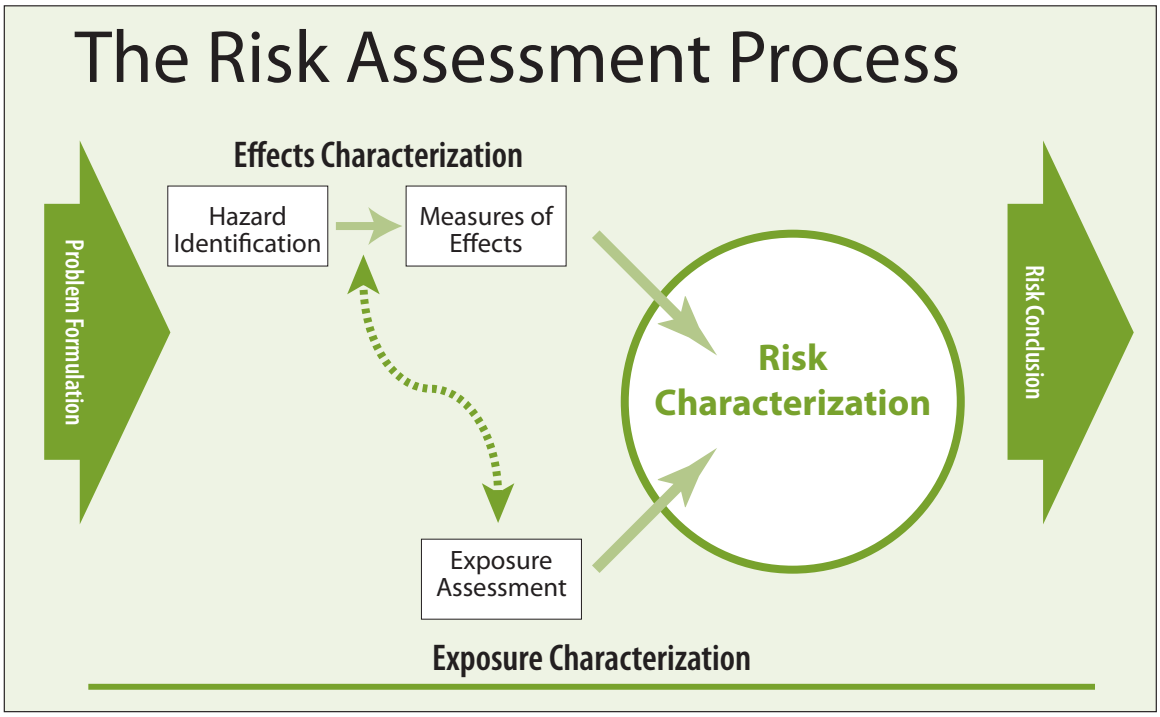


Problem formulation sets the stage for an ERA, Wolt said. When dealing with new and novel technologies, Wolt said, a litany of concerns can be listed, but problem formulation enables identification of the ones of greatest consequence. It also outlines the analysis plan for the analytical phase of the ERA—what studies are needed to address issues of consequence and ecological entities of concern. It also allows the identification of the relevant starting point (known as a tier) and the appropriate endpoints of concern.

Hazard, Wolt continued, is an intrinsic biological, chemical, or physical property with the potential to cause harm. In an ERA, he said, we want to understand the hazard or perceived hazard, then we can understand the effect. Adverse *effect* is a consequence of exposure. It is commonly addressed as toxicity, he said, but more broadly it is any adverse consequence of exposure. Wolt noted that while the instance of hazard is often interpreted as risk, hazard and exposure need to be integrated to get a sense of actual risk.

Exposure is often overlooked, Wolt said, if a formalized ERA process is not conducted. To look at exposure, one has to establish the causal chain linking deployment (e.g., putting a novel plant into the environment) through exposure to effect. In other words, the assessor must determine what

Figure 13: Effects Characterization and Exposure Characterization



is known regarding the duration, intensity, frequency, and route of exposure.

Nontarget organisms are what ERAs are being applied to, Wolt said. With GE crops to date, he said, this paradigm has been applied mostly to insect-resistant crops where the novel product is insecticidally active, and scientists are interested in how it might impact nontarget insects. Thinking more broadly, Wolt said, scientists must consider all of the environmental entities of concern that could be affected by (in this case) GE species. These might include individuals, populations, communities, ecological services, and functions.

The GE ecological risk considerations we would factor into a GE crop risk assessment, Wolt said, start with product characterization, which is part of the problem formulation phase. This is often overlooked by GE product developers, Wolt said; they fail to recognize that a lot of product characterization information is needed, including that regarding host and donor familiarity, the activity and specificity of the novel product, protein equivalency, and expression. Wolt said the assessor must then factor that information together with risk characterization, which includes an assessment of effect (given the hazard), exposure, and risk. Together this all leads to a risk conclusion. The risk conclusion, Wolt said, describes the adequacy of the risk characterization and is generally conservative, in balance with the given uncertainties.

Environmental risk assessment for GE crops is complex, Wolt said, due to the facts that genetic engineering is a relatively recent technology and biological information is not fully quantifiable. There are two levels of concern, he explained. The first are the stressor-mediated effects on individu-

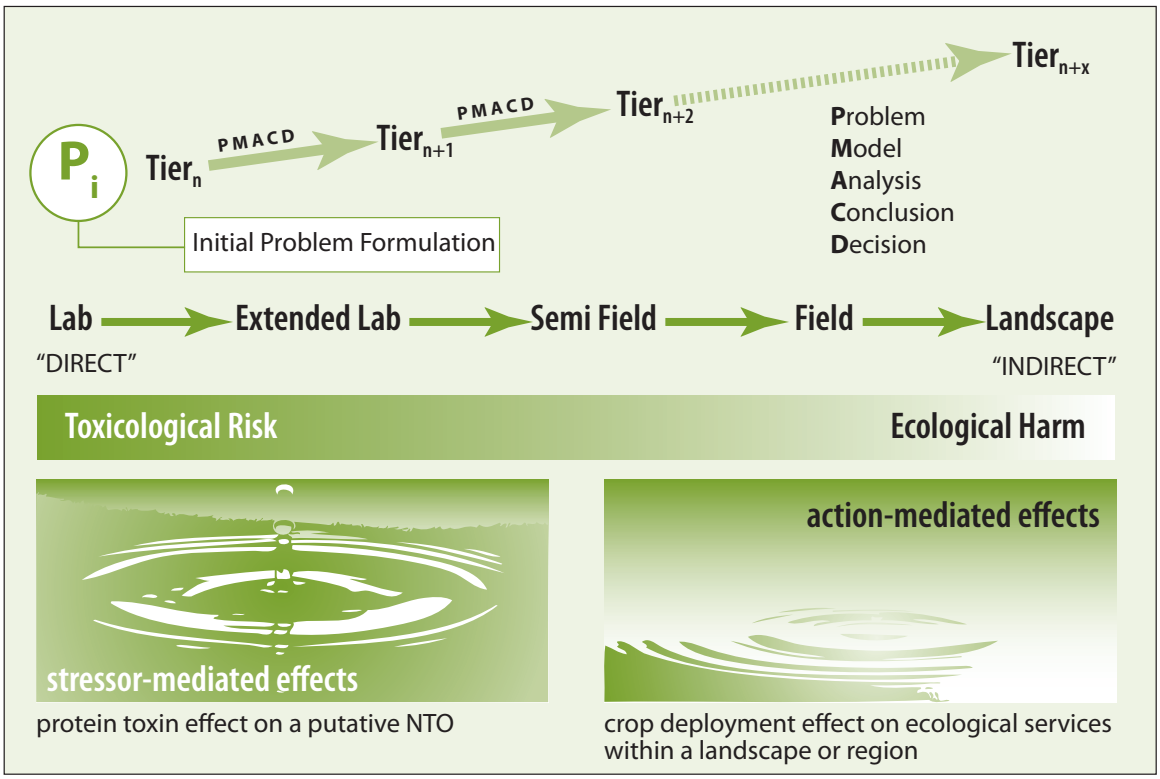
als and populations (i.e., those that arise directly from the novel product); the second are the action-mediated effects of deployment on populations and communities (i.e., those that result in the broader environment from the deployment of the novel product).

Wolt said the general philosophy toward ERAs for GE crops is as follows:

- They should describe the weight of evidence based on a comprehensive evaluation of the data.
- They should proceed from general understanding to specific entities of concern.
- They must support findings with quantitative data and analyses to the fullest extent possible.
- Risk-based findings should focus on harm that may be manifested and exposures that are environmentally relevant.
- They should seek a determination of reasonable certainty of no harm to the environment.
- Stressor-mediated effects provide insight as to action-mediated effects.

Wolt then shared a schematic showing the assessment continuum and conceptual shift within a tiered scheme of ecological risk assessment (Figure 14). It shows, he said, how individuals conducting ERAs for nontarget organisms for GE crops are trying to understand them from the point of view of stressor-mediated effects (in lab settings) and action-mediated effects (in the field or landscape level). ERAs involve a tiered pro-

Figure 14: the assessment continuum and conceptual shift within a tiered scheme of ecological risk assessment



cess, he explained, through which we must find the appropriate level to study. We must decide, he said, if further research is warranted and, if so, what the nature of it would be.

Wolt said he believes this ERA process is broadly applicable to GE crops and GE trees. It has been used for a range of technologies, systems, and environments. He said assessors must continually look at process improvement, especially as the process is applied to new technologies. Also needed is some idea of how well the information translates to community- and landscape-level environments. He noted, too, that assessors need to implement appropriate tiered strategies and decision points. And he questioned if ERAs and testing are consistent with the product-development paradigm that is likely to be used (or is being used) for GE trees.

In the interest of time, Wolt skipped his final few slides and summarized his main points. We've seen the ERA process used for a variety of GE products over the years, he said. There has been a positive record of successful evaluations and subsequent safe use of the commercial products. Wolt said he encourages the use of ERAs because the process allows for focused, objective, science-based decision making. It has sufficient flexibility to encourage the use of a wide number of testing strategies, modeling paradigms, and analytical tools, to come up with a synthesis of ecological risk for the products of biotechnology. And, he concluded, it forces scientists to ask the appropriate questions at the appropriate times. That is, it constrains and focuses the risk assessment on consequential concerns—real risks.

After Wolt's talk, one participant asked how environmental risk assessment takes into account the depth or value of the different harms that may occur. Wolt said the ERA process does not qualify the level of harm, as that is a policy issue, not a scientific issue. Another participant argued that scientists should seek to assess benefits to nontarget organisms, not just harms. Wolt agreed and said this same framework could be used to assess the benefits. He noted that the EPA conducts benefit analyses separately from harm analyses. Another participant noted that APHIS considers benefits under its Endangered Species Act obligations.

Multi-Level Stochastic Modeling for Risk Assessment

Presentation by Alan Gelfand, Ph.D., Duke University

Alan Gelfand is J.B. Duke Professor of Statistics and Decision Sciences at Duke University. His talk focused on stochastic modeling and Bayesian analysis for risk assessment, which could be used in the development of GE trees.

Gelfand said he would skip talking about risk assessment generally, since Jeff Wolt just covered that topic. Gelfand did mention, however, that actions imply consequences, and inputs imply outputs. We need to specify and identify inputs, he said, as well as consequences and threats. And we have to identify the things we are imagining to be affected by genetic engineering.

Ideally, he said, we would like to characterize the risk or chance of an adverse outcome in a probabilistic fashion. That requires a notion of stochasticity, and a stochastic model.[2] That in turn leads to formalizing the scope of the model. The sophistication of the modeling, Gelfand said, frames the input-output scenarios you can examine. We all probably start small and eventually build. Within any framework, Gelfand said, the general ideas he will describe in this talk will apply.

So, what is Bayesian analysis for risk assessment? Gelfand said it is simply Bayesian inference applied to action/consequence scenarios.

And what is Bayesian inference? Gelfand explained it as follows: Say we want to determine the probability of event B happening. That can be noted as *P(B).* Then some new event—call it A—comes along, and we want to revise the chance of the event B to *P(B|A)* so we can get an adjusted probability. We must implement this adjustment through Bayes rule. Bayes rule considers the chance of A happening given B and the chance of A happening if B didn't occur. It is expressed in this equation:

$$P(B|A) = \frac{P(A|B)P(B)}{P(A|B)P(B) + P(A|B)P(B)}$$

In stochastic modeling, Gelfand said, we replace events with random variables, and replace simple probabilities with probability distributions. Say X is an exposure level, and Y is an adverse health outcome. Then you want to look at *f(Y|X)* (i.e., the distribution of Y health outcomes given X exposure). This looks like a regression setting, he said. So, where is Bayes in that?

We need to take a bigger view, Gelfand said. He suggested we think about the entire collection of unknowns in this scenario, and call that U. Then we should think about the data we can bring to bear, and call that D. The way we usually specify models, he said, is to explain the data we've seen given the things we don't know. Those unknowns might include the parameters in our models, regression coefficients, variances, variance components, and/or random effects. We can express such a model as *f(D|U)* (i.e., the distribution of the data given the unknowns).

Gelfand said we should add another step to this, however, whereby we incorporate all of our *a priori* knowledge about the unknowns in our scenario. We can represent that with *f(U),* he said.

2 *Stochasticity* means, essentially, randomness—a lack of any predictable order or plan. *Stochastic* can be defined as involving a random variable or variables.

Figure 15: learn about the process given the data

What we really need

- Consider an ecological process with a real data set of latent (unobservable) activities say X, e.g. stomatal conductance, carbon assimilation, carbon allocation, etc.
- Process can be in time, in space
- Since the process is not deterministic (or we don't/couldn't ever know enough for this to be the case), let $f(X)$ denote the distribution that produces process realizations
- Let Y be the data we can collect associated with the process, i.e., Y is driven by X so we envisage $f(Y|X)$
- Again, turn the Baysian crank and obtain $f(X|Y)$

Then, Gelfand continued, Bayes rule allows us to turn this into the distribution of our unknowns given the data. That is, we can infer about what we don't know from what we have observed. This is the Bayesian perspective, Gelfand said. It is represented as $f(U|D)$ (i.e., the distribution of the unknowns given the data). There is subjectivity in these things, of course, he said—in both the *a priori* knowledge and in the model building itself.

The U values, Gelfand said, can be model parameters, such as regression coefficients and variances. But they can also be future values or forecasts, or even interpolations (in the sense that we might know what's going on in a portion of the study region and can interpolate to other portions).

Most important, Gelfand said, what we can get from this not just a point estimate and a standard error, but an entire distribution of what we don't know. That substantially enriches the inferential possibilities, he said.

With an entire distribution of unknowns, Gelfand explained, we can address much more useful scientific questions. We can talk about consequential threats, he said, such as exceedances (i.e., the probability of an outcome exceeding some specified threshold). Or we could talk about quantiles (i.e., specifying the outcome level so that the chance of exceeding that is some sort of percentage). So, he said, there is a richer range of possible inferences when we have an entire distribution of what we don't know.

Gelfand then restated the distinction between classical analysis and Bayesian analysis. Classical analysis, he said, would ask us to focus on the model for the data, given the unobservables. And it would ask us to explain what we have seen given what we don't know. That's the usual sampling distribution idea, he said. He added that this seems backward to him, and Bayesian analysis seems more natural, in that it asks us to explain what we don't know given what we have observed. The more data we collect, he added, the more we can continue to revise what we don't know.

Figure 16: hierarchical models add text here to fit...

Not so simple—need a hierarchical or multi-level model

- Defining X; what is the scope of our modeling?
- Specifying $f(X)$. Can never provide this model precisely; use process knowledge combined with process uncertainty to provide a model $f(X|\theta_{model})$
- Similarly, can never provide $f(Y|X)$ precisely. Again. $f(Y|X,\theta_{data})$
- What about θ_{data}, θ_{model}? How would we know them? Are they fictitious? $f(\theta_{data})$, $f(\theta_{model})$
- So, full model is

  $f(Y|X,\theta_{data}) \cdot f(X\theta_{model}) \cdot f(\theta_{data}) \cdot f(\theta_{model})$
- And Bayes rules yields

  $f(X,\theta_{data},\theta_{model}|Y)$

Next we have to think about decision making, Gelfand said, and how to connect a distribution under a model into a decision. To do this we would turn to notions of utility theory, he said. We'd have to specify the losses for taking certain actions given certain states of the unknowns. To do this we would build loss functions or general utility functions. He explained that $L(u,a)$ denotes the loss for taking action a when u is the true state of the unknowns. We can envision taking actions of various types, he said. In other words, we have the unknowns out there, the data we collect in the face of that, and then actions we can take based on the data we've collected. This is a standard decision theory framework, he explained, and there is an optimal solution. The optimal solution is discovered through a Bayesian decision rule. If working with posterior distributions, he said, we can address things in this fashion. The challenge is to identify the utility. We have to decide what the costs and benefits are, and some sort of tradeoff is going to have to be captured in our utility. It's subject-matter specific and has to be teased out through informed discussion, he said.

Gelfand asked participants to think about an ecological process in which much is unobservable. For example, he said, you cannot actually see stomatal conductance, carbon assimilation, or carbon allocation. You can collect relevant data, however (e.g., light availability, leaf area). So, he said, you have to imagine we have the process operating and the data that we can see. Then, we can "turn the Bayesian crank" and learn about the process given the data. (See Figure 15.)

This is not a simple thing to do, Gelfand said. It has unknowns in it, and even the data given is going to have unknowns in it. In any case, he said, we create a full model, and the Bayesian paradigm allows us to turn things around to define everything we don't know given what we've observed. (See Figure 16.)

Gelfand then shared a few additional ideas about modeling. All models are incorrect, he said, but some are useful. For a complex ecological process, he said, the model will take the form of a directed graphical model. If we want to assess our models, and how confident we are in using them for inference, some believe it is a question looking at the parameters. Gelfand said he believes instead we should look at predictions, forecasts, and interpolations—things that mean something regardless of the model.

In conclusion, Gelfand said, uncertainty is the critical issue. We cannot make simple quantitative statements without assessing the uncertainty that goes with them. One of the advantages of Bayesian analysis, he said, is that you can propagate the uncertainty associated with all parts of the specification.

Full-Group Discussion of Ecological Effects

After the four presentations in Panel 1, Norm Christensen, Professor of Ecology at Duke University's Nicholas School of the Environment, summarized key points from the presentations and then moderated a full-group discussion.

Christensen began by saying that scientific discoveries are not typically limited by ignorance, but by a denial or ignorance of ignorance. The challenge, he explained, is to identify what we don't know. Christensen then summarized, in a shorthand way, the five types of ecological risks that Allison Snow presented. He said they include: creating new bad things, making bad things worse, harming the bystanders, disrupting more complex communities of things, and causing irreparable loss to species diversity and the functioning of ecosystems. He said Sarah Reichard's talk on invasiveness raised for him three questions that ought to be part of the research agenda: Can we anticipate patterns of invasiveness that arise from cumulative effects and trophic cascades? Do we understand the features that make something complex? And, do we understand the environment in which an invasion may take place?

Jeff Mitton, Christensen said, talked about conifers and the unique issues relating to longevity and extensive pollen drift. His talk raised the question, Can we understand genetic change specific to GE trees in the context of the broad complexities of genetic change? The focus of transgenics is often to develop a particular performance feature, he said, but there are other features that will affect the fitness or invasive ability of a species.

Christensen then noted that Jeff Wolt raised the issues of how well we understand the components of risk and how we define hazards. The hazards and risks may include direct effects and stressors, Christensen said, but also indirect effects, in complex ecosystems. Other questions raised by this talk, he said, include: What is our basis for assigning risk? What if we have risk-risk tradeoffs? And what's our comparator—regular cropping systems?

With Alan Gelfand's presentation, Christensen continued, we learned much about the power of Bayesian inference. He challenged us to be more systematic about what we don't know, Christensen said, and argued that it's the power of prediction that is important. The point about models, he continued, is whether we can embed a model for both analysis and understanding. That's made more difficult by the fact that trees have very complex life histories.

Christensen then opened the floor for discussion among all workshop participants, and the discussion focused first on the potential unintended effects of genetic engineering. One participant asked whether there is evidence of pleiotropy among the genes being introduced into GE crop plants.[3] Another participant responded that scientists know pleiotropy takes place, but they have not defined its limits. He said it needs to be looked at on a gene-by-gene basis. Another person said peer-reviewed policy articles have been published that catalogue the unexpected effects that have arisen from GE plants. What is unclear, he said, is whether transgenic lines exhibit more pleiotropy than conventionally bred lines. No research program seems to be addressing that, he added. Later, another participant argued that

3 *Pleiotropy* occurs when a single gene influences multiple phenotypic traits.

transgenic plants have in fact been shown to exhibit higher levels of pleiotropy than conventional plants. Another participant said he read a paper a few years ago that revealed a *Bt* corn to have unexpectedly higher levels of lignin than regular corn. He added that when genes are transferred into wild populations with heterogeneous genetic backgrounds, it becomes even more difficult to predict what effect they might have, as they may be affected by alleles at other loci.

Another participant argued, however, that the rules of breeding are not breached by genetic engineering. There has been an enormous amount of experience to date with things like GE soybeans, he said, which seem to behave just like conventional soybeans. A body of empirical evidence exists, he said, showing a lack of meaningful unintended effects for that crop, as well as for corn. Regarding the study of lignin in *Bt* corn, he said, questions were raised about how that analysis was conducted.

Another participant noted, too, that breeding efforts in conventional agriculture have sometimes turned up unexpected detrimental effects. She recalled one situation in which potato breeders inadvertently transferred toxic alkaloids into eating potatoes, and the problem wasn't identified until many years into the breeding program. Something similar occurred in celery production, she said, wherein individuals harvesting a certain kind of celery broke out in rashes. She asked Alan Gelfand if he felt Bayesian inference would supply adequate decision-making tools given the possibility of these kinds of unintended consequences. Gelfand responded that quantitative data is needed to make decisions, and he said we are in a "data-starved environment" in relation to information on GE trees. With adequate quantitative data, he said, the tools exist to make decisions.

One participant then asked Jeffry Mitton several questions. Let's assume we find an allele in ponderosa pine that promotes drought tolerance, this person said, but in other places that allele has negative effects given changes in frequency. But let's say that allele could be commercially useful in loblolly pine, he continued. Let's also assume that doing some of the science to meet regulatory requirements can be very expensive and hard, particularly if done event-by-event as we do today. As a scientist, he asked, what kind of studies would you demand be done to prove the safety and efficacy of such a gene transfer? Mitton responded that that sort of experiment should be relatively low risk, because you would just be introducing one more allele into a species. But he said he would have a lot of questions about the gene action involved and the impact of the transfer. Is there something special about the background physiology of ponderosa pine that allows it to work? he asked. Would it have the same consequences in a different species?

Finally, another participant asked Alan Gelfand what process can be used for specifying and prioritizing the unknowns. Gelfand responded that that information can be distilled in different ways so as to frame models to define what we know. He further said he advocates the development of models by interdisciplinary teams, to ensure that all the unknowns are identified.

Panel Two: Gene Flow and Hybridization/Introgression

Panel 2 consisted of three presenters who discussed issues relating to gene flow, hybridization, and introgression. Steve DiFazio of West Virginia University spoke about methods for tracking pollen flow and seeds; Nathalie Isabel from Natural Resources Canada discussed alternative approaches for monitoring gene flow from GE trees; and Amy Brunner of Virginia Tech talked about transgenic systems for sterility in *Populus*.

Approaches for Tracking the Physical Movement of Pollen and Seed from Tree Plantations

Presentation by Steve DiFazio, Ph.D., West Virginia University

Steve DiFazio is Assistant Professor in the Department of Biology at West Virginia University. After acknowledging his co-authors, contributors, and funders,[1] DiFazio summarized the key points of his talk:

- Dispersal does not equal gene flow.
- Gene flow does not necessarily imply risk.
- Effective gene flow matters, and it is best measured directly.
- Measuring gene flow is extremely difficult for trees due to large spatial and temporal scales.
- An ecological frame of reference is required for transgenic risk assessment for trees.
- The context of gene flow is of paramount importance.

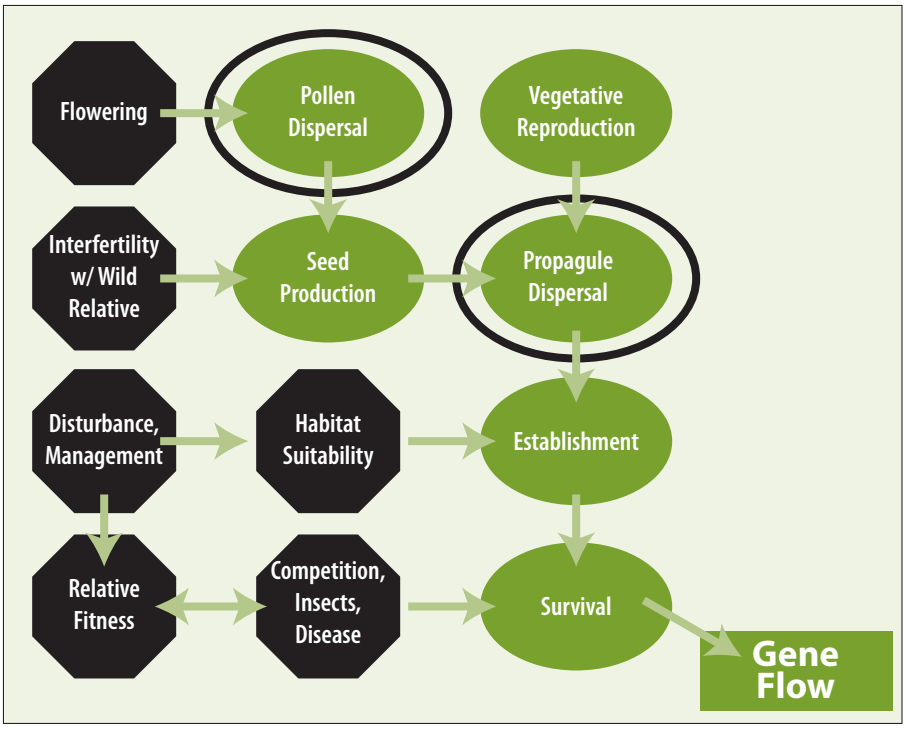
DiFazio then went through each point in turn. To illustrate that dispersal does not equal gene flow, he shared a slide (Figure 17) showing that dispersal is only one component in a complex set of interactions involved in gene flow. These interactions are driven in part by management and ecological factors, DiFazio said, as well as biological phenomena such as seed production, vegetative reproduction, and flowering. Gene flow could actually be inhibited at any step in this process, he said. In short, it is important to take a holistic view.

In addition, DiFazio continued, gene flow is only one part of the risk equation. Hazard identification is also very important and context specific. But, he added, gene flow is important and may constitute a hazard if wild relatives are compromised by hybridization. Of course, gene flow can sometimes be beneficial, he said, even with transgenics; the American chestnut is an example in which scientists may actually want to encourage gene flow.

DiFazio then shared methods for estimating gene flow. The simplest, most direct approach, he said, is to include a unique marker in the transgenic plant and then assay for the marker. Unfortunately, assaying is not a simple task, DiFazio said, because the probability of encountering the marker at long distances is quite low. So, some sort of Bayesian framework or iterative process may be required to optimize sampling efforts. Another issue, DiFazio said, is that this method actually assesses dispersal and may or

1 DiFazio's coauthors included Steve Strauss, Gancho Slavov, and Stefano Leonardi. Other contributors included Steve Garman, Tom Adams, Shuping Cheng, Tracy Allen, Jace Carson, Rick Meilan, Ben Straub, Gokcin Temel, and Eliza Walthers. The work DiFazio discussed was funded by the USDA's Biotechnology Risk Assessment Program, the Department of Energy's Biofuels program, the Tree Genetic Engineering Research Cooperative (now TBGRC), and the National Science Foundation's Frontiers in Integrative Biological Research.

Figure 17: Dispersal ≠ Gene Flow

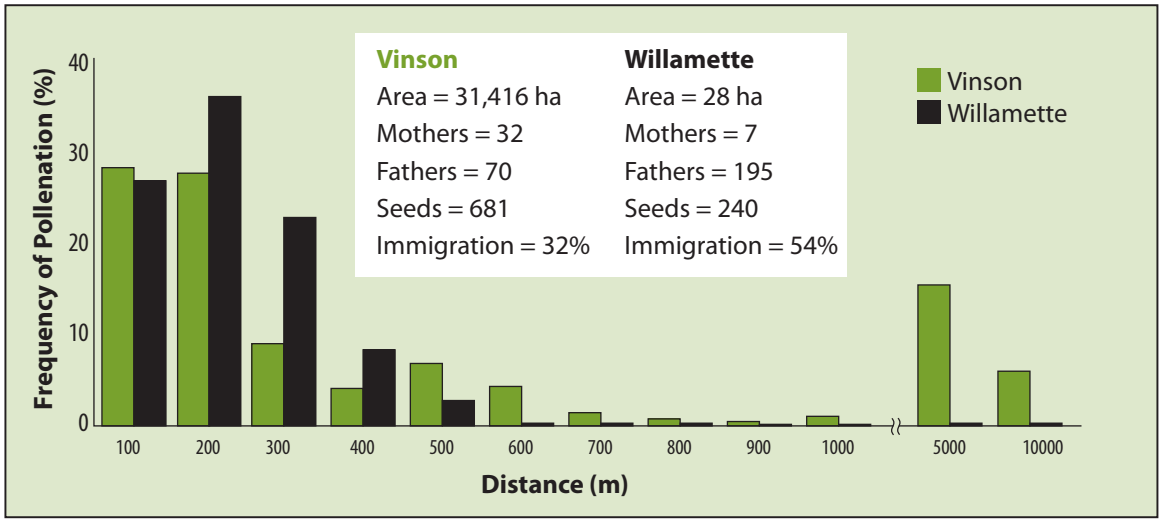


may not indicate gene flow itself. Whether actual gene flow takes place depends on a variety of factors (Figure 17), and rates of gene flow depend in part on the lifecycle of the organism. And with trees, he said, the time it takes for gene flow to occur is likely much longer than, say, the duration of a typical research grant.

Indirect estimates using population genetics approaches can be used to measure gene flow, DiFazio said. In this method, the degree of homogenization between two populations is seen as a function of gene flow, and the number of migrants over time is indirectly proportional to the degree of differentiation of the populations. Such indirect estimates are powerful, DiFazio said, because they enable an average picture of gene flow over a long period of time and for a large number of populations. However, this method cannot be used to measure gene flow over a short timeframe. Also, DiFazio continued, the assumptions of population genetics models are often violated in real populations. One of the assumptions, for example, is that there's an equilibrium between migration and genetic drift. That's not likely to be the case when introducing a new large source of germplasm to the landscape, he said, as with a transgenic tree plantation. And because the lifecycle of trees is so long, it is going to take a very long time to reach an equilibrium situation. So, DiFazio said, using evolutionary timeframes and population genetics models is not going to be the most useful approach for estimating gene flow in a transgenic risk assessment.

Mechanistic models of dispersal are another approach, DiFazio said. This method has been used very successfully in the past to estimate both seed and pollen dispersal in crops and trees. It involves measuring the physical characteristics (e.g., terminal velocity) of seeds and pollen, and using weather data and models of particle dispersal and laminar flow to estimate a dispersal curve. Using such a method, DiFazio said, very long-distance dispersal events appear possible and even probable, as has been shown by Claire Williams and colleagues (Williams et al. 2006). Once again, however, this method estimates dispersal and not

Figure 18: Pollen Dispersal Curve: Empirical Data



actual gene flow. To get to gene flow, he said, one would need to incorporate elements such as competition, interfertility, and habitat suitability. Also, he added, because these models are very complex, they are computationally intensive for landscape-scale simulations.

The final approach DiFazio discussed was parentage analysis. This process involves genotyping all of a population of plants to determine the parentage of a given seedling. This method can be used to measure contemporary gene flow, DiFazio said, and it is relatively assumption-free. At the same time, it is time-consuming, expensive, and limited to small populations.

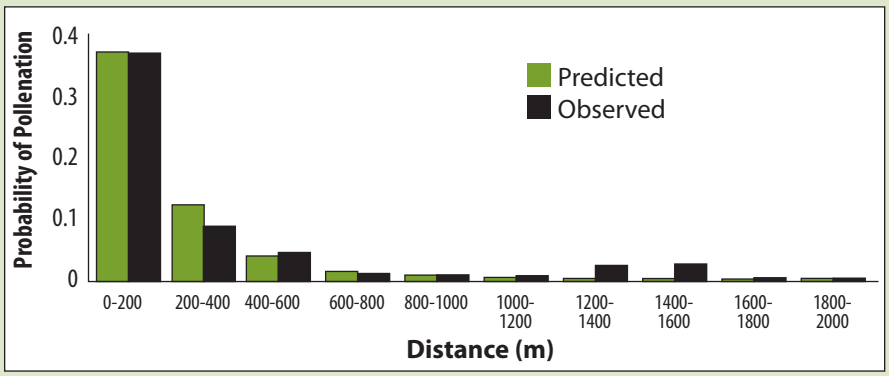
DiFazio then provided an example of the type of data that can be garnered from a parentage analysis. He showed a slide with data on pollen flow from two wild populations of *Populus trichocarpa* (black cottonwood) in the Pacific Northwest. (See Figure 18.) The X axis represents distance, while the Y axis represents frequency of pollination. The two populations, represented with white and black bars, had very different characteristics. One was a low-density population in eastern Oregon; the other was a high-density population in western Oregon. Despite the differences between the two populations, DiFazio said, the observed effective pollination distances declined to background levels at about the same point, and the pollination curves were relatively concordant (DeFazio et al., submitted). So, DiFazio said, this is an effective way to estimate a pollination dispersal curve for a wide variety of situations.

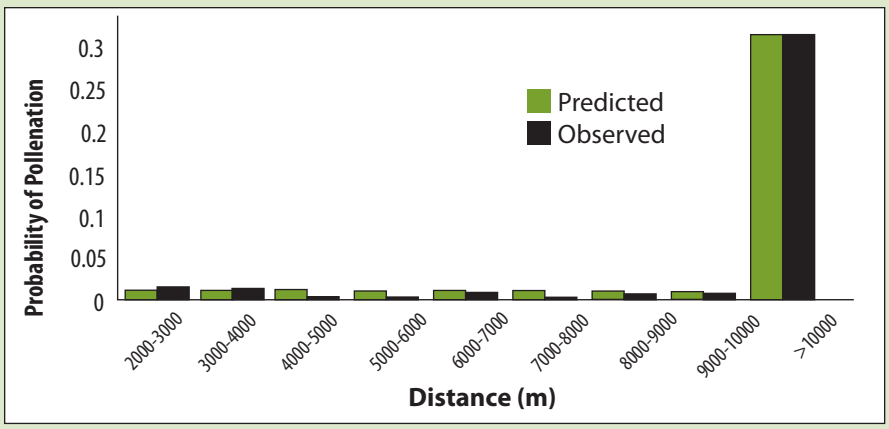
Once you have this empirical data, DiFazio continued, you can fit it to a model to estimate the probability density function of pollen dispersal. In Figure 19, the X axis is distance, and the Y axis is the probability of dispersal to that point. This is a mixed model, DiFazio explained, wherein a mixing parameter (p) is estimated simultaneously with the parameter that describes the negative exponential component of the function (β). This allows accommodation of a long tail of uniform probability that extends many kilometers. In this case, he said, the exponential part of the curve declined to background at 600 meters, and the tail extended to 37 km. So, effective pollination is occurring between parents that are 37 km apart (DeFazio et al., submitted). The bottom line

Figure 19: Fit to Fixed Model

$$f(x) = p\left(\frac{1}{\beta}e^{-\frac{x}{\beta}}\right) + (1-p)\frac{1}{u}$$

$\beta = 179$ m
$u = 36{,}910$ m
$p = 0.56$





Exponential portion declines to background at 600 m

Tail extends to 37 km

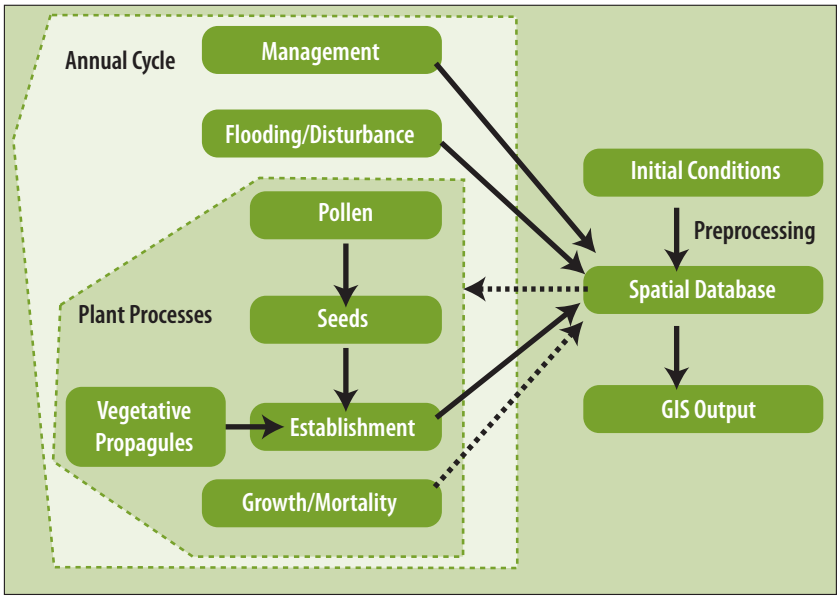
from this study, DiFazio said, is that gene flow distances in trees are extremely long, and the tails of the distribution curves go on almost indefinitely. And, he noted, it is very difficult to devise an integrated experimental approach for assessing changes that occur at that large a scale.

DiFazio then discussed simulation modeling. Simulation modeling is needed, he said, because of the long generation times and large spatial scales associated with trees and forests. He said simulation models allow for the integration of multiple factors that might affect gene flow, such as effective competition with wild populations, disturbance regimes, habitat creation, management scenarios, biotic interactions, environmental stochasticity, and insect pressure.

DiFazio said he and his colleagues developed a simulation model that incorporates many of these factors. It's called the STEVE model—Simulation of Transgene Effects in a Variable Environment. (See Figure 20.) It includes a module that allows for the simulation of management regimes, flooding, and disturbance. Part of it is parameterized by field experiments such as the one DiFazio described regarding pollen flow. The model is integrated with a GIS database, thereby allowing the incorporation of data from any landscape for which GIS layers depicting tree populations are available.

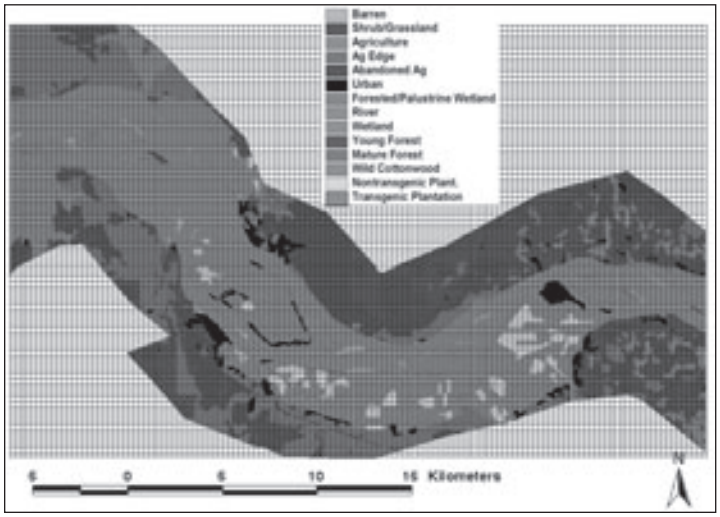
Figure 21 shows an actual landscape that DiFazio's team modeled. Wild populations of cottonwood and male and female cottonwood plantations are represented using different colors on the map. The model divides the landscape into a grid, he explained, whereby each 10x10 meter section is assigned a particular habitat type. The different habitats are then indicated on the map.

Figure 20: Simulation of Transgene Effects in a Variable Environment



DiFazio and his colleagues performed a sensitivity analysis for relative fertility and the competitiveness of transgenics relative to wild types. They found that, at full fertility of the transgenic plant, competitiveness has a significant effect on transgene spread. As fertility is reduced, there is a drastic drop-off in transgene spread, even in the face of high competitiveness. DiFazio concluded that gene flow is mitigated by reductions in fertility. So, he said, one must consider competitiveness and potential dispersal simultaneously. The amount of gene flow taking place from the plantation to the wild was important in determining the amount of potential spread. In short, gene flow *does* matter, and must be considered simultaneously with the fitness effects of transgenes.

Figure 21: actual landscape that DiFazio's team modeled



DiFazio then talked about fractional factorial sensitivity analysis. This involves taking numerous factors into account at once, and it allows the researcher to consider the importance of different factors for a scenario in which there is, for example, a small field trial vs. a large area of commercial cultivation. DiFazio said they discovered that scale does matter, as different factors were important for each scenario. Fertility and

competitiveness were important for both, as might be expected. However, vegetative reproduction was found to be important at the large-scale commercial plantation level, but not at the field-trial level. In contrast, long-distance dispersal of pollen was important at the field-trial level, but not as important in the case of large-scale commercial cultivation (DiFazio et al. submitted) This suggests, DiFazio said, that it is difficult to make inferences from small-scale field trials to larger-scale commercial plantations.

Spatial context matters as well, he said. The model reveals that wild relatives may out-compete transgenics for habitat. Even with the large-scale cultivation of transgenics, DiFazio said, wild plants produce so many more propagules that they survive through competitive exclusion. Over time, then, one would expect to find increasingly fewer transgenic plants as one moves away from the plantation.

In conclusion, DiFazio said:

- Direct measures of dispersal are going to be most effective for estimating transgene flow from tree plantations.
- Dispersal is a small but important piece of the picture.
- The total process of gene flow must be considered, and preferably in an ecological rather than an evolutionary timeframe, with the spatial context taken into consideration.
- The effects of competition from native populations must be considered.

In response to a question after his talk, DiFazio clarified that the simulations took into account both "regular" transgenic trees and those engineered with reduced levels of fertility. In response to a question regarding wild plant propagule pressure, he said that the model has density-dependent mortality, and the transgenics were most of the time in a minority compared to the wild plants, so they were eliminated by competitive exclusion. Finally, DeFazio clarified that in the field study he described early in his talk, the team detected actual gene flow only to 21 km, but the mixed model predicted that gene flow would occur out to 37 km.

Alternative Approach to Monitor Gene Flow and Introgression from GMOs to Wild Relatives

Presentation by Nathalie Isabel, Ph.D., Natural Resources Canada

Nathalie Isabel is a Research Scientist with the government agency Natural Resources Canada. She spoke about the Canadian geopolitical context, short-rotation plantations (the case of *Populus* species), risk assessment, an empirical study of local hybridization, and a metapopulation modeling approach to assess gene flow and introgression. Isabel began by thanking her co-authors: Patrick Meirmans, Jean Bousquet, Marie-Claude Gros-Louis, and Manuel Lamothe.

The regulatory framework in Canada, Isabel said, provides a coordinated system of regulations based on the novelty of a trait or a substance rather than on the method used to make it. It's a case-by-case approach, she said, and regulations vary by province. Isabel said her talk would focus on Quebec, where the poplar breeding program is the largest in Canada and where her research has been conducted.

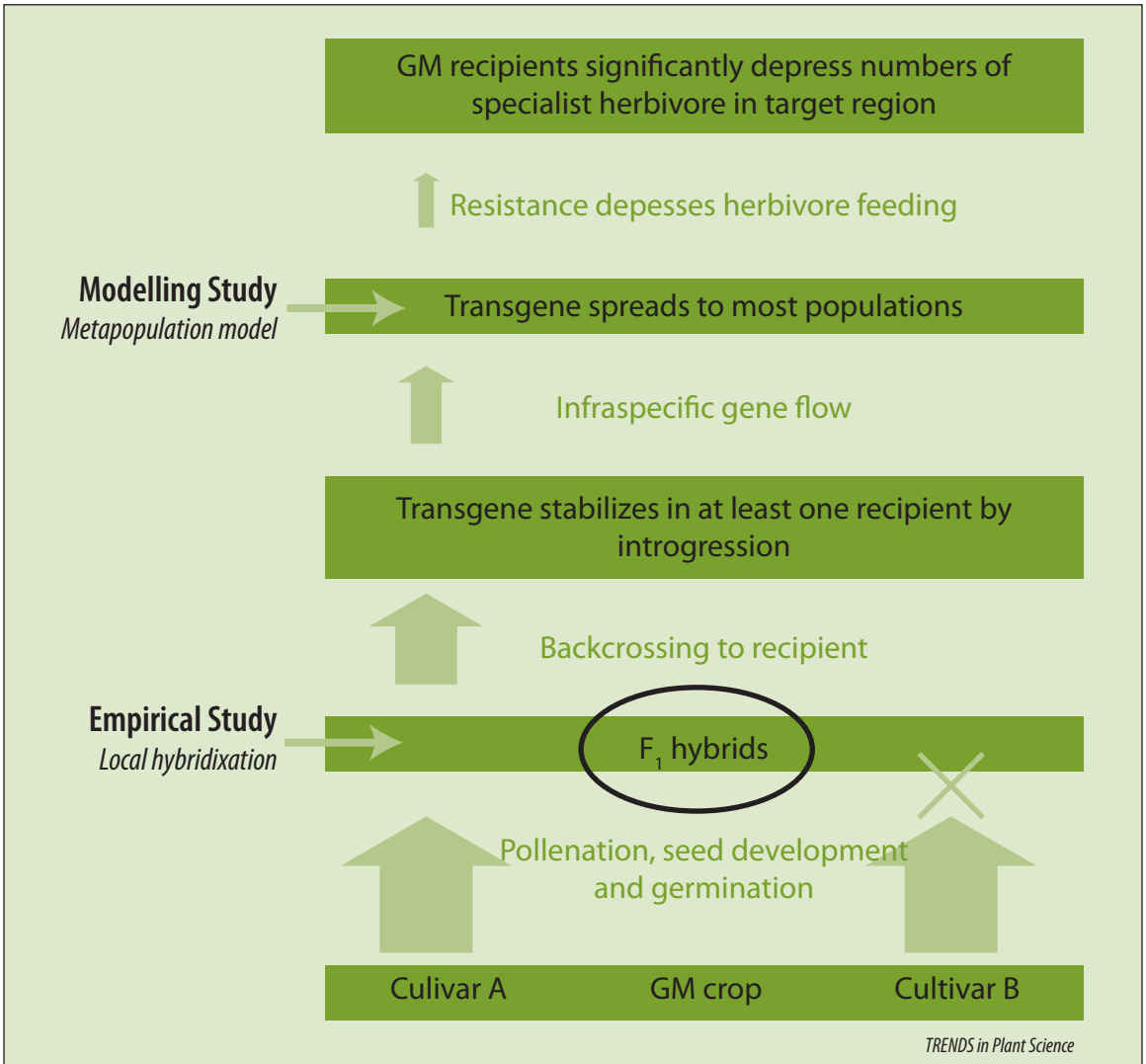
The poplar breeding program started in the early 1970s, Isabel said, and has involved two native species—*Populus deltoides* and *P. balsamifera*—and three exotic species—*P. trichocarpa, P. nigra,* and *P. maximowiczii.* All of these species are interfertile, she said, at least under controlled conditions. Isabel also noted that poplar is a dioecious species—that is, it has separate male and female trees.

The breeding program in Quebec seeks to develop genotypes adapted to various bioclimatic conditions, Isabel said, including resistance to various diseases. More than 5,000 clones are being evaluated, and 40 have been selected so far. *P. maximowiczii* is used most for growth and productivity, she said. Generally, the program selects for growth, productivity, and cold tolerance. The trees with exotic components are developed for two entirely different contexts, Isabel explained: plantations located in the northern boreal forests, and those situated on former agricultural lands in southern Canada.

A unique field trial of GE trees in Canada was established by the Canadian Forest Service (CFS) in 1997, Isabel said, and is being conducted under strict reproductive isolation. Isabel explained that a colleague of hers from the CFS has conducted a study on a GE poplar to look at the persistence in the soil of recombinant DNA (Hay et al. 2002). The GE trees are female poplar clones of European origin. Isabel said the trees are not particularly interesting for use in Canada, but the trial will provide useful data regarding the stability of the transgene and changes in the phenotype over time (Lachance et al. 2007).

Isabel said she and her colleagues did some brainstorming to consider what the next generation of GE trees would look like. They determined that such trees will likely have genotypes that are very well adapted to their bioclimatic conditions, and that genetic engineering could tailor genotypes identified through association studies and/or functional genomics (Groover 2007). A risk assessment of transgene escape for poplars, Isabel said, requires a detailed knowledge of the possibility of backcrossing between natural populations and hybrid clones with exotic components. So, she continued, they decided to gather baseline data on the clones in the breeding program. In particular, they are interested in following the

Figure 22: Risk Assessment (Wilkinson's Approach)



"GMO risk assessment strategy should combine both empirical and modelling studies and includes all steps of the integration process"

(Wilkinson *et al*, TIPS 2003)

spread of exotic alleles from the poplar plantations into the native poplars, in order to understand the potential introgression of transgenes into wild populations.

Mike Wilkinson proposed an approach for risk assessment several years ago, Isabel said. (See Figure 22.) He stated: "GMO risk assessment strategy should combine both empirical and mod-

eling studies and include all steps of the introgression process." (Wilkinson et al. 2003). Isabel said she and her colleagues thus decided to use both empirical studies and modeling.

Isabel then described the results of their research, which took place from 2002 until 2006. For the empirical study of local hybridization, she said, they sought to estimate the rate of spontaneous hybridization from plantations of exotic trees into adjacent natural populations of native species. They had access to plantations of poplars with exotic components producing flowers and pollen in the boreal context in Matane, which is in northern Quebec, and also in Sorel, which is in southern Quebec.

The two recipient species are the two native species, she continued—*P. balsamifera* and *P. deltoides*. The donor species are all of the hybrids between the natives and the exotics that exist on the plantations. The non-plantation poplars at the Matane site are exclusively *P. balsamifera*, while at the Sorel site they are primarily *P. deltoides* with a few *P. balsamifera* mixed in. Their intent, Isabel said, was to have a minimum number of years of data collection and seed collected from a minimum number of native mother-trees.

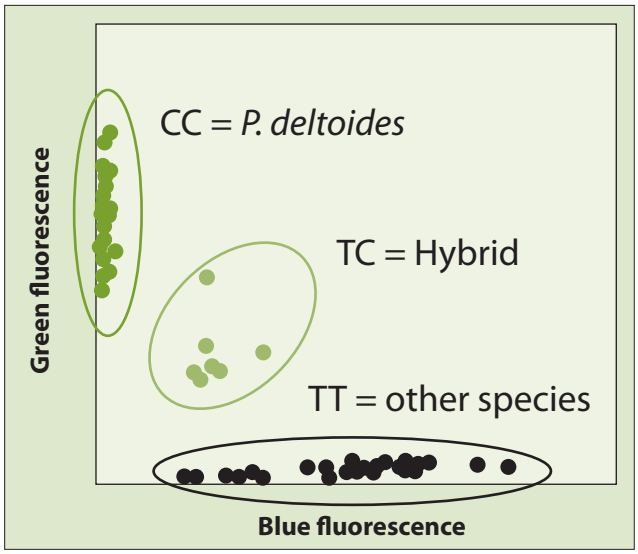
In Figure 23, Isabel explained, the dots represent native trees from which the researchers harvested seed, in order to look for hybridization. For the genotyping, she continued, they used species-specific markers based on an SNPstream assay of gene-loci (mainly transcription factors) (Meirmans et al. in press)[2] and then they compared seeds collected on the native mother-trees before and after germination.

The results of this empirical study, Isabel said, were as follows. In Matane, with the *P. balsamifera* mother trees, most of the paternal alleles came from *balsamifera* as well, with a few from *deltoides* and *trichocarpa*, but very few *nigra*.

In Sorel, only four trees are *balsamifera*, and the study showed that up to 62% of alleles came from fathers other than *balsamifera*. What's curious, Isabel said, is that there is no variation from year to year, but instead essentially the same amount of hybridization each year. In Sorel with the *deltoides*, Isabel said, they found 3% or less hybridization. This result is similar to what they have found in a different study of larch, she said.

In Sorel, Isabel continued, they wanted to know if there was a spatial pattern of distribution of the hybridization rate in a given seedlot, derived from a given native mother-tree according to its position relative to the plantation. No pattern was observed. For example, no hybrid seed was detected in the seed collected from some of the

Figure 23: SNP Stream



2 SNPstream is a trademarked system that enables researchers to automate and scale up the genotyping process.

native mother-trees next to the plantation. But some hybrid seed was found on mother-trees one kilometer away.

When they compared hybridization rates between seeds and seedlings, Isabel said, there were almost no differences. So it appears there is no selection against hybrids during germination.

Isabel and her colleagues have decided to focus now on the fate of a transgene, in parallel. This is because, she said, they have to be aware that when a wild population is in contact with a GM crop, it is also in contact with other populations. So, they have developed a metapopulation model, and they have expanded upon the standard model of population genetics to look at introgression with GMOs. The main result from that, Isabel said, is that the effective rate of introgression depends on the population structure of the wild relative.

So, Isabel concluded, they will continue the study of long-range hybridization and gene spread, and will look for advanced generations of hybrids in natural populations. *P. nigra* was introduced years ago by the first immigrants, Isabel said (Richardson et al. in press). She and her colleagues will also study the potential of genes to spread through the whole range of the native species (*P. balsamifera*) by studying population structure, long-distance gene flow, and molecular evidence of adaptation.

In a brief discussion period after Isabel's talk, one participant posed this question: If a transgene could be created that would increase the niche breadth of, say, a wild cottonwood by 5%, wouldn't that be a benefit to society, since cottonwood is a keystone species? Isabel said that is a society decision, not a scientific decision. The participant suggested that some data could be gathered to help answer the question, however.

Isabel said in response that she and her colleagues have realized that transgenic poplars may not been grown in the forestry context in Canada. If they are grown at all, she said, it would likely be on agricultural lands. So, they have decided to do a study in Saskatchewan of gene flow between shelterbelts on farms (planted with multiple genotypes of poplars with exotic components) and a small fragmented natural stands of *P. balsamifera*. These genotypes were primarily derived from *Populus petrowskyana*, which was introduced by the first immigrants, she explained. (They are a cross between *P. nigra* and *P. laurifolia.)*

Transgenic Systems for Sterility in Populus

Presentation by Amy Brunner, Ph.D., Virginia Tech

Amy Brunner is Associate Professor of Molecular Genetics in the Department of Forestry at Virginia Polytechnic Institute and State University (Virginia Tech). She spoke about transgenic systems for sterility in poplar species. At the outset, Brunner acknowledged the many individuals involved in the work she was to present, including Jingyi Li (University of California-Berkeley), Cathleen Ma (Oregon State University (OSU)), Olga Shevchenko (OSU), Hao Wei (OSU), Rozi Mohamed (Universiti Putra), Brooke Montgomery (OSU), Rick Meilan (Purdue), and Steve Strauss (OSU).

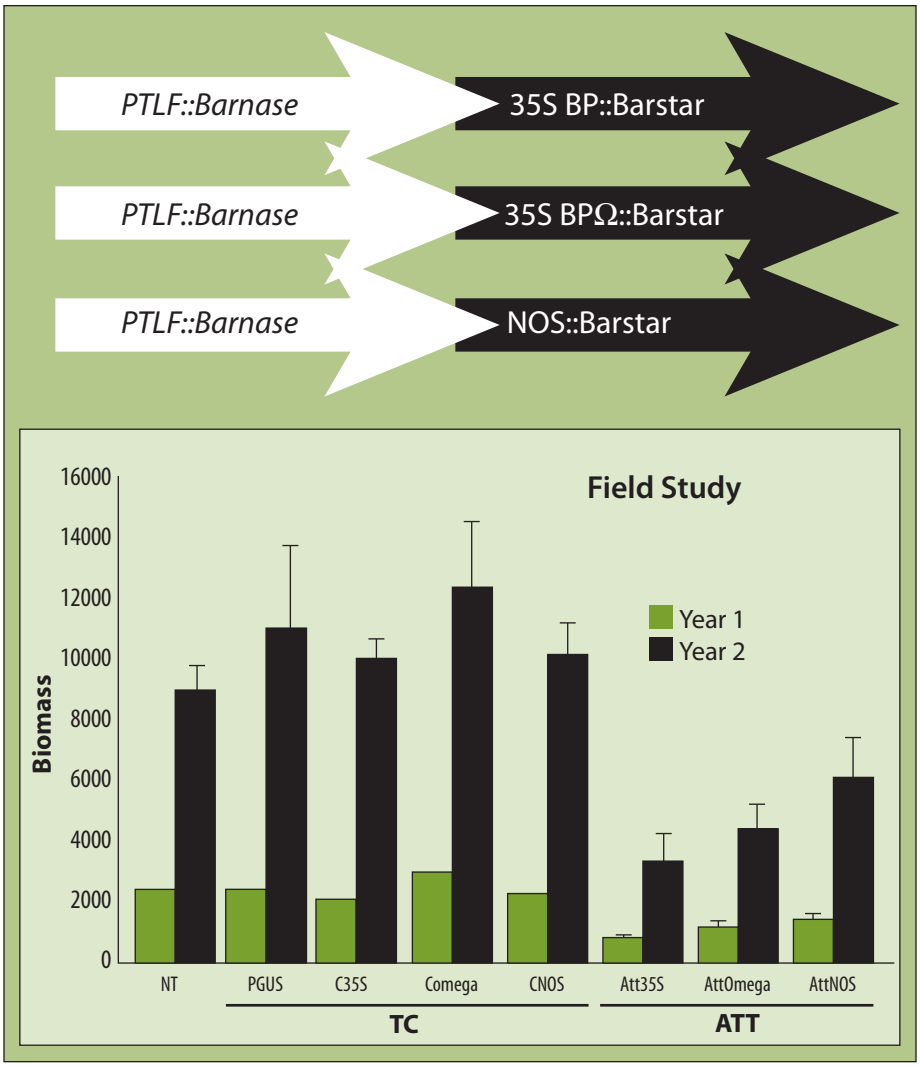
Brunner first shared two general lessons regarding sterility, which she said are probably intuitive but nonetheless worth reiterating. First, she said, much of the research on sterility in trees is based on work done previously with annual plants. The success of a sterility transgene in an annual plant, however, may or may not indicate success in a tree. Second, there is a long lag time between when scientists first develop an interesting approach to sterility and when they can verify that it has worked in a tree. And they must not only determine if a tree is sterile, but also whether or not a sterility transgene affects tree growth. Wei et al. (2006) have shown that growth performance in a greenhouse may not be indicative of performance in the field.

Brunner then described a number of sterility methods (reviewed in Brunner et al. 2007). One of the first sterility methods ever described, she said, was reproductive tissue ablation. More recently, a new method using transgene excision has generated publicity. In this approach, transgenes are excised before the gametes are released (Luo et al. 2007). In both cases, she said, promoter fidelity is important—and is very problematic in trees. Why? Because trees live for so long, they encounter a large variety of different biotic and abiotic events that alter gene expression. Moreover, trees increase greatly in size and complexity over a long time period, and age- or size-related changes in gene expression occur, as well as changes associated with the annual seasonal cycle of growth and dormancy.

To illustrate the issue of promoter fidelity and also the difference between greenhouse and field performance, Brunner described research conducted involving a floral promoter directing the expression of an ablation transgene. She said they knew the *PTLF* promoter would predominantly direct expression of the cytotoxin BARNASE in the flower, and that it would show low levels of vegetative expression. What they tried to do, Brunner explained, was attenuate that low level of vegetative expression by inserting a companion transgene that expressed the inhibitor of BARNASE, called BARSTAR.

In the greenhouse study, she said, growth rates were good, and relative growth rate and biomass were equivalent in *PTLF::BARNASE* transgenics and controls. But in the field, the plants that carried the PTLF transgene were impaired in growth compared to the nontransgenic and transgenic controls that did not contain *PTLF::BARNASE*. (See Figure 24.) Shown on the left are three constructs that contain the *PTLF::BARNASE* transgene and a *BARSTAR* transgene driven by one of three different weak-to-moderate constitutive promoters (35S basal promoter, 35S basal promoter with omega enhancer, and NOS). Control transgenics contained only a *BARSTAR* transgene

Figure 24: BARNASE – BARSTAR Sterility System (Wei et al. 2006)



or the reporter gene construct PGUS (Wei et al. 2006).[3]

Another common sterility method, Brunner said, involves RNA-mediated gene suppression. This method utilizes RNA interference and artificial micro RNA transgenes. Another method is protein-mediated gene suppression. In the latter, she said, you can alter the sequence of the target gene to create a dominant negative mutant transgene that will inhibit the activity of the wild-type protein. Another method is to engineer a "zinc finger" to repress a target gene.[4] Or, it's possible to overexpress a gene that acts as a floral suppressor. The ultimate method, Brunner said, which is not yet feasible, is targeted mutagenesis or gene replacement.

Brunner then discussed concerns regarding sterility methods. First, she said, suppression approaches are not equivalent to null mutations. Second, the flowering pathway contains significant genetic redundancy. The question remains,

3 Shown on the left are three constructs that contain the PTLF::BARNASE transgene and a BARSTAR transgene driven by one of three different weak-to-moderate constitutive promoters (35S basal promoter, 35S basal promoter with omega enhancer, and NOS). Control transgenics contained only a BARSTAR transgene or the reporter gene construct PGUS (Wei et al. 2006).

4 A "zinc finger" is a configuration of a DNA-binding protein that resembles a finger with a base, usually cysteines and histidines, binding a zinc ion.

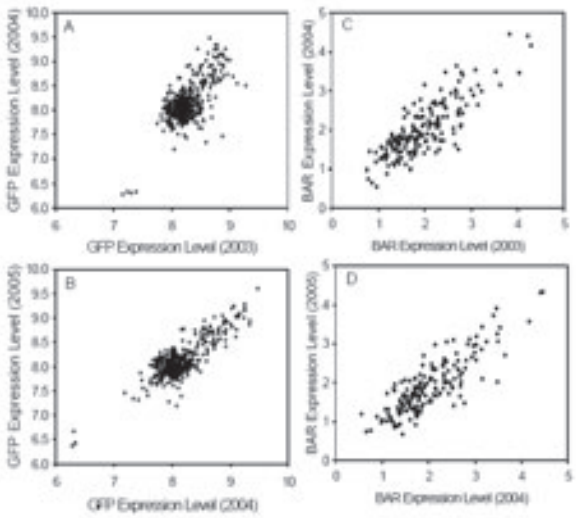
therefore, whether the simultaneous strong suppression of multiple genes can overcome that redundancy and the remaining low level of target gene expression. A third concern, she continued, is that over-expression of proteins may have other undesirable or pleiotropic effects.

Brunner then gave an example of unintended side effects that may occur when attempting to engineer sterility. In this case, Brunner and her colleagues over-expressed a floral repressor in poplar (Mohamed 2006). None of the plants flowered, as expected based on homologous gene function in annual plants. But the transgenic trees also exhibited delayed spring bud flush, which they did not expect based on studies of annual plants which do not undergo winter dormancy.

Another concern about sterility methods, Brunner said, is the following: With current technology, whenever scientists introduce a new genetic construct into plants, they ultimately generate a population of transgenic trees that exhibit variation in transgene expression due to variation in the chromosomal location of the transgene. Such variation makes it challenging to create a reliable means to identify sterile transgenic trees long before they reach flowering age.

Brunner next talked about the stability of transgene expression. She said first that statistically robust studies of transgene stability in trees in field environments are lacking. However, one study does provide some insight. It was the Ph.D. thesis of Jingyi Li, a former student of Brunner's and Steve Strauss's, who generated a large number (404) of primary transgenic events and studied them over three years in the greenhouse and the field. Li also reintroduced a subset of primary transgenics into tissue culture and regenerated "secondary" transgenics via organogenesis. The

Figure 25: Strong Correlation of transgene expression level between years



R = 0.7-0.86
P < 0.01

idea, Brunner said, was to observe how stress induced by tissue culture organogenesis and field environments affected the stability of transgene expression. Ultimately, a total of 2,256 primary and secondary transgenic individuals were planted in the field study. The specimens included two types of reporter transgenes—green florescent proteins (GFPs) driven by the Cauliflower mosaic virus 35S promoter, and an herbicide-resistance gene (BAR) driven by the poplar RbcS promoter.

Brunner said the study found a strong correlation in transgene expression level among years, for both types of reporter transgenes. In other words, the transgenes appear to be stably expressed over time in a field environment (Li 2006). (See Figure 25.)

In a separate study, Jingyi Li also looked at RNAi, Brunner said—specifically, RNAi targeting a resident BAR transgene that had previously been introduced into poplar. This part of the research

Figure 26: Stability of RNAi (Li 2006)

- **2 year field study of 56 RNAi transgenic events (4 ramets/event)**
- **Resident *BAR* transgene targeted**
- **RNAi directed at the coding sequence (PTGS): 80% of transgenic events showed > 90% suppression of *BAR* expression.**
- **RNAi targeting the promoter (TGS): 6% of events showed > 90% suppression**

effort was a two-year field study of 56 RNAi transgenic events, with four ramets (i.e., clones) per event. They looked at two types of RNAi constructs, one targeting the coding region (i.e., post-transcriptional gene silencing (PTGS)), the other targeting the promoter (i.e., transcriptional gene silencing (TGS)). The coding version was much more efficient at causing strong suppression than the promoter construct, Brunner said.

Brunner said this study found that, regardless of the RNAi method, there was a good correlation of target gene suppression among years. The study also assessed some events during the seasonal change in temperature (because some research on annual plants has shown that RNAi suppression is repressed during cooler temperatures). It also looked at the resident BAR gene they were targeting for suppression that was driven by the *Arabidopsis* RbcS promoter, which showed some expression variation during that time period. But still the RNAi event stayed suppressed through the whole period, Brunner said (Li 2006). (See Figure 26.) Thus, this study showed that RNAi-mediated suppression can be highly stable in field-grown trees over multiple years and seasonal changes.

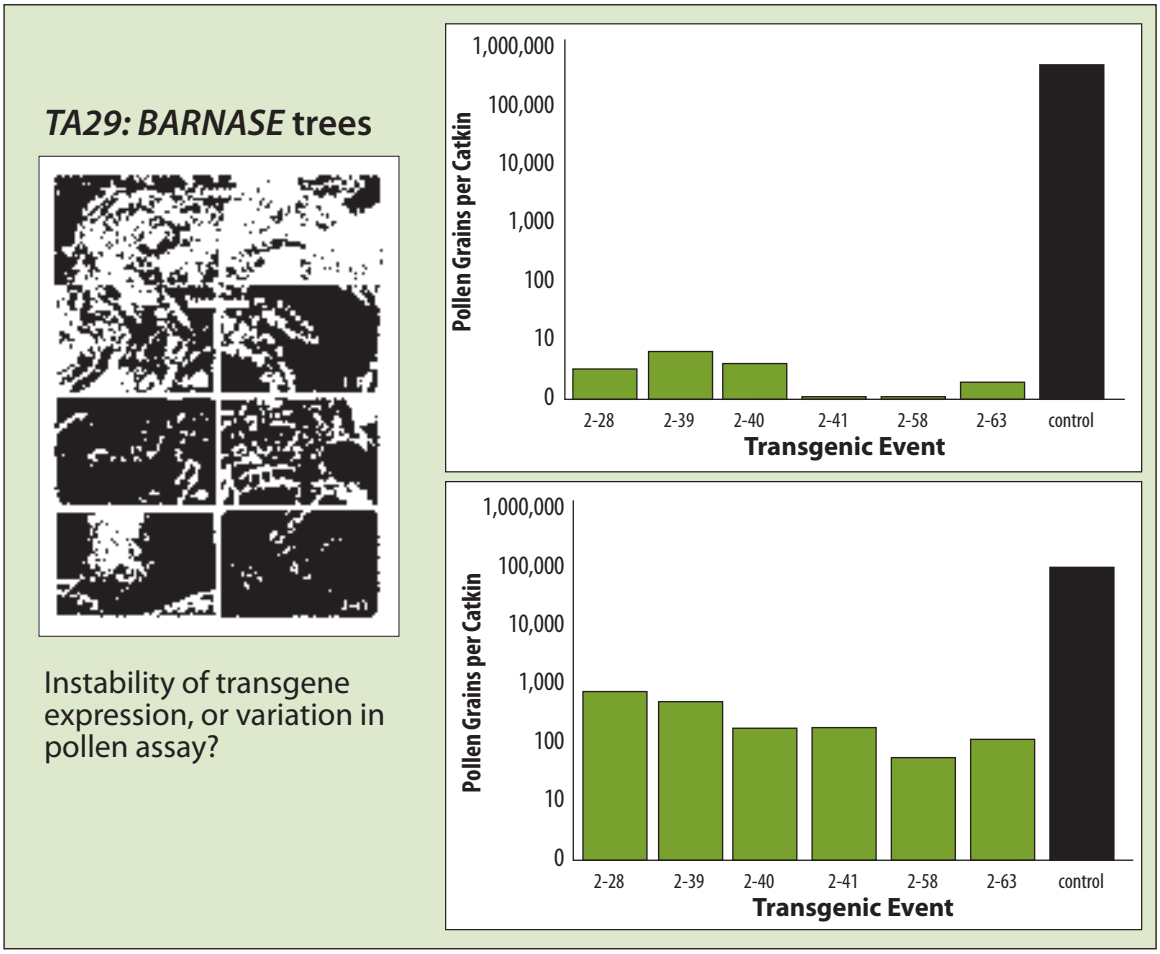
The following questions remain to be answered, Brunner said: How applicable is the stability of an innocuous reporter gene or herbicide transgene to the stability of a sterility transgene? Also, how applicable is the stability of an RNAi targeting a resident transgene to the stability of an RNAi targeting an endogenous gene?

To give an example of potential issues that may arise, Brunner shared data from a field study involving various plant genes that affect gibberellin (GA) signaling, causing a kind of dwarfism, or shortening of the internode. In this study, Brunner said, 12.7% of the plants had one or more branches that had reverted to wild-type conditions. They don't know the cause, she said, though one possibility is the localized somatic silencing of the transgene. They saw similar results in a greenhouse setting in transgenics overexpressing the poplar gene, *PTLF* (unpublished data).

One of the first sterility transgenic poplars produced at OSU, Brunner said, involved the introduction of a transgene that had the tobacco anther-specific promoter TA29 driving expression of the cytotoxin *BARNASE*. In two successive years, they looked at pollen count. As illustrated in Figure 27, they saw high levels of suppression both years, but with differences each year (Brunner 2007 and unpublished data). She said they do not know if the difference was just variation in the pollen assay or an actual difference in the transgene's expression. The assay process is very difficult and could be the culprit, she said. Brunner noted that, depending on the mode of transgene action, it can be very difficult to assess sterility. And it remains unclear, she said, what level of accuracy is acceptable.

Another early transgene tested, Brunner said, was an antisense version of PTLF, the poplar homolog of the floral meristem identity gene *LEAFY*. In

Figure 27: Hierarchical Models



this case, she explained, they observed mutant flowers consistently across years, but not complete sterility (unpublished data). She suggested that gene suppression directed at multiple genes might be more effective, though that still needs to be determined.

After Brunner's talk, one participant asked if she had tried to breed the plants with the mutant flowers, to see if they result in normal progeny. Brunner said they had not. She said it would have been especially challenging with the *PTLF*-antisense transgenics, because of the gradient of mutant floral phenotypes produced that showed various levels of conversion of normally unisexual female flowers to bisexual flowers, male flowers, and vegetative-like structures.

Another participant suggested that delayed bud flush could be useful, in some cases. He noted, for instance, that eucalyptus breaks dormancy if there's early warm weather, and then a cold snap will kill it. Delayed bud flush could be helpful in those situations.

Full-Group Discussion of Gene Flow and Hybridization/Introgression

After the four presentations in Panel 2, Norman Ellstrand, Professor of Genetics and Director of the Biotechnology Impacts Center at the University of California, Riverside, briefly summarized the presentations and then moderated a full-group discussion.

Ellstrand noted first that the panel as a whole dealt with the exposure component of risk, which involves first dispersal, then gene flow, then introgression. He noted that Steve DiFazio's talk reviewed dispersal and gene flow, and included an excellent model. He said the presentation caused him to reflect that this group has come together at an interesting time: The genetic improvement of tree plantations has received less attention recently than in the 1980s, and yet it is returning to the fore because of global needs for carbon sequestration and biofuels. These needs are likely to drive huge shifts in scale, Ellstrand said, which will have serious implications for exposure. For example, he said, while the effects of small plantation stands on large native forests are somewhat understood, we may in fact end up seeing huge plantation stands affecting small native stands.

Ellstrand then noted that Nathalie Isabel talked about an alternative approach to monitoring gene flow and introgression, and about *Populus* experiments conducted in Canada. Of particular interest there, Ellstrand said, were the excellent retrospective studies that have been done. It's wonderful when scientists don't have to set up new experiments to answer questions, he said, but rather can examine the flow of genetic markers in areas where the history of hybridization is well known and understood.

All of the gene flow that takes place, Ellstrand continued, leads to questions about systems for sterility. He said Amy Brunner did an excellent job reviewing in 20 minutes what was covered in a 300-page report on biological confinement conducted for the National Research Council (NRC 2004). One of the key questions raised in that report and in Brunner's talk, Ellstrand said, was whether genomic background or environment would play a roll in the expression of these genes. Brunner's final questions were very important, he said, as it may not be necessary for many transgenes to be confined at all. But where confinement is needed, he asked, how much is necessary? Is it adequate if we achieve 98% sterility that behaves well over a variety of genomes and environments? This is where science and policy come together, Ellstrand said, because scientists must understand what level of accuracy in sterility the public and policymakers find acceptable.

In the full-group discussion, the first participant who spoke echoed Ellstrand's comments about the interface between science and policy. The issue of sterility is where the policy, legal, and ecological issues come together, this participant said. As we have learned from the talks today, he continued, the scale of gene flow among trees can be enormous—spanning many kilometers. But under current federal regulations, *no* release of a transgene to the environment is acceptable until that gene is "deregulated," or proved to be as safe as its conventional counterparts. As a result, this person said, scientists cannot conduct any field research, because it's impossible to achieve zero gene flow in a field experiment environment. And yet it's by doing field work, he argued, that we will find answers to crucial research questions.

Other challenges to research, this participant said, include the skill it takes to work with mating and pollination in trees, and the length of time required. The timeframes involved in researching trees are often longer than research grants or even academic careers, he said, so key issues are not being researched in academic laboratories. Amy Brunner touched on a few ways to address sterility, this participant continued, and there are other possibilities, depending on whether you want to have flowers intact for pollinators, for example. But, he concluded, it is very challenging to think about how to develop any kind of effective sterility system under the current regulatory regime.

Another participant then asked Brunner if she thought scientists could develop a poplar that does not flower at all but instead could be propagated clonally. Brunner said that may be possible, though it will take a long time to develop. Once scientists learn more about pathways and how genes interact, she said, we may be able to determine how they will react if we down-regulate or over-express them. But, she said, research thus far on annual crops has not uncovered any "magic bullet." Another participant asked if poplar propagules are important to the ecosystem, such that nonflowering poplars might not be ecologically desirable. Another participant said that a large number of insects do feed on female *Populus* flowers.

Referring to Figure 24 (see page 44), one participant asked why that sterility study showed stable results in the greenhouse but not in the field. Brunner said it was likely due to variation in promoter activity and the fact that the cytotoxin is very potent. Promoter sequences can be very sensitive and can easily be turned up or down in response to environmental stimuli in the field, she said. The questioner then asked how sterility can be made stable over time, given its sensitivity to environmental conditions. Brunner said field testing and replication will be essential to answering that question, as will research into a variety of sterility methods. Another participant said it is probably unrealistic to expect 100% stable sterility. Additional parallel research is needed, he said, to look at the effects of "leakage," or low levels of escape of nonsterile transgenic plants.

Another participant asked whether the potential for gene transfer across tree species boundaries had been studied. A participant responded that that is being studied in a FIBR project funded by the National Science Foundation. That project is looking at natural hybrid zones, he explained, and introgression from one species to another. (It is not focused on transgenics or plantations, but rather on natural populations under natural conditions.) The initial findings, he said, show extensive introgression taking place (Martinsen et al. 2001; Whitham et al. 2006). Nathalie Isabel noted that she and her colleagues are going to study a *Populus* clone that is used in farm shelterbelts, to assess gene flow between those shelterbelts and natural native populations. Later, another participant pointed out that once the reproductive barriers between species are bridged and a gene crosses into a different species, it then becomes an intra-specific gene flow issue. That is, the transgene would likely spread rapidly within that new species.

A participant then suggested that if 100% sterility will be difficult to achieve, perhaps scientists should instead focus on assessing the survival chances of transgenes that escape. He argued there would be strong negative selection against any new gene persisting in the environment, and perhaps that should be sufficient. Another participant replied that the relative proportion of wild to transgenic trees is an important consideration, as Ellstrand noted. Later, another participant said that the former idea of "swamping" transgenic

alleles as a way to contain them is being tested in sunflower.

One participant then mentioned two methods for containing transgenes that he said deserve attention but are not being widely studied. The first is a strategy proposed by Jonathan Gressel in Israel that uses stacked constructs to reduce fitness (Gressel 1999). The second is virus-vector technology, wherein a virus is genetically engineered to cause the plant to produce the protein of interest; the plant itself is not actually genetically engineered. The transgenic virus is short-lived, however, because the transgene is such a load on the virus genome (making up 20% or so) that the virus "throws off" the transgene in a few short generations.

Finally, one participant asked if any scientists are using woody shrubs in research as a substitute for trees, given that they may reproduce more quickly than trees but are more similar in makeup than annuals. It appeared that the answer was "no," in part, Brunner said, because poplar is as good a woody plant as any for studying what is needed. Also, she said, it's challenging to transfer a sterility gene into any woody plant, so it would not be any easier with a shrub.

Panel Three: Fitness

Panel Three was composed of three presenters who addressed issues relating to fitness. Al Lucier of the National Council for Air and Stream Improvement spoke about the modification of trees for fast growth; Rick Meilan of Purdue University talked about manipulating the lignin content of Populus to improve its use as a biofuel feedstock; and Laura Georgi of Clemson University talked about efforts to create a blight-resistant transgenic chestnut tree.

Modification of Trees for Fast Growth

Presentation by Al Lucier, Ph.D., National Council for Air and Stream Improvement (NCASI)

Al Lucier, Senior Vice President of NCASI, spoke about factors to consider in designing research on the potential ecological consequences of modifying trees for fast growth. At the outset, Lucier acknowledged that Peter Farnum, as well as Les Pearson and Maud Hinchee of ArborGen, provided valuable ideas for his talk.

Fast growth is a trait of interest and value to forest managers, wood buyers, and society, Lucier said. Why? First, because faster tree growth allows forest managers to concentrate wood production on lands that are best-suited to that purpose while managing other lands less intensively for multiple objectives. Second, high growth rates can lead to lower production costs and better value for wood buyers. Third, fast-growing, short-rotation trees may be excellent feedstock for liquid transportation fuels and other forms of biomass energy. In summary, high rates of tree growth on soils that are well-suited to intensive management may enhance the economic viability of woody biomass production and the ecological sustainability of managed forest landscapes. Lucier suggested that substantial support for this proposition can be found in scientific and forest policy literature.[1]

Lucier then showed the cover of a recent issue of *Science* (Figure 28), showing a huge Great Dane next to a tiny Chihuahua. The cover photo illustrated the central idea in an article titled "A Single 1GF1 Allele is a Major Determinant of Small Size in Dogs"[2] Wouldn't it be interesting, Lucier said, if a single gene could make a poplar tree grow as big as a redwood?

Lucier then made three points about characteristics of tree growth in context of a conceptual model in which growth is a function of genetic (G), environmental (E), and management (M) factors.

1. Tree growth is a complex trait with several spatial and temporal dimensions

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1 For example, see the paper *Restoring the Forests* by David Victor and Jesse H. Ausubel – Published in *Foreign Affairs* November/December 2000, Vol 79, Number 6, pp. 127-144. This paper is posted at *http://phe.rockefeller.edu/restoringforests/*).

2 Nathan B. Sutter, Carlos D. Bustamante, Kevin Chase, Melissa M. Gray, Keyan Zhao, Lan Zhu, Badri Padhukasahasram, Eric Karlins, Sean Davis, Paul G. Jones, Pascale Quignon, Gary S. Johnson, Heidi G. Parker, Neale Fretwell, Dana S. Mosher, Dennis F. Lawler, Ebenezer Satyaraj, Magnus Nordborg, K. Gordon Lark, Robert K. Wayne, and Elaine A. Ostrander *Science* 6 April 2007 316: 112-115 [DOI: 10.1126/science.1137045] (in Reports)

(e.g., height, diameter, wood density, seasonality of growth, and age dependent changes in growth processes).

2. Interactions among G, E and M factors have important effects on tree growth.

3. Tree growth is affected by genes that regulate a wide variety of processes including photosynthesis, carbon allocation, nutrient use efficiency, stress resistance, and many others.

Lucier suggested that these characteristics of tree growth should be considered when developing testable hypotheses about the ecological consequences of a specific "growth gene" deployed in a specific tree species. Examples of questions to consider include:

1. What processes does the gene regulate?

2. What dimensions of growth are affected?

3. What kinds of interactions among G, E and M factors are likely to influence gene flow and ecological consequences?

Lucier also suggested that scientists interested in ecological consequences of transgenic trees should be aware of a list of 17 plant traits developed by USDA APHIS to guide assessment of ecological risks that may be associated with transgenic plants. The traits include: growth habit, lifespan, vegetative vigor, ability to overwinter (or overseason), days to flowering, days to maturity, seed parameters, proportion surviving from seedling to reproduction, outcrossing frequency, impact on pollinators, pollen parameter, fertility, self-compatibility, asexual reproduction, seed dispersal factors, symbionts, and stress adaptations.

Lucier then posed two research questions about the potential effects of genetic factors on ecological consequences of growth genes deployed in transgenic trees.

1. If a transgenic tree pollinates another transgenic tree of the same clone, would normal barriers to inbreeding constitute a substantial barrier to gene flow? Lucier speculated that this question would be important to address in models of gene flow from clonal block plantings of transgenic trees. A model parameter relevant to this question is the fraction of transgenic pollen involved in crosses within the clonal block that produce infertile seeds or selfed progeny with low fitness.

2. If a transgenic tree pollinates trees of different clones, would the growth effects of the growth transgene be consistent or variable? Lucier suggested that this question could be addressed within the next decade through investigations involving grafting of transgenic trees onto mature rootstock (to induce early flowering) and mass controlled pollination of non-transgenic clones.

Regarding the environmental (E) and management (M) factors in the model, Lucier emphasized that they are variable over space and time. He suggested that E and M factors (and their interactions) must be considered when modeling potential ecological consequences of deploying a growth gene in trees. Examples of important E factors include weather, soil type, and vegetation type. Important M factors include weed control, tree spacing control, and nutrient amendments.

Lucier then posed two questions about the E and M factors:

1. Which combinations of E and M factors promote large (or small) differences between the growth of trees with a specific growth gene ($growth_t$) and growth of trees without the growth gene ($growth_0$)?

2. Are the effects of a growth gene on tree growth and fitness likely to be the same in natural stands as in intensively managed plantations?

Lucier suggested that answers to these questions will depend on characteristics of tree species and growth genes. For example, loblolly pine is very intolerant of shade. Differences between $growth_t$ and $growth_0$ are likely to be greatest in recently disturbed environments where loblolly seedlings are growing in full sunlight (e.g., in a recently harvested area that has been prepared for replanting). In contrast, differences between growtht and growtho are likely to be small for suppressed loblolly seedlings growing very slowly in the shady understory of a closed-canopy forest. This example suggests that models of gene flow from transgenic loblolly pine plantations should consider the extent and proximity of different kinds of environments where progeny of transgenic trees might become established.

Lucier then discussed the challenge of defining specific ecological consequences to be considered in a research program. He noted that some stakeholders believe that the presence of transgenes in the environment per se is a consequence of concern because it represents a departure from a more natural state. Other stakeholders are more interested in consequences that have regulatory endpoints (e.g., harm to an endangered species) or substantial effects on ecological or economic functions of wild populations.

Lucier noted that land use history has had major effects on wild populations of tree species in many regions. For example, current wild populations of pines in the US South comprise the progeny of trees that survived extensive deforestation during the 18th and 19th centuries. Lucier suggested that effects of gene flow from transgenic trees on functions of wild populations of southern pines will need to be interpreted in context of this history. Lucier also discussed the need to consider explicitly how intra-specific effects of gene flow affect inter-specific competition and other ecological processes.

Lucier speculated that retrospective field studies of the genetic and ecological consequences of conventional tree breeding programs might provide insights into potential consequences of gene flow from transgenic trees into wild populations. For loblolly pine, he suggested that excellent opportunities for retrospective studies of gene flow rates might be associated with the following historical circumstances: (i) a loblolly pine genotype known as 7-56 has been planted extensively and carries a rare mutant allele of the CAD gene, and (ii) fast-growing loblolly pine genotypes from North Carolina that have been planted widely in Arkansas and Oklahoma.

In closing, Lucier summarized his remarks:

1. The potential ecological consequences of deploying a transgenic "growth gene" depend on several genetic factors that may influence gene flow and fitness effects. Models of ecological consequences need to represent the mode of action of the growth gene, the dimensions of tree growth affected by the gene, and the potential for interactions among several genetic factors.

2. The fitness effects of a growth gene always depend on environmental and management factors. Explicit consideration of environmental and management factors (and their interactions with each other and genetic factors) is a complex but essential task in modeling the ecological consequences of gene flow from fast-growing transgenic trees.

3. Ecological studies of fast-growing transgenic trees should be designed to support assessments of both benefits and risks associated with specific deployment scenarios. Risks of interest to many stakeholders include potential effects on endangered species and ecological functions of wild populations. Progress in risk assessment requires development of testable hypotheses about parameters in models of gene flow and fitness effects.

In the short discussion after Lucier's talk, a participant asked about the function of the CAD gene. It was noted in response that CAD (cinnamyl alcohol dehydrogenase) is an enzyme involved in the last step of the lignin synthesis pathway. The natural mutant CAD gene in 7-56 provides only 50% of the normal level of enzyme activity.

Another participant asked whether scientists were looking to "split the pie" differently in their development of growth genes, or "expand the pie." In other words, are the growth genes being studied aiming to reapportion a tree's energy, or to increase the total amount of energy taken in? Lucier said he believes reallocation is most likely. Another participant said another option is to optimize basic biochemical systems; that is, adjust gene expression levels to be more efficient in certain processes.

Yet another participant said he believes that forest managers do not want more growth, per se, but rather more wood formation. To make wood, he said, the cells in the secondary meristem have to decide to either divide or differentiate. If they divide, they make more cells, if they differentiate they make more xylem. What scientists want to do is stimulate those cells to do both of those things more, he said. It is that simple decision point on which researchers ought to focus, he said.

Manipulating Lignin Biosynthesis to Improve Populus *as a Bio-Energy Feedstock*

Presentation by Rick Meilan, Ph.D., Purdue University

Rick Meilan is Associate Professor of Molecular Tree Physiology in the Forestry and Natural Resources Department at Purdue University. He spoke about efforts to manipulate lignin biosynthesis in poplars to improve their utility as a biofuels feedstock.

Meilan set the stage by sharing a few quotes. First, he reminded participants of President Bush's statement in his 2006 State of the Union address about America's addiction to oil. In that speech, Bush also said: "We'll also fund additional research in cutting-edge methods of producing ethanol, not just from corn, but from wood chips and stalks, or switch grass. Our goal is to make this new kind of ethanol practical and competitive within six years" (Bush 2006). Meilan said his talk would focus on the technology needed to achieve that goal (i.e., the utilization of cellulosic feedstocks).

Meilan then shared several other notable and related quotes, shown in Figure 31. Indiana is the fifth-largest producer of corn in United States, he said. Farmers in the state grow corn for human consumption, for feed for cows and pigs, and for export. But Indiana would have to import corn, he

Figure 31: Other Notable Quotes

"Most of the world's oil is concentrated in places that are either hostile to American interests or vulnerable to political upheaval and terrorism, and demand for oil will increase far more rapidly than we expected just a few years ago. Within 25 years, the world will need 50 percent more energy than it does now."

"With the end of 20 years of low oil and gas prices, investment in alternative fuels has surged. As more is invested, innovation in technology and production will drive prices down further. That is why it is so important to get the first cellulosic ethanol facilities up and running."

Senator Richard G. Lugar, 13 March 2006

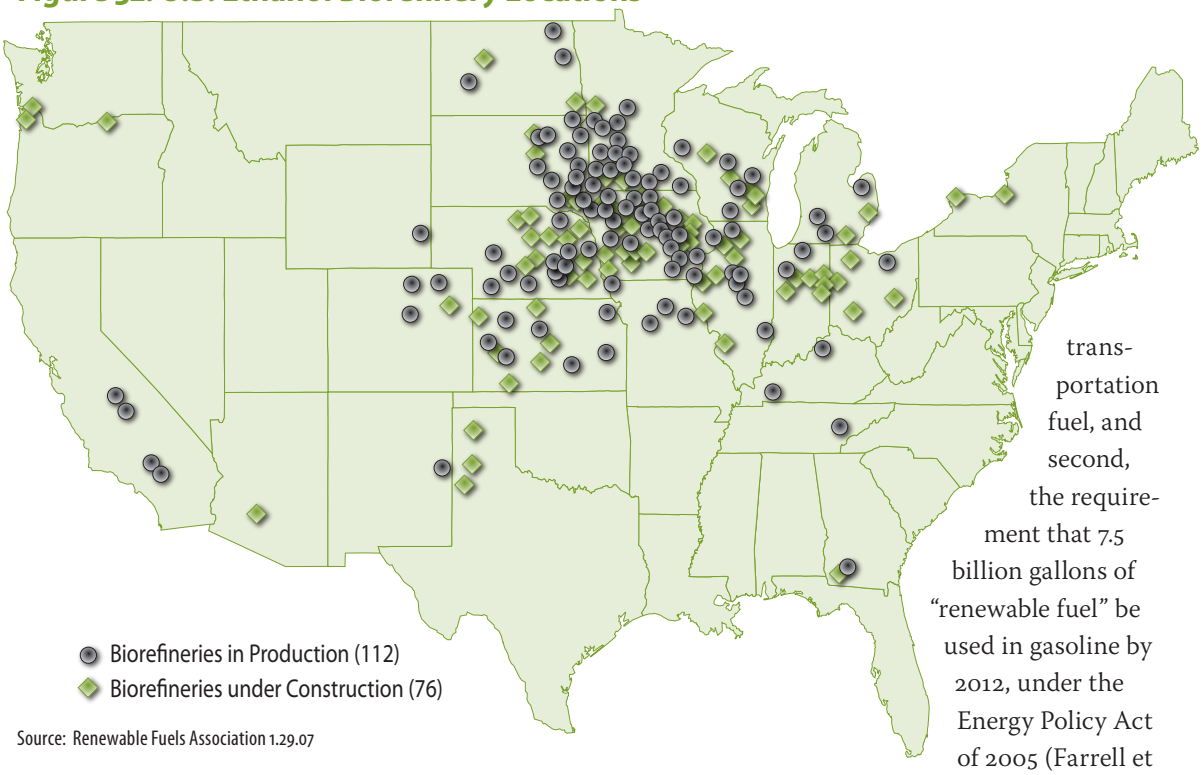
"At the beginning of last year, Indiana had only one ethanol plant. Today, six ethanol plants and two other biodiesel plants are under construction, with more on the way."

Indianapolis Star, 18 March 2006

"If Indiana wants to support only corn-based ethanol production, we would have to import corn. What we need is a whole set of plants that are well-adapted to particular growing regions and have high levels of productivity for use in biofuel production."

Ag Communications, Purdue University

Figure 32: U.S. Ethanol Biorefinery Locations



Source: Renewable Fuels Association 1.29.07

said, if corn is to be the only feedstock for ethanol production in the biofuel facilities that are currently under construction.

Figure 32 shows ethanol biorefinery locations in the U.S. and those under construction, Meilan said, though it does not include those being planned (Renewable Fuels Association 2007). Where are the feedstocks going to come from for all of these facilities? Meilan asked. Clearly it cannot be corn alone, he said.

The demand for ethanol is growing, Meilan said. In 2004, 4 billion gallons of ethanol were blended with gasoline; this volume is equivalent to 2% of all gasoline sold in that year (Department of Energy 2005). In the future, much larger quantities of ethanol are very likely to be produced, Meilan said, because of two federal policies: First, a $.51 tax credit per gallon of ethanol used as a transportation fuel, and second, the requirement that 7.5 billion gallons of "renewable fuel" be used in gasoline by 2012, under the Energy Policy Act of 2005 (Farrell et al. 2006).

The Department of Energy's Genomes-to-Life research roadmap, Meilan continued, includes a "30 by 30 plan" that proposes substituting 30% of liquid transportation fuels with ethanol by 2030 (DOE 2005). That's a tall order, Meilan said, since in 2004 the figure was only 2%. To achieve 30%, he said, we will need significant biomass from cellulosic feedstocks, not just starch from corn kernels.

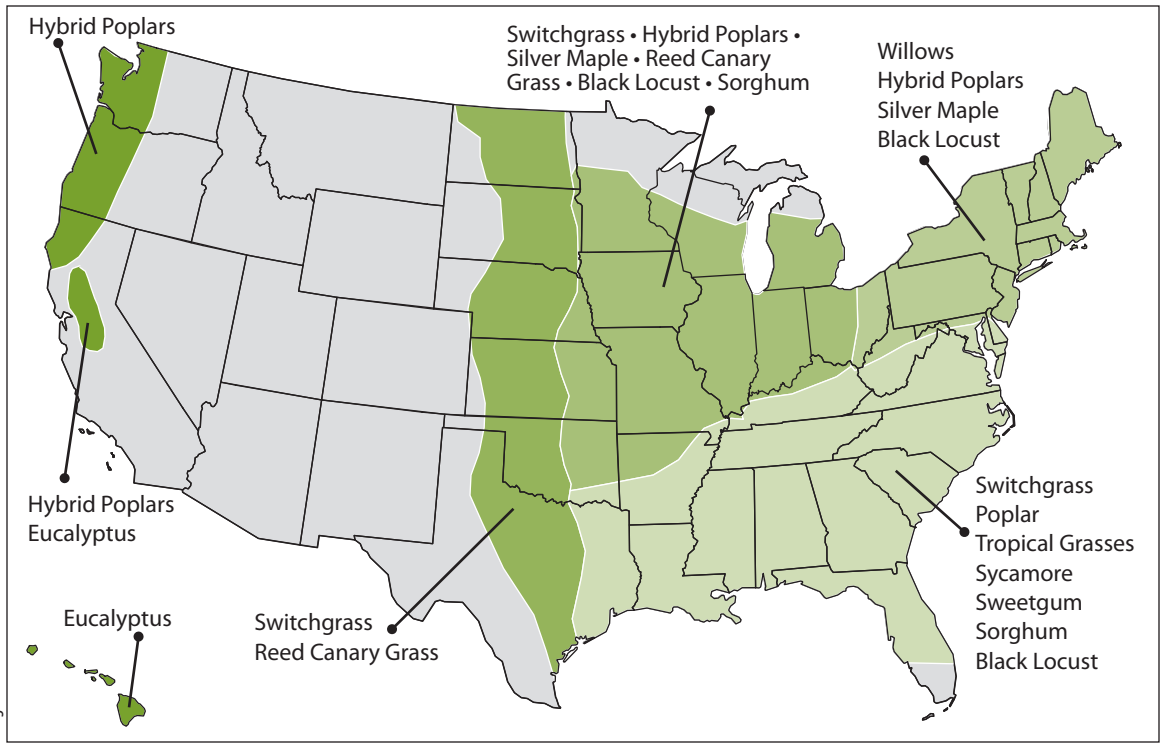
Right now we rely on corn for ethanol, Meilan said, because we know how to do it—the technology is available. Corn supplies the sugar needed for fermentation. And the yeast involved in fermentation cannot break down complex carbohydrates, he explained, just simple sugars. So, what will be the feedstock for new ethanol facilities coming online soon?

Meilan shared a map (Figure 33) put together by Lynn Wright and her colleagues at the Oak Ridge National Laboratory (Wright 1994). The map illustrates the suite of biomass crops they believe will be needed to achieve energy self-sufficiency and transportation needs, Meilan said, including: hybrid poplars, eucalyptus, switchgrass, reed canary grass, silver maple, black locust, sorghum, willows, poplar, tropical grasses, sweet gum, and sycamore. All of these species are well adapted to growth, Meilan said. But, he explained, there are two important parameters when considering a feedstock for ethanol production: biomass yield, and how efficiently the sugar in the biomass can be converted to ethanol. All of these species produce sugar, Meilan said, but the most abundant form of that sugar—cellulose—is relatively inaccessible using current technology.

Meilan then provided some basic information about plant polymers, to illustrate the magnitude of the challenge. Starch and cellulose are both made up of glucose units, he said, and starch is one of the primary constituents of corn kernels. All crops contain cell walls, which comprise an abundant supply of cellulose. The linkages between the glucose units in starch are different than those in cellulose, however, Meilan explained. The shape of a starch molecule is saw-toothed, so it cannot be packed very tightly. By contrast, the cellulose molecule is more linear and

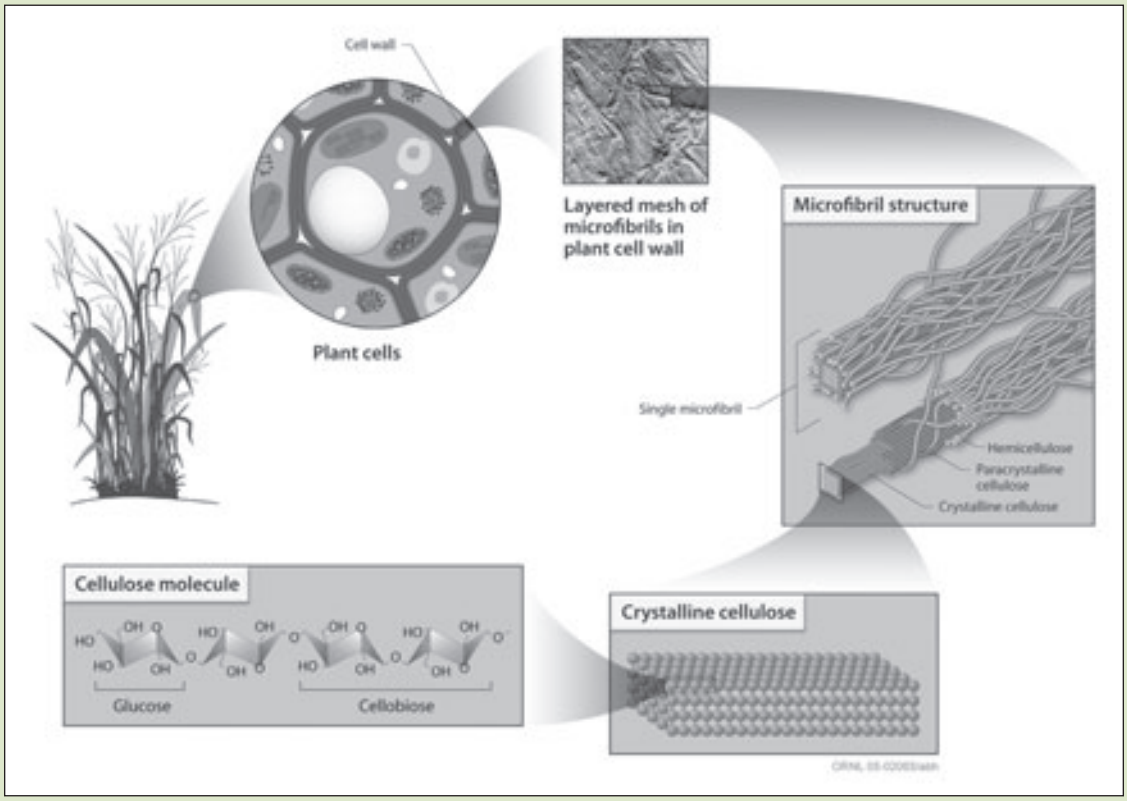
Figure 33: A Suite of Biomass Crops will be Needed to Achieve Energy Self-Sufficiency

"…with only modest changes in usage, farmland and forests could yield more than 1.3 billion dry tons of biomass each year, enough to reduce present oil demand by nearly one-third" (USDA and U.S. DOE, 2005)



Wright et al. DOE-ORNL

Figure 34: Plant Polymers



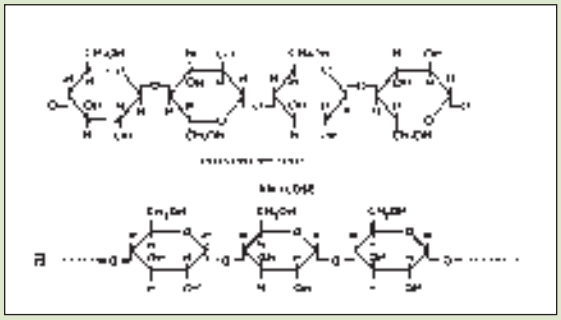
Source: Genomics GTL Roadmap, Office of Science, U.S. Department of Energy, August 2005 (*http://doegenomestolife.org/roadmap/index.shtml*).

Starch

- **Made of glucose units**
- **Currently used to ferment ethanol**

Cellulose

- **Also made up of glucose**
- **Wound into microfibrils**



Source: Physiology of Woody Plants by T.T. Kozlowski and S.G. Pallardy, 1997, Academic Press.

strong, and the plant weaves these strands of cellulose together in cable-like structures call microfibrils, hundreds of which are woven together in every plant cell wall. Given these differences in their structures, cellulose is a more efficient way to store glucose than starch, Meilan said. Gram for gram, the energy density from cellulose is much higher than of starch. (See Figure 34.) Thus cellulose feedstocks in general, including trees, have the potential to yield more bioenergy than starch from corn.

But a third important biopolymer is lignin, Meilan said, and this is where the problem lies. Lignin is a complex, cross-linked phenolic polymer that interferes with the degradation of plant cell walls. In fact, lignin is itself a structural component of plant cell walls, and is cross-linked to the microfibrils. Cell wall structure is best understood as being like lathe and plaster in an old house, Meilan said. The cellulose cables, or microfibrils, are like the wood strips of lathe, and the lignin is like the plaster, overlaying the microfibrils. So, Meilan said, one must strip away the lignin to get to the cellulose, which, in turn, can then be hydrolyzed into simple sugars (i.e., glucose). Thus lignin is needed in a plant for strength, but it prevents access to the underlying glucose units.

What Meilan and others want to do, he said, is alter the composition of the lignin to increase the accessibility of the cellulose, which can be cleaved into the simple sugars that can be fermented by yeast to produce ethanol.

The basic steps in bioethanol production, Meilan continued, include plant growth, feedstock preparation, pretreatment, hydrolysis of the solid, and ethanol fermentation. Pre-treatment may take the form of acid treatment or steam explosion, among other methods, Meilan said, none of which are particularly effective at present.

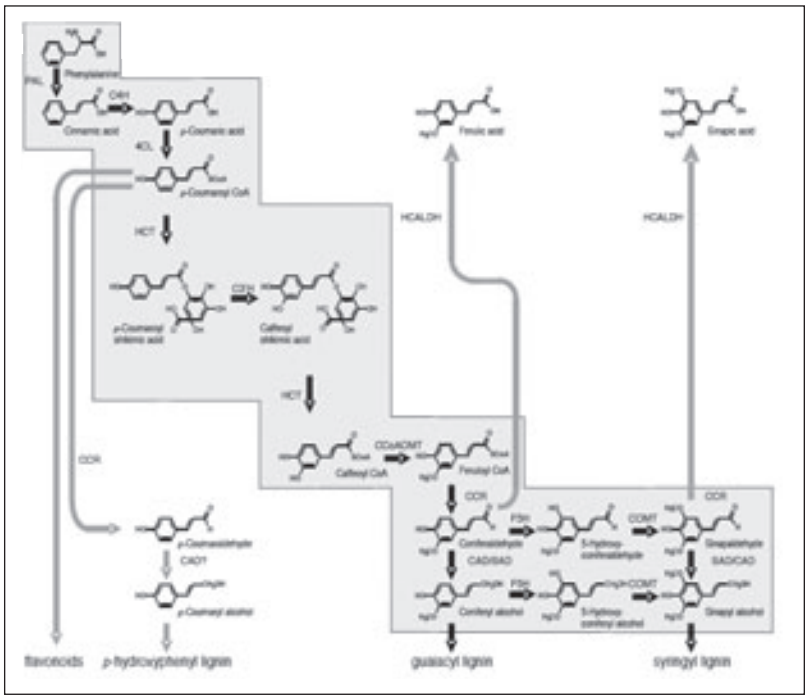
The average relative composition of cell walls includes a bit less than one-third lignin, Meilan said. The remaining two-thirds are split between cellulose and hemicellulose.

Some scientists believe, Meilan said, that reducing lignin content may lead to increases in cellulose content. But critics argue that reductions in lignin will compromise the structural integrity of the plant and make it more susceptible to pathogens and diseases.

The three basic building blocks for lignin (called "monolignols"), Meilan said, are as follows: syringyl (S) units, guaiacyl (G) units, and hydroxyphenol (H) units.

Meilan then shared Figure 35, which illustrates the complicated biosynthetic pathway for lignin. What Meilan is seeking to do, he said, is alter this pathway at key places. An enzyme called C4H, Meilan explained, is the entry point into the biosynthetic pathway. One idea is to down-regulate the expression of that CH4 gene, which would reduce everything downstream, including the amount of total lignin.

Figure 35: Lignin Biosynthesis



Source: Clint Chapple, Biochmeistry Department, Purdue University

There's another key enzyme called C3'H, Meilan continued. If you down-regulate this, you can cause a backup of the precursors. That would cause an overflow into one branch of the pathway, leading to a lignin that is comprised largely of hydroxyphenyl units. So the plant would primarily rely on H units for making lignin.

And there's yet another possibility, Meilan said: up-regulating F5H would prevent the accumulation of guaiacyl units and cause the accumulation of syringyl units, so the plant will primarily make S lignin. Conversely, by down-regulating F5H, there will be an accumulation of guaiacyl units, so the plant will primarily make G lignin.

The point of all this, Meilan explained, is to induce the plant to make a specific type of lignin—a pure S lignin or a pure H or a pure G, or perhaps specific ratios of two of the three. Then, he said, we can determine the composition that is easiest to extract, so we can gain access to the underlying cellulose.

Figure 36 shows the various possibilities just mentioned. It shows the target genes, the strategy, and the predicted outcome. Meilan emphasized that he and his collaborators are seeking to manipulate lignin composition, not content.

Meilan then explained that he and colleagues Clint Chapple and Mike Ladisch at Purdue have received a $1.4 million grant from the Department of Energy (DOE) to do this—to modify the composition of lignin in poplar. He said they are seeking to create transgenic poplars that have four key enzymes up- or down-regulated. They will then field-test them and assess the extent to which their cellulose can be liberated. Such assessments will involve conducting cell-wall deconstruction analyses of wild-type and lignin-modified transgenic lines. Finally, he said, once they determine the optimal lignin composition, they will develop a metabolic fingerprint for the plant with the ideal lignin make-up, and use that for high-throughput screenings of wild populations, to find wild (not transgenic) plants that have that ideal composition. In this way, non-GE plants can be propagated for biofuel or used for conventional breeding.

In summary, Meilan said, ethanol production capacity is increasing rapidly. A suite of alternative bioenergy crops will be needed, especially cellulosic feedstock. Trees have several advantages, particularly *Populus*. However, lignin composition affects the ease with which cellulose can be extracted from *Populus*. Meilan said that perhaps he and his colleagues can apply what they learn from *Populus* to other species as well.

In closing, Meilan acknowledged his collaborators, Clint Chapple and Mike Ladisch, and his

Figure 36. Target Genes for Manipulation in Poplar and Their Expected Impact on Lignin Content and Composition

Target Gene	Strategy	Predicted Outcome
C4H	RNAi down-regulation	Decreased lignin content
C3'H	RNAi down-regulation	Decreased lignin content, high H subunit content
F5H	RNAi down-regulation	Lignin content unaltered, low S subunit content
F5H	Up-regulation	Lignin content unaltered, high S subunit content
F5H COMT	Up-regulation RNAi down-regulation	Lignin content unaltered, high 5OHG subunit content

funders: the DOE's Plant Feedstock Genomics program, and Discovery Park Energy Center at Purdue University.

In the question-and-answer period that followed Meilan's talk, one person noted that removing too much lignin from a tree will cause negative fitness effects. He asked what is likely to happen if the composition of lignin, but not the content, is altered. Meilan said he is hopeful that composition changes will not result in deleterious fitness effects, but he said that needs to be researched and tested. He and his colleagues plan to assess plants with modified lignin composition for structural integrity, wood strength, and susceptibility to pests and diseases.

Another participant said he thought much research had already been done to identify the types of lignin that are easier to break down. He said, for instance, that it had been shown that the more syringyl lignin in a plant the more easily it will hydrolyze. Also, he said, another scientist has reduced the lignin in poplar by a factor of two and is testing those trees, and they are expected to be useful as a source of biomass. The real problem, this person said, is that not enough land is available to grow enough poplar to make it useful as a feedstock. Meilan agreed that some preliminary work had been done on hydrolysable linkages. But he said no one had made a systematic attempt to achieve and analyze pure S lignin, pure G, or pure H. Also, Meilan said, his project is seeking to get to the point where transgenic technology is ultimately not needed. Meilan also said he agrees that poplar—or any other single species—cannot be the sole feedstock for biofuels. A variety of species will be required, he said.

Another participant pointed out that growth and lignin have proven to be negatively correlated. That is, if you increase lignin, you decrease the growth rate. So, there may be a substantial increase in growth rates, he said, if lignin content is decreased.

A participant then asked how changing the lignin content or composition is likely to affect products of the phenylpropanoid pathway, which are important to the ecology of the poplar. Meilan said that reducing overall lignin content would likely have deleterious effects, but he thinks changing the composition may not. It also may depend on *how* the composition is modified, however, which he said is why he will be looking at a variety of steps in the pathway.

Finally, a participant noted that there are so many possible genes that would be useful to modify and study, and this project seemed to be only scratching the surface. Meilan agreed, and said they would like to do a high-throughput screening in order to help breeders find the most useful genotypes, so they don't have to do any more long, expensive field trials than are necessary.

Blight-Resistant Transgenic Chestnut: Pie in the Sky or Peavey?

Presentation by Laura Georgi, Ph.D., Clemson University

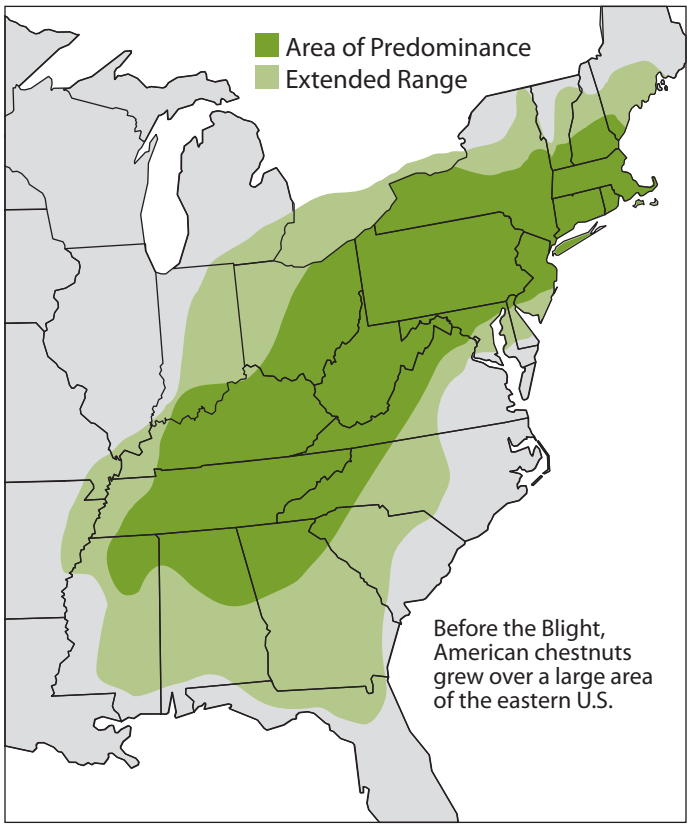
Laura Georgi is a post-doctoral researcher in the Department of Genetics and Biochemistry at Clemson University. Her presentation addressed the development of a blight-resistant transgenic chestnut. Georgi said she would cover the ecological implications of the chestnut restoration effort, the effects on fitness, the potential for gene flow to other species, the potential effects on nontarget organisms such as fungi, and critical needs for research. She noted that a "peavey"—mentioned in the title of her presentation—is a long tool used for breaking up logjams.

Georgi began by providing a brief overview of the chestnut blight story. Before the blight, she said, the American chestnut (Castanea dentata) was plentiful in much of the eastern United States. (See Figure 37). It was a large, abundant tree that grew quickly and was a competitor, she said, almost to the point of being a weedy species. The blight—caused by the fungus Cryphonectria parasitica—was introduced from Asia in the early 20th century. It spread throughout the chestnut's range in about 50 years and had a dramatic effect on the landscape. American chestnuts are very susceptible to the fungus, Georgi said; Asian Castanea species are less so. The fungus killed the trees via girdling.[3]

Many chestnut trees killed to the ground by the fungus continue to sprout from the stump, Georgi said. There is hypovirulence caused by a number of mycoviruses.[4] While this hasn't solved the problem in America, she said, it has mitigated the disease somewhat in Europe.

The American Chestnut Foundation has been carrying out a traditional genetic improvement program for 24 years, Georgi continued, building on the work of other organizations before them.

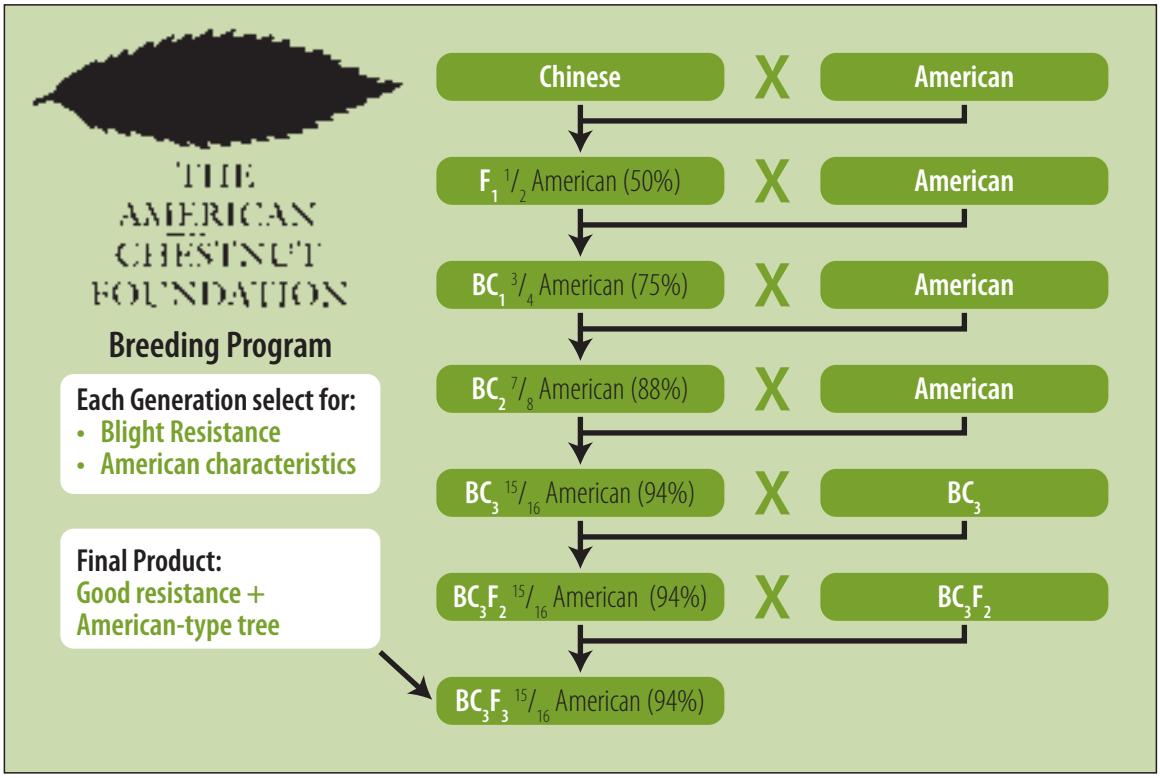
Figure 37: American Chestnut Range



3 See *www.acf.org* for more on the blight story.

4 *Mycoviruses* are viruses of fungi. Infected fungi are hypovirulent. In other words, a disease of the fungus makes it less pathogenic to the tree.

Figure 38: American Chestnut Foundation Breeding Program



Their program involves a backcross process to select for blight resistance, she said. (See Figure 38.) The objective is to develop a largely American tree (15/16ths) with the resistance of the Chinese species. The Foundation has some lines advanced to this level, Georgi said; unfortunately, as of last fall the number of resistant trees developed was not as high as hoped.

At the same time, Georgi said, scientists have been working for a number of years to develop a blight-resistant chestnut via genetic engineering. The primary investigators have been Scott Merkel at the University of Georgia, and Bill Powell and Chuck Maynard at the State University of New York College of Environmental Science and Forestry. These scientists first studied a synthetic gene inspired by a frog antimicrobial peptide (Powell et al. 1995). Georgi said she had some concerns about this method, as the peptide is not a particularly specific antimicrobial, and would likely have detrimental nontarget effects. Plants have longstanding associations with symbiotic fungi, she explained, and we do not want to interfere with those.

The researchers are now investigating the gene from wheat oxalate oxidase (OxOx), Georgi said, which is a natural defense protein found in grains. It does not aim to kill the fungus, she explained, but to degrade a virulence factor produced by the fungus. That is, it would make the chestnut resistant to the blight fungus, but not to other fungi that do not produce oxalic acid. Some work has been done with this gene in Populus, she added (Liang et al. 2001).

With the OxOx gene, Georgi said, the research-

Figure 39: OxO transformed American chestnut in the field (SUNY-ESF) (June, 2006)

ers are using a soybean vascular-specific promoter, since that is the tissue in which they want to express the resistance gene. It is possible to select for the OxOx in transgenic tissues directly by challenging it with oxalic acid, she said, but to do that you must use a nonspecific promoter, which appears to make the trees less fit. Georgi noted that other available promoters include three American chestnut vascular promoters; wound-induced promoters from Chinese chestnut and poplar, and a wound-induced vascular promoter from Eucalyptus.

Georgi explained that the explants used in this transformation system are somatic embryos that are derived from immature zygotic embryos. The scientists have successfully transformed the chestnut using this method and have acclimated it to the field. It has been planted under all applicable regulations on a research farm. (See Figure 39.)

Efforts are also underway, Georgi said, to clone blight-resistance genes from the Chinese chestnut. The genetic map of an F2 Chinese-American cross was published in 1997, she said. However, the genetic map locations of the genes need to be better defined. The markers Georgi has been working with, she said, are three cM (centiMor-

gans) away from the map locations for blight resistance.[5]

Georgi then noted that C. dentata is not the only Castanea species native to the U.S. It overlaps extensively with trees known as chinquapins—C. pumila var. ozarkensis and C. pumila var. pumila. Thus there is some concern about transgenes from a GE chestnut introgressing into one of these species.

Research on GE chestnuts are still in the "pie in the sky" phase, Georgi said, and much more work remains to be done. Maynard and Powell have created and acclimated one transgenic line, she said, but they do not yet have enough trees to do effective testing.

Georgi noted too that chestnut researchers are in an odd position, because while other scientists working on GE trees are seeking to prevent gene flow, GE chestnut researchers will want to promote gene flow. The goal, she said, is to release the chestnut from the fungal stranglehold it is in, and the challenge is to do it safely. The chestnut is a very competitive species when not blighted, she added, so one question is, has the ecosystem changed so much that it can no longer support the chestnut? That is unclear, she said, and remains to be researched.

In closing, Georgi acknowledged the individuals who have done most of the work on the chestnut: Paul Sisco, American Chestnut Foundation; Scott Merkle, University of Georgia; Charles Maynard and William Powell, State University of New York College of Environmental Science and Forestry; Haiying Liang and Albert Abbott, Clemson University; Tom Kubisiak, USDA Forest Service; Robert Bernatzky, University of Massachusetts; and John Carlson, Pennsylvania State University.

In the discussion period after her talk, one participant asked if scientists have observed any reduced pathogenicity of the blight. Georgi said no, only the hypovirulence phenomenon. Unfortunately, she said, the most hypovirulent strains are not competitive in the environment.

Another participant asked about the remaining genetic diversity of the chestnut. Georgi said the species was not very genetically diverse even before the blight; in fact, Castanea dentata was the least variable of all the chestnut species. However, stump sprouts do exist through its range, she said, and full-grown trees planted outside its range (i.e., in the Pacific Northwest) also do survive.

In response to another question, Georgi noted that chestnuts begin to flower after several years.

Finally, a participant asked if the research could be too successful—if scientists could inadvertently create an invasive chestnut species. What might limit the chestnut population besides the blight fungus? she asked. Georgi said other fungi could attack it, such as inky root rot. She agreed, however, that the potential invasiveness of any new chestnut will have to be carefully assessed.

5 In other words, the markers and the trait occur together most of the time, but the markers do not perfectly co-segregate with the trait. Recombination between the marker and the trait can be expected to occur in 3% of meioses. So, while they are linked, there's a fair amount of physical distance between them on the chromosome.

Full-Group Discussion of Fitness

John Burke, who is Associate Professor of Plant Biology at the University of Georgia, served as moderator of the discussion after Panel 3. He first shared some thoughts of his own about fitness, building on the three previous presentations.

Burke said the aspect of fitness of importance to him is not the performance of transgenic trees in managed settings, but rather the effects of those trees on volunteers and/or hybrids outside of managed settings. Transgenes are fed into natural ecological systems by dispersal, Burke said, and then they are either filtered or amplified by selection. Those transgenes may or may not have an ecological impact after escape, however. Burke explained that "fitness" is used as a proxy for the potential for ecological impact, but ecological impact itself is the sticky issue. If a gene is deleterious, he continued, it is not much of a concern unless there is overwhelming pollen flow, because selection will purge populations with deleterious alleles. But if a gene increases fitness under natural conditions, he said, then it could have an ecological impact.

Thus in population genetic theory, Burke continued, rates of gene flow are not as important as selection in terms of the establishment of alleles in populations. Highly advantageous (or highly fit) alleles, even if they escape rarely, will spread with a high frequency within their population and then move to other populations. Alleles with low fitness, by contrast, will be selected out of the population and will not have much effect. So, Burke said, the outcome is entirely fitness-dependent once there is an escape.

This leads to the question of what level of containment is necessary, Burke said. With a highly fit or advantageous allele, he said, any escape at all presents a substantial concern, because it will spread rapidly. Also, if the allele crosses a species boundary, it will then be governed by intra-specific gene flow.

That said, Burke continued, one of the real problems scientists now face is the inability to conduct realistic field trials. Estimating fitness is not terribly challenging if field tests can be conducted, he said, but if researchers cannot allow a tree to flower, it becomes quite difficult. Unique ideas such as the miniaturization studies that Peter Farnum suggested will have to be attempted, Burke said. A further challenge is how to assess the likelihood of harm if a transgene does escape. Even if there is spread, Burke said, there will not necessarily be a negative ecological impact.

In the discussion that followed Burke's comments, one participant said some of the modeling literature incorrectly presumes that once an advantageous allele escapes, it will spread quickly. Those models are not stochastic, this person said, and they assume that all transgenes are highly advantageous. In fact, he said, if you have large reservoirs of nontransgenic trees, hundreds or thousands of years might pass before there is any noticeable increase in the frequency of a transgene. That is, the nontransgenic genes will "swamp" the transgenes for a long time. Burke agreed, and noted also that the models do not take into account context- and environment-specific factors. Such variability is very difficult to model, he said.

Another participant commented that fitness must be assessed in both plantation settings and wild settings. A tree that is fit in a plantation setting may behave quite differently in a wild setting, he said; thus it is important to test the plant

in a situation that mimics escape. Burke agreed and noted that he and his colleagues are not concerned about performance under cultivation, but rather only in the wild. And that, he said, is where the problem lies in terms of conducting effective experiments under current regulations.

Discussion then turned to Rick Meilan's study of the lignin composition of trees. One participant said he was intrigued by the idea of using transgenic systems to identify the trait of interest, and then seeking out nontransgenic trees that carry that trait. He asked how deploying nontransgenic trees would be different—from an ecological risk perspective—than using the transgenic trees. Meilan responded that it would not be different, except from a regulatory perspective. That is, a transgenic tree would have to be reviewed by the USDA, while a nontransgenic tree with the same trait would not. Meilan noted too that the most challenging part of his project will be developing the metabolic profile to identify trees in the wild that have the optimal lignin composition. That process has a lot of potential pitfalls, he said. Another participant said it may be possible that trees with the optimal lignin content do not exist in the wild, because they are not fit.

A participant asked Meilan whether, in down-regulating certain genes, he was affecting both copies of the gene. Meilan said that yes, they have designed the RNAi constructs to down-regulate both copies.

Discussion then turned to the work being done to develop a transgenic chestnut. In response to a question, Laura Georgi clarified that the OxOx gene is not toxic to the fungus, but rather decreases the concentration of a product of the fungus. One participant asked if she and her colleagues were working to ensure they did not create a "super-chestnut"—one that would be resistant to diseases it would have been susceptible to under pre-blight conditions. Georgi said they were keeping that in mind, though they would like to develop a chestnut that could grow more widely in the South, where previously it had been restricted to higher elevations due to *Phytophthora cinnamomi*, another exotic pathogen that apparently arrived in the 18th century.

Day One Recap Panel and Discussion

Toward the end of Day One, a panel of five workshop participants answered key questions from moderator Peter Farnum. The purpose of the panel was to help assimilate the information shared during the presentations and begin to discuss what research is most needed going forward. The panelists included Ron Sederoff, Ph.D., Distinguished University Professor of Forestry and Co-Director of the Forest Biotechnology Group at North Carolina State University; Cassie Philips, J.D., Vice President of Sustainable Forests and Products at Weyerhaeuser Company; Michael Wach, Ph.D., Chief of the Regulatory and Environmental Analysis Branch in APHIS's Biotechnology Regulatory Services office; Anita Klein, Ph.D., Program Officer with the Plant Genome Program at the National Science Foundation; and Jessica McGlyn, Senior Program Officer at World Wildlife Fund.

Farnum first asked the panelists to comment on what they saw as the main areas of potential ecological risk regarding GE trees, and on the areas of research needed regarding those risks.

Ron Sederoff of N.C. State spoke first. He said what is needed most is a better understanding of the baseline—of the ecological history and existing status of the forests and trees of interest. Such baseline information could then be used to evaluate potential changes going forward. We tend to view forests as static, he continued, but they have not been so for hundreds of years. Chestnut is a good example; it used to have a large range, but within a small number of tree generations the Appalachians became blanketed in oaks and hickories. Likewise, he said, the Atlantic coastal plain was originally covered with pines, then essentially clearcut, and now contains pine plantations. Landscapes like these are not stable genetic systems, Sederoff said. They continue to change, and climate change will exacerbate those changes. We know trees are highly heterozygous, Sederoff continued. They are rich in genetic variation, and selection drives dramatic changes at all stages of development. So, he asked, is one more gene with a modest positive or negative effect going to have a significant impact on a forest that is only a few generations old? In short, he said, a clearer perspective on ecological history and genetic composition is sorely needed.

Cassie Philips of Weyerhaeuser said that from her standpoint the critical issue is how to get GE trees "deregulated" (released from the regulatory system) so they can be used commercially. Scientists must keep in mind the USDA's legal standards and procedures for deregulating GE trees when designing research, she said. To get a plant deregulated, Philips said, a developer must meet the substantive standard in the Plant Protection Act, and, most likely, follow the procedural rules in the National Environmental Policy Act (NEPA) as well. The latter will require the development of an environmental impact statement (EIS). Philips said those legal hurdles lead her to believe that researchers need to spend more time working on very basic issues and questions, including those that are not likely to reveal any serious risks. All minor and major questions will have to be addressed in an EIS, she explained, so the USDA can defend their decision. Philips said it would be helpful for workshop participants to scope the range of potential issues, including those that are potentially low risk, so scientists can be sure to address them. Because this is a matter of deregulating, Philips continued, the *proponents* have the burden of proof. If developers do not have all the answers, the plant will likely not be deregulated. Philips then noted that a Swiss expert committee on biosafety published a report last year that she thinks could serve as a good model for GE trees.

Michael Wach of APHIS spoke next and described the differences between the data needed for a field permit and that required for deregulation. For a field testing permit, he said, the key issue for the agency is confinement. If you can prove that the transgene is confinable, Wach said, you will be able to get a permit and do your field trial. If you want your trees to bloom and flower, he continued, you need to tell the agency right away, because an EIS will likely be required and should be started as soon as possible. The issues are a bit different for a product to be deregulated, Wach continued. In that case, APHIS needs to know what the tree is doing. It's not an issue of gene flow; if the plant is going to be deregulated, it's a given there will be gene flow. Instead, the issues are: Is the GE plant more or less competitive than its nontransgenic counterpart? And, will it in any way harm agriculture or the environment? So, Wach said, research permits and deregulation raise separate sets of questions.[1]

Anita Klein noted first that she was speaking for herself, not on behalf of her employer, the National Science Foundation. Klein said she had been thinking about the day's presentations in terms of research priorities. What seems important, she said, is modeling the effects of long-lived species, which have a slower succession of genotypes and reduced rates of evolution. For each type of GE tree, Klein continued, research on potential ecological impacts is needed. For example, she said, research is needed on the impact of sterility on plantations and natural forest ecosystems. Klein also said she likes the idea of extrapolating from known cases of nontransgenic gene flow that have resulted from the past 400 years of transcontinental travel. In terms of the traits that may be introduced, Klein said the "low-hanging fruit" include *Bt* and Roundup Ready varieties. Finally, Klein noted that plant breeders have often made annual crops less fit but more suited to human needs. Trees are somewhat different, she said, in that we need them to be fit enough to live a long time before harvesting. Also, creating GE trees differs from traditional tree breeding, because scientists will be able to make changes so much faster than has traditionally been feasible. These issues need to be considered in the research process, she said.

Jessica McGlyn from the World Wildlife Fund began by saying that while she agrees that regulatory issues must be addressed, she also believes market acceptance issues will be a challenge in the future. Anyone who has dealt with the export of GE crops to Europe, she said, knows that market acceptance ought to be considered up front in research. McGlyn then said that the environmental community is interested in four main issues relating to GE trees. The first is confine-ability. There must be safeguards such that natural populations of trees are not negatively affected, she said. The second issue is "in-stand" impacts on target species, nontarget species (e.g., *Lepidoptera*), and ecosystem services (e.g., soil nutrient flows). Spruce budworm is endemic in forest stands in the West, for example. What happens if that budworm is constantly exposed to *Bt* rather than having it applied as a spray periodically? The third issue, she said, which was not addressed in the presentations, are the ecological implications of shifting entirely to more-efficient trees. What are the implications for stand structure if we start growing cold-tolerant eucalyptus in North Carolina? she asked. Loblolly plantations support significant biodiversity, she said, but stands of transgenic eucalyptus may not. The

[1] For a thorough description of the federal regulatory system governing agricultural biotechnology, see the Pew Initiative on Food and Biotechnology's report *Issues in the Regulation of Genetically Engineered Plants and Animals* (2004) (*http://pewagbiotech.org/research/regulation/*).

fourth issue, McGlyn said, which is outside the scope of today's meeting but still worth mentioning, is the socioeconomic impacts of transgenic trees. What if transgenic trees become the popular choice the way GM soybeans have? she asked. In closing, McGlyn noted that today 15% of forests are Southern plantations, and that is predicted to double by 2020. What if instead of loblolly plantations the region has only GM eucalyptus plantations? What are the implications at the stand level for biodiversity? These questions need to be considered by researchers, she said.

Farnum's second question to the panelists concerned establishing priorities for research. What criteria should be used for establishing such priorities, he asked, and why? Facilitator Abby Dilley pointed out that some participants had already mentioned market acceptance and "low-hanging fruit" as possible criteria.

Anita Klein noted that the National Science Foundation has a focus on basic biology. To justify a research plan to the NSF, she said, a scientist must establish why the study should focus on trees as opposed to some other organism. If answers can be found more quickly with an annual plant, she said, why use trees? Other key words at the NSF, she said, are theory, resilience, and conceptual biology—that is, they seek to draw research priorities according to theoretical, conceptual, fundamental theory in biology.

Mike Wach said that APHIS does not evaluate the quality of a researcher's science. Rather, their sole focus is, can the research be done safely?

Cassie Philips said her main criteria would be that the research is necessary for APHIS to build a defensible record for deregulating a GM tree. She suggested first combing through the literature to see what has been done, in order to identify, and then fill, any gaps.

Ron Sederoff said it is impossible to foresee what problems will arise down the road, in part because many of them will be policy-based, not science-based. Genetic engineering often inspires objections that are philosophical and values-based, he said, not science-based. Sederoff said it's important to prepare for a hostile, rapidly changing political environment. To do that, he argued, researchers must focus on very fundamental issues, such as the principles of adaptation, growth, metabolism, and evolution of the trees in our forests. Because we don't know what to prepare for, Sederoff said, we must collect the basic information that will allow us to answer whatever questions arise. Sederoff also noted that a very significant issue on the horizon (as Rick Meilan said in his talk) is the need to grow biomass to feed biofuel facilities. He said he's concerned that a large proportion of the world will be clear-cut before we are done.

Later, another participant echoed Sederoff's comments about the need for more basic, baseline information. What is needed, he said, is a better understanding of the genetics of natural populations. He said scientists should ask question such as: What is the magnitude of fitness variation in natural populations? What is the magnitude of selection coefficients among the genes in a genotype? How many genes impact adaptation for a particular character (e.g., growth rate)? And, how are the fitness differentials of several or many genes put together?

Jessica McGlyn said that, in terms of criteria, environmental groups would be more comfortable if any GE trees planted were proven to be sterile. That is her number-one priority, she said, in the absence of other information about the safety of these trees.

Cassie Philips said the research around environmental risk should be collaborative and open

and in the public domain. Universities, nonprofit groups, and the private sector should work together from the outset where possible, she said.

Mike Wach noted that APHIS's website includes completed applications for field testing and deregulation, which anyone can view to see what is required. Wach also encouraged participants to be sure they have the resources necessary to conduct transgenic research safely before they even apply. A successful program requires staff, funding, facilities, and equipment, he said.

A participant then asked Wach for confirmation that APHIS would only approve a plant if it is shown to be harmless. He was interested to know if the agency would, instead, give approval in conjunction with containment strategies. In response, Wach first clarified that APHIS does not "approve" anything. Instead, they deregulate—or remove from the regulatory system—plants that are found to be no more of a plant pest risk than the conventional plant from which they are derived. So, he said, a plant does not have to be risk-free, but it must be no more risky than the original nontransgenic plant. Wach added that deregulation "in part" is a possibility, though it has not been done thus far.

Other participants then offered ideas for criteria as well. One person said, for instance, that researchers ought to focus on answering the questions that will need to be considered in the deregulation process. He said practical, testable hypotheses were essential. Another participant said researchers should consider both the potential risks and the potential ecological and economic benefits of GE trees.

Another participant said that, given the policy and social reality, and given that research is difficult and expensive, the costs of basic transgenic technology must be reduced. To do that, he said, researchers should first focus on improving transformation efficiency (i.e., perfecting the process of mutagenesis). Second, he said, researchers should focus on developing traits with high value. And third, he noted that continuing to seek deregulation on an event-by-event basis will be inefficient and time consuming. He said scientists should focus on central hypotheses and study transgenes in classes, not individually. For instance, he said, scientists could look solely at genes that increase fitness, or that improve insect resistance.

Day One Wrap-Up

Participants then switched to considering how to organize their discussion on Day Two of the workshop. As a starting point for discussion, meeting organizers presented the following matrix, to illustrate the types of GE trees currently being researched and moving toward commercialization in the United States. It was suggested that workshop participants could use the matrix to help them organize into small discussion groups on Day Two.

Participants then commented on various aspects of the matrix. One argued, for instance, that disease resistance should also be considered for loblolly pine, eucalyptus, and poplar, as large plantations of clones of those species will invite potential disease problems. Another person said eucalyptus is currently being tested for both fast growth and modified lignin. Other participants noted that the matrix only covers what is being addressed in the U.S., and that much public research is being conducted in China, where numerous GE trees have been planted and will likely be commercialized. (A steering committee member noted, in response, that the committee chose to focus only on the U.S. for this workshop.) Another participant said there could conceivably be "Xs" for every gene, not just every type of gene; for instance, multiple genes for growth in loblolly pine may be employed.

Finally, several participants said that more fundamental issues should be addressed by the workgroups on Day Two, such as gene flow generally, and fitness generally. A steering committee member said he did not disagree, but that in each of those general situations it seemed logical to focus on the most relevant species/trait combination as examples. Another participant said that basic, baseline information is also needed on each individual species. For instance, he said, a compendium of information needs to be compiled for poplar, loblolly pine, and eucalyptus. Other participants said model systems present another important area of research, as do ecological impacts such as nutrient cycling and landscape-level effects.

The meeting organizers promised to take all of these ideas into account in organizing workgroups for Day Two. With that, the workshop adjourned for the day.

Figure 40: Biotech Tree Research Considerations & Priorities

1. Consider	**2. Prioritize**	Loblolly Pine	Eucalyptus	Chestnut	Poplar
• Gene Flow • Containment • Fitness • Ecological Impacts • Baseline Models	Fast Growth	**X**	**X**		**X**
	Modified Lignin	**X**	**X**		**X**
	Cold Tolerance		**X**		
	Disease Resistance			**X**	

Day Two

At the beginning of Day Two, facilitator Abby Dilley said participants would be split into four workgroups—one each on the topics of gene flow/containment, fitness, ecological impacts, and baselines/models. The workgroups were asked to identify specific research questions that need to be addressed and, to the extent possible, specific research designs. Dilley also suggested that participants think about how they and their institutions could collaborate on the research opportunities identified.

Dilley then shared a slide summarizing the criteria for research mentioned in the discussion on Day One, for participants to keep in mind in their workgroup conversations. The slide indicated that research on GE trees should:

- provide information on traits broadly, rather than event by event
- be based on testable hypotheses
- address the question, "Can this trait or gene be introduced safely?"
- generate data to address substantive standards and regulatory requirements
- focus on high-value traits
- demonstrate the value of GE trees

This list spurred a short discussion of additional criteria that participants felt were important. One participant said, for example, that scientists should consider how trees are both different from and the same as annuals, and how forested lands are both different from and the same as agricultural lands. Another person suggested that studies take into account issues of scale, since GE trees may be adopted widely. He said scientists should put themselves in the shoes of the auto industry circa 1909, and consider, "What if this technology really catches on?"

Finally, a participant said scientists must consider how their research relates to important national problems, such as the looming energy and climate change crises and the need for alternative fuels. Another person agreed, and argued that federal agencies should work together to create a regulatory framework that will enable the deployment of GE energy crops, including trees. The government body with the most funding to invest in such an effort, he said, will likely be the Department of Energy. Another participant noted that several federal agencies have already been charged with working together on the issue of feedstocks for biofuels, and have drafted a preliminary "biofuels feedstock implementation plan." Funding for such work, she said, will come from the DOE, the NSF, and a joint USDA/DOE research program, among other sources.

On a related topic, a participant asked how the research discussed in this workshop will be funded. One of the workshop organizers said one option is to collaboratively develop a package of research proposals and then seek funding from Congress and granting agencies such as the NSF. Foundations, the private sector, and individual agencies could also be approached, he said. He noted that an organized, consensus-based proposal for collaborative research may be more likely to secure significant funding than disparate and competing small proposals.

With that, meeting organizers split participants into the four workgroups. Each group was tasked with discussing the following questions: What are the primary research questions that are particularly important for this topic? Which of those are the most important? And, what are some designs or elements of designs that could be employed to answer those questions? The workgroups met for two and a half hours. The following is a summary of the report-outs from each group, and the full-group discussion periods that followed each.

Group 1: Gene Flow and Containment

Norm Ellstrand served as the spokesperson for Group 1, which discussed gene flow and containment. He said the group identified four potential research projects.

First, Ellstrand said, Group 1 proposed conducting comprehensive studies regarding the efficacy of biological confinement. He said the group developed the outline of an experiment that would involve multiple organisms—including an angiosperm and gymnosperm and wind pollination and insect pollination—to test some of the molecular biology-based fertility control tools. These tools must be tested under field conditions, Ellstrand said, and they may not work in all environments and all phenotypic backgrounds, so some redundancy in confinement is needed.

Thus, the group felt that a marker system should be developed to make the transgenic trees readily identifiable from a distance, even via remote sensing or satellite imagery. (Chloroplast pigmentation might be one option, Ellstrand said.) Such an optical marker would be stacked onto the transgene, Ellstrand explained, and would provide an efficient and effective way to keep track of the modified trees in the field.

Ellstrand said this kind of study should assess the influence of the genotype on different genomes, so it would need to involve at least two genomes within a species, and also multiple genotypes within each environment. There was some discussion of using herbaceous models, he said, but the group ultimately decided against that. They felt the studies should be conducted in woody plants, and ideally in the types of GE trees that are now being developed. In short, Ellstrand said, the group recommends that sterility traits must be field released to be effectively assessed, but they should be released on small acreages, closely monitored, and inextricably linked with an optical marker.[1]

The second type of project that Group 1 proposed, Ellstrand continued, was a landscape-scale, long-distance gene flow study involving non-GE trees. Group members agreed that short-distance mating events have been studied enough, he said, but that large-scale assessments of background gene flow would be helpful. In such a study, Ellstrand explained, researchers would identify a large stand of a tree of interest and then measure the gene flow "rain" that originates from it in one of several ways. For instance, "trap plants" could be placed in the middle of the prairie to see what pollinates them; pollen could be collected from great distances to see if it's viable; and paternity studies could be done, to genotype all plants in a population and identify hybridization at a great distance. The idea, Ellstrand said, is to understand what very large populations are producing and distributing at a distance.

Third, the workgroup proposed a retrospective analysis. Such a project would identify a pre-existing system in which the date of introduction is known, Ellstrand explained, and then use multilevel markers to measure the spread of the species. For example, one could look at the introduced *Populus* species in Canada, he said, or the 7-56 loblolly pine allele in the U.S. Such a study could be used as a model of what happens when a novel gene is placed in the environment and has a chance to "cook" over several decades, Ellstrand said.

[1] Kiang, Y. T., J. Antonovics, and L. Wu. 1979. The extinction of wild rice (Oryza perennis formosana) in Taiwan. *Journal of Asian Ecology* 1:1-9.

Finally, Group 1 proposed studying a variety of scale-related issues through modeling. Ellstrand said such models could analyze issues such as: the differences between small plantations and very large plantations; the differences between the gene flow relationships of large plantations and those of small natural populations; the effects of GE trees on non-GE tree plantations; and the effect of management methods on gene flow. Regarding the latter, Ellstrand referenced a system studied in Taiwan in which populations of native wild rice dwindled (for a variety of reasons), after cultivated rice was brought in from China and planted widely. Apparently the researchers identified effects on the wild populations not only due to gene flow from the larger nonnative populations, but from management activities such as the alteration of water flows and habitat.

Ellstrand then mentioned several side issues that came up during Group 1's discussions. Participants noted, for instance, that there will be variation depending on the species, including differences in pollination, taxonomy, seed dormancy, and age of reproductive maturity. Selfing frequency and vegetative reproduction, for example, are issues associated with domesticated gene penetration into natural populations. Increased selfing or asexual reproduction, Ellstrand said, results in higher levels of linkage disequilibrium, which affects introgression rates. The impact of density is also important, he noted.

Finally, Ellstrand said, the group touched briefly on policy-related issues. In particular, they noted that different traits should perhaps be associated with different levels of confinement. In some situations confinement will be unwanted, Ellstrand said, as with the chestnut. (Though he said the gene mechanism in any transgenic chestnut should be analyzed carefully, as some may be more desirable than others.) At the same time, he said, some transgenic traits may cause no harm even with a "drip drip drip" of genes into the environment roughly equivalent to the mutation rate. For these, he added, sterility traits could be included to limit the spread, if desired. But then other traits, he concluded, should perhaps be completely contained—either never grown outside or never grown in food plants. Plants containing pharmaceutical compounds are one example, he said.

In the full-group discussion after Ellstrand's report-out, one participant asked what the group meant by "mutation rate." He noted that the term could mean either the accumulated frequency of mutations, or the rate at which mutations take place. Another participant said scientists often can't measure the background mutation rate in any case. A workgroup member said he was thinking of the rate at which mutations take place, which might be somewhere in the 1 in 1,000,000 range.

One participant then challenged the suggestion that "pharma traits" should not be grown outside. He said plants naturally contain all sorts of beneficial proteins that are pharmaceutical in nature. A workgroup member said they had in mind only true medicines, such as human growth hormones, not natural products such as tannins.

Participants then made a variety of other comments regarding the report-out. One person said, for instance, that confinement might simply be addressed by growing—in two-year rotations—trees that do not flower for ten years. Another person said that quantum changes in technology (i.e., mutagenesis) must be sought to ensure complete sterility in the future. Ellstrand agreed and said the proposed marker system could facilitate studies that would lead to such technology breakthroughs. Another person said scientists should conduct studies with the marker gene but without the sterility gene, to make sure the marker works.

This last point led to a discussion of whether federal regulators would allow the release of a transgenic marker. Mike Wach from APHIS said no one had ever proposed such a study, but if they did it would require an EIS under NEPA, through which the potential plant pest and environmental impacts of the marker would be assessed. Also, he said, the researchers would probably need to have a way to retrieve the progeny. One participant then suggested that the scientific community request the deregulation of marker genes broadly, so as to facilitate the study of gene flow.

Another participant said the reality of research on confinement systems is that scientists will want to look at several options, or maybe even hundreds. And they need to make the trees flower, which means release of transgenes into the environment. So, they may need to request the deregulation (or partial deregulation) of numerous transgenic events. This person asked if a mechanism exists for deregulating numerous events simultaneously. Wach said comprehensive permits sometimes cover hundreds of transgenic constructs being put into a single variety in an experimental setting, and he said APHIS could certainly review such a proposal in terms of multiple containment methods. Ellstrand clarified that the workgroup was not contemplating allowing GE trees to flower indefinitely, but rather to let them flower only long enough to enable an analysis of sterility.

Group 2: Fitness

Al Lucier served as the spokesperson for Group 2, which discussed fitness. He said Group 2 recommended activity in three areas: consensus documents, experiments, and models.

First, Lucier said, the group envisioned creating documents like those published by the Organization for Economic Co-operation and Development, in which everything known about the baseline ecology and biology of a particular species is compiled as a reference. Such documents could serve as the foundation for risk studies, Lucier said, and could be prepared for eucalyptus, loblolly, chestnut, and poplar. Lucier said workgroup members recommended the development of two other consensus documents as well: one on methodologies for ecological risk assessment, and another on the implications of regulatory policy for academic research and product development. Lucier said the latter is needed because the barriers to entering the field are so high for small companies.

Lucier then described the experiments envisioned by Group 2. In general, he said, the group advocated comparing the growth rates and competitiveness of transgenics and nontransgenics in managed and unmanaged environments. This concept could have several permutations, he said. For example, researchers could assess the growth and competitiveness of a cross between two transgenic trees with the same transgene. They could look at the growth and competitiveness of a transgenic/wild cross. And/or they could assess the growth and fitness of a transgenic species itself, as vegetatively spread.

Proxies and correlates of fitness would need to be measured, Lucier said, such as seed dormancy and viability (measured in the lab); pollen viability and size (measured in the lab); photosynthetic efficiency (measured in the field); and early seedling growth (measured in the field).

Regulatory strategy would have to be determined early, Lucier continued. As an example, a researcher might first apply for a permit with the option to allow the plants to flower, and then establish the field sites and collect preliminary data. Then perhaps the regulatory agency would need to decide whether to allow the trial to continue based on what has been learned from the initial data. If the trial could go forward, the researcher could then assess post-flowering measures, such as seed production performance and growth performance at later ages.

Lucier said the workgroup talked in some detail about experiments involving transgenic plants crossed with wild species. The general approach, he said, would be to cross a transgene into a nonselected background, and then test the resulting seedlings in nonmanaged sites to look at fitness correlates. To do this, he continued, a researcher could top-graft a scion from a transgenic plant onto a nontransgenic rootstock and force it to flower, then cross it with the wild type. The researcher could cross the transgenic pollen back onto the nontransgenic scion as well, he said.

Group 2 also talked broadly about models and hypotheses, Lucier said. One of the hypotheses, he explained, is that domestication leads to low fitness in natural environments. This might be a good subject for a consensus document, he said, and it would be interesting to know how that concept is relevant or not relevant to trees, which are relatively undomesticated.

The group also addressed a tiered approach to research, Lucier said. If research on a transgene gives no indication of increased fitness, he asked, does that imply no ecological impact? That seems

like a reasonable supposition, Lucier said, but it needs to be tested. Because it is extremely difficult to assess ecological endpoints, he added, surrogates for fitness may be useful. Lucier also said scientists need to better understand natural populations and landscapes that may be affected by the release of a transgene.

Lucier said the group also talked about learning by doing. If it is too difficult to conduct research, he asked, will it mean the suppression and decline of the whole field? This is obviously not the intent of the regulatory system, he said, but to some degree it could be the effect.

He said the workgroup also discussed the notion of parallel paths of research. On the one hand, he said, research may be done to support a particular permit or the deregulation of a particular product. On the other hand, more general insights may be needed to inform the evolution of the regulatory process and the advancement of science. These two types of research may intersect, Lucier said, but it may be helpful to think of them as on parallel paths. For instance, public money should perhaps not be used to support product development, but it could be used to finance research into general concepts and principles.

Finally, the workgroup discussed the concept of partial deregulations or deregulations with contingencies, Lucier said. They felt that monitoring could be an important component of such approaches. But more thinking needs to be done, he said, to determine how they would work.

At the outset of the full-group discussion following Lucier's report-out, another workgroup member emphasized that the group advocated research on the effects of transgenes on performance in an unmanaged environment; they did not tackle the ecological implications of increased or decreased fitness.

Another participant said she could envision environment-specific situations in which no increase in fitness is measured. For instance, she asked, how could research account for the fact that invasive species are not weedy in their natural habitats? A workgroup member said the group agreed these studies need to be replicated numerous times and in different environments, to achieve multiple outcomes. Another participant said it would be important to see how other, similar transgenes have spread, to help anticipate environmental variation in a newly transformed species.

One participant then discussed the idea of a tiered regulatory system. He said that requirements that seem reasonable for annuals, like determining fitness effects, can be onerous or impossible for trees. He thus viewed a tiered system as essential. He noted that the USDA invites public comment on changes to their regulations, and he suggested this group comment together to suggest the establishment of a specific tier for trees.

Participants also spoke briefly about the impact of regulatory requirements on small developers. One noted that the Pew Initiative on Food and Biotechnology conducted a workshop on that subject, and the general conclusion was that the barriers are almost insurmountable for developers of specialty crops. Keith Pitts from Pew noted that a summary of that workshop is available on Pew's website (Pew Initiative 2007). Another participant said there are economic, access to talent, and intellectual property barriers as well.

Finally, a participant commented that models may be necessary to assess seed-based outcomes, since some species don't flower until they are ten years old or more.

Group 3: Environmental Effects

Marilyn Buford, National Program Leader for Silviculture Research at the USDA Forest Service, served as the spokesperson for Group 3. Buford said the workgroup identified several possible starting points for assessing environmental effects. Among them were the gene level, the trait level, the community level, and the trophic level. They decided the best place to start was to look at functional changes, Buford said. That is, what are the functional changes to the environment that can result from the introduction of a transgene? And, do those changes matter, in an outcome sense? The group also decided that growth, reproduction, and defense could be looked at for each plant or plant group.

Buford said the workgroup noted that environmental impacts are not necessarily negative. Some may be positive, and those should be identified. Also, she said, they decided that seedlings can be useful in research, but that researchers should keep in mind that seedlings do not have the same ecological impacts as trees.

Group 3 identified several "big-picture" functions that forests and trees perform, Buford said, as follows:

- water quality and quantity
- habitat for wildlife, endangered species, aquatic species, and humans
- recreation and spiritual fulfillment
- goods and services, including timber, non-commodity goods, ecosystem services, climate change mitigation, soil health, etc.

In that context, Buford said, the workgroup discussed indirect outcomes, including fitness, weediness, and a loss of sensitivity to abiotic stress, which can create problems in itself or can increase the risk of damage.

As the workgroup looked at these broad functions, Buford said, they talked about how best to study effects. They did not end up identifying a single study or experiment. Instead, she said, they developed a framework of issues to consider in designing studies.

First, Buford explained, they considered that studies would need to take place at the following scales:

- Greenhouse or pot scale
- Microcosm or nursery seedling scale
- Experimental planting scale
- Commercial plantation scale
- Landscape scale

Beginning at the experimental planting scale, she said, there is interaction and integration with all other scales. Depending on the trait(s) or gene(s), some issues might be appropriate to study at the greenhouse scale, but eventually one would need to move into a larger planting scale. The idea is to focus on the functions that may be affected, Buford said. A researcher who is interested in foliage, she said, would study general and specific insects that chew on foliage. By looking at those insects, they could learn larger lessons about environmental impact. Other surrogates might include soils, water, nutrient cycling, and so forth. A lot of the large-scale studies would have to be done with models, she said.

Overall, Buford said, the workgroup felt that this kind of research should be integrated, open, and collaborative. They noted the example of the FACE study, she said. In that case, a large committee identified questions to be answered and types of studies to be done, and then individual scientists applied for grants and carried out the actual work. Some studies would require significant interaction with regulatory agencies, Buford said. She added that mitigation strategies should be designed in parallel with the studies, to handle any unforeseen escapes of the transgene.

The controls would have to be carefully chosen, Buford continued, and they might not be non-transgenic. They could be sysgenic, she said, or varieties such as the loblolly pine with the 7-56 allele. Loblollies planted well outside of their original range could also be used as controls (e.g., in New Zealand and China).

Finally, Buford noted that Group 3 talked a bit about barriers and benefits to ecological research. The barriers they identified included funding, the regulatory system, intellectual property issues, liability, technical progress, social acceptance, and vandalism. The benefits they noted included educational outreach, the acquisition of basic scientific knowledge regarding genes in ecosystems, and the ability to answer key questions.

In the full-group discussion that followed Buford's report-out, one workgroup participant said that studies should include both transgenic and non-transgenic comparators, including distinct commercial varieties. That way, he said, new varieties could be compared to what is being grown in the landscape now. On another topic, he noted that funding for research on environmental effects will likely need to come from a range of agencies, so an interagency funding mechanism may need to be created.

Another workgroup participant said Group 3 also discussed the idea of consensus documents. Specifically, they suggested compiling what is known regarding how to assess environmental effects, such as impacts on soil, wildlife conditions, and so forth.

Finally, Buford said it was suggested that ecological research on GE trees presents the opportunity for building a dedicated study location in one or more of the key forest systems in the United States.

Group 4: Baselines and Modeling

The report-out for Group 4 was separated into two parts. Allison Snow presented the group's discussion of baselines, while Steve DiFazio described their discussion of modeling. A full-group conversation followed each part.

Snow said Group 4 seemed to agree that existing plantation forests would be the most appropriate baseline for assessing the effects of transgenic trees. Regular plantations clearly have effects on the environment, she said—though some have more than others. Snow said the group was interested in ecological effects within existing plantations, as well as how those plantations affect surrounding areas, including unmanaged stands of forests and protected areas with special status (i.e., National Parks). All of that makes up the baseline, she said.

To understand and measure this baseline, Snow continued, scientists could study the following variables relating to existing, nontransgenic plantation forests:

- genes from the planted tree species
- the establishment and invasiveness of the planted tree species
- biodiversity (i.e., the effect on birds, the food chain, *Lepidoptera*, and threatened and endangered species)
- the effects of scale and time (i.e., the size of the plantations, and how long they have been there)
- pests and pathogens
- pollution (water quality, air quality, and soil quality)
- the cycling of nutrients, including water and carbon

In short, Snow said, the workgroup sought to identify those factors on which plantation forestry might have an effect—on managed natural stands, unmanaged stands, and natural or protected areas. They called this an "ecological synthesis."

The National Science Foundation has a Program for Ecological Synthesis, Snow said. It takes existing knowledge and people from different disciplines and applies them toward a common goal. Snow suggested that perhaps the NSF could organize a workshop to identify gaps in existing knowledge about plantation forestry.

After Snow's report-out, another workgroup member emphasized that much of the research contemplated by the group has already been done, in particular by NCASI, and just needs to be compiled in one place. He said the group agreed that if scientists are going to evaluate transgenic trees and plantations, they need clear and comprehensive data on existing plantations, to use as comparators.

A participant asked if the workgroup considered the need for baseline data on single-clone plantations. Snow said the group did not, but rather considered what exists today in intensively managed plantations. Another participant said that, over the past 10-20 years, plantations have moved from opened pollinated, to closed pollinated, to containing only the best few families of trees. Single-clone plantations are next, he said, so it's important to recognize that information may be needed on a range of plantation types.

One participant said he couldn't resist observing that plantations perhaps also ought to be compared to what otherwise might be there. Are we comparing plantations to old-growth forests, he said, or to row-crop agriculture? The larger eco-

logical and landscape context can be important, he said.

Snow then commented that plantations may actually enable the protection of natural forests. Nathalie Isabel said that is how they promote plantations in Canada; they say plantations help to release pressure on natural forests, because more wood can be grown in a managed plantation than in an unmanaged forest stand. One participant asked whether there is any evidence of that, or whether the success of managed plantations might actually increase the amount of natural land converted to plantations. In Canada, Isabel said, forested land is mostly public, so the government decides what will be converted. Another participant said that studies show, in general, increasing harvest from plantations over time and decreasing harvest from natural stands. But he said there's no way to measure cause and effect. Another participant pointed to the example of Europe, which was once largely clearcut, but natural forests returned once the continent turned to plantation forestry.

Steve DiFazio then presented the workgroup's discussion of models. He said the group first identified questions amenable to addressing with models. The obvious question that could be assessed, he said, is the rate and extent of transgene introgression.

Models are most useful if they don't have to project too far into the future, DiFazio said, and if the researcher is comfortable with broad predictions. One solid use of a model is to look at the relative importance of model assumptions, he said. By altering the levels of different variables, one can look at the importance of each and ultimately set a research agenda. Models are also helpful in estimating degree of uncertainty over time, DiFazio said, and they force a formalization of assumptions.

DiFazio said Group 4 talked a bit about the processes that should be included in a model for transgene flow. They included:

- the whole pollination system (e.g., fertility, dispersal, pollination)
- dispersal of seed or vegetative propagules
- disturbance and habitat creation
- establishment
- competition/mortality (including inter- and intra-specific competition)
- management decisions
- biotic interactions

The species to be modeled are assumed to be poplar, loblolly, and eucalyptus, DiFazio said, as these are the species for which commercialization is being promoted. Poplar and loblolly have been modeled somewhat, he said. Eucalyptus has not, but DiFazio said it would be simpler to do because it has been introduced into the U.S. and has no wild relatives.

Validation and falsification are essential, DiFazio continued (i.e., comparing predictions from different models). DiFazio said that he is biased toward spatially explicit simulation models, but that researchers must look at how other models converge or diverge from them.

To do the validations, DiFazio said, the difficult experiments described in the gene flow report-out must be conducted, otherwise the model is useless. The model must be tested, he said, and some landscape-scale estimates of gene flow must be gathered if the models are to be reliably validated at the scales that are most important for transgenic risk assessment.

Finally, DiFazio said the group talked about how case studies could be used. Because flowering

transgenic plantations do not currently exist, he explained, we may have to use analogous systems as case studies, such as the introduction of exotic species and natural hybrid zones.

The short full-group conversation after DiFazio's report-out covered a range of topics. First, one participant said he remained unclear as to whether APHIS would ever allow the dispersal of tree transgenes under permit, or whether that could occur only after deregulation.

Another person said it is important to be cautious about what is possible with models, as it's going to be particularly difficult to parameterize fitness, for example. Proxies for fitness will have to be used, he said, and scientists will need an iterative process whereby they continually gather new information to improve the model.

One participant asked about a scenario in which scientists would insert a transgene for a particular trait into a plant, and then remove the transgene (but keep the trait) through breeding. If that were possible, he asked, what would be the regulatory status of that plant? Another participant said that has been attempted, but it is not technically feasible, as a single copy of the gene always remains at the lock site.

Earlier the topic of specific mutagenesis had come up, and at this point a participant explained how he thought it could be done. He said you would start with an RNAi construct and take the level of expression of the gene way down, then mutagenize the plant and look for knockouts. Then you would outcross and keep your knockouts. In that way you could remove the transgenic elements and achieve the desired modification via acceptable mutagenic practice, he said. If you just mutagenize (without using RNAi), he explained, you get a recessive mutation in a heterozygote and you can't see it. If you suppress it first, you can see the mutation because it has greater penetration. Another participant commented that homozygous plants are not of particular interest to tree breeders, due to viability issues.

Discussion of Next Steps

The final discussion of the workshop concerned how to move forward with the research ideas presented during the previous two days. First, one participant echoed an idea raised at the beginning of Day Two. Given that significant funding is needed to carry out this research, he said, and that funding institutions may be more likely to fund broadly supported, interdisciplinary projects, workshop participants ought to develop a consensus document describing research needs and priorities relating to genetically engineered trees. This person further suggested that workshop participants then jointly take that document to potential funding agencies, to make the case for funding.

Many participants expressed support for this idea. It was noted by several that building consensus itself takes time and money, and can be hard work. Yet many seemed to believe it would be worth the effort. One participant said she could support such a document only if it were written in the spirit of "*if* GE technology is applied to trees, then these sorts of ecological risk assessment activities should be conducted," rather than, "GE technology *should* move forward, and we should accelerate its development by conducting these risk assessment activities."

Participants also discussed further one of the research activities mentioned during the workshop—that of developing consensus-based baseline documents for each tree species that may be modified via genetic engineering. A number of participants expressed support for this idea. An APHIS representative suggested that participants seek to have such documents peer reviewed, as that would make them most useful to the agency under current law. One participant said the information could be compiled in a book form, with a peer-reviewed chapter for each key species.

Another APHIS representative, speaking generally about GE trees, encouraged participants to "consult early and often" with the agency on any proposed research. He emphasized that the agency has an open door policy and is eager to talk with scientists about their work early in the process, so pitfalls can be avoided.

A participant noted that APHIS is in the process of preparing to issue new regulations governing GE plants. She said this presents an opportunity to think creatively about how the law should apply to GE trees, as it might be logical to have trees treated differently than regular agricultural crops. Another person concurred and said he hoped participants would help work toward a more realistic regulatory system governing GE tree research—one that would not tie the hands of researchers as much as at present.

A participant noted that a number of agencies want to have research questions relating to GE trees addressed and should be able and willing to provide funding. In Canada, one participant said, special funding exists to help regulators compile baseline data and do basic research. Another person agreed there is public value in conducting risk assessments on this technology, so such work is rightfully funded by the public purse.

Another issue that arose again was the idea of having a few select test sites for transgenic trees, where researchers with different expertise could be brought in to do a variety of studies. It could be modeled on the FACE program, one person said.

On another topic, a participant cautioned that the debates about transgenic trees and about using trees as feedstocks for biofuel facilities will focus a lot of attention on the plantation forestry industry in the U.S. The notion that we are on the brink of

significantly expanding plantation forest acreage is going to be an eye-opener for a lot of people, she said.

Finally, a number of participants thanked the organizers for an excellent workshop, and said they appreciated the diversity of participants at the table and the candor of the discussions. In closing, the meeting organizers thanked everyone for their participation and thanked the funders and sponsors for their support. They said they looked forward to continuing this important work together in the future.

Appendix A

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Appendix C

Abbreviations

APHIS	Animal and Plant Health Inspection Service, USDA
DOE	U.S. Department of Energy
EIS	environmental impact statement
EPA	U.S. Environmental Protection Agency
ERA	environmental risk assessment
GE	genetically engineered
GFP	green fluorescent protein
GM	genetically modified
GMO	genetically modified organism
IFB	Institute of Forest Biotechnology
NCASI	National Council for Air and Stream Improvement
NEPA	National Environmental Policy Act
OSU	Oregon State University
PIFB	Pew Initiative on Food and Biotechnology
USDA	U.S. Department of Agriculture

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