Finding of No Significant Impact and Decision Notice

Animal and Plant Health Inspection Service

Issuance of Permit to Release Genetically-Engineered *Populus* Species and Hybrids

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has received a permit application (APHIS number 06–250–01r) from Oregon State University to conduct field tests using clones of *Populus* species and hybrids. Permit application 06–250–01r describes 95 genetic constructs that can be categorized by their intended traits: reproductive sterility genes, genes affecting stature or reduced light response, genes aimed to modify tree physiology, and activation tagging mutants aimed at the development of "experimental domesticates." A subset of these transgenic *Populus* hybrids was previously approved for planting under permit 95–031–01r. An EA was prepared for this permit and the trees were allowed to flower. Permit 95–031–01r expired and was subsequently renewed under permit 00–151–01r. Under permit 00–151–01r additional constructs were added to the field test. These additional trees were not allowed to flower. The permittee has requested that plants added under permit 00–151–01r and plants planted under Notifications (04–096–04n, 05–236–03n, 05–236–04n, 06–069–05n, 06–096–04n, 06–109–08n, 06–125–104n) be incorporated under this new permit (06–250–01r) and also be allowed to flower. A description of the field tests may be found in the attached Environmental Assessment (EA), which was prepared pursuant to APHIS regulations (7 CFR part 372) promulgated under the National Environmental Policy Act. The permit is scheduled to go into effect in February 2008 in Benton County, Oregon.

An EA was prepared and submitted for public comment for 30 days, as announced in a notice published in the *Federal Register* on July 18, 2007 (72 FR 39378–39379, Docket No. APHIS–2007–0018). APHIS received five comments during the comment period and addressed them, where appropriate, in an attachment to this document.

APHIS proposed three different alternatives for the proposed field tests requested in the permit application:

- the denial of the permit (Alternative A)
- the granting of the permit with no Supplemental Permit Conditions (Alternative B)
- the granting of the permit with Supplemental Permit Conditions containing duplicative safety measures and reporting requirements (Alternative C)

Based upon analysis described in the EA, APHIS has determined that the action proposed in Alternative C will not have a significant impact on the quality of the human environment because:

1. The test site is on land controlled by Oregon State University and is expected to provide adequate physical security.

2. There are no sexually compatible wild trees within 20 miles. Sexually compatible trees are located in the Cascade Mountains at 3000 – 4000 feet above sea level.

3. Flowering of the trees in the field test will be earlier than for the native aspens in the Cascades due to the effect of the difference in elevation on flowering time.

4. There are two ornamental trees that are sexually compatible located in an area within 2000 feet of one of the field sites. However, these are in a landscape setting where grass is mowed beneath the trees thereby making it unlikely that progeny could establish in the vicinity. The trees will be monitored for the production of seedlings from the unlikely event of crossing with trees in the field test, to ensure that no progeny establish if these two trees are fertilized from pollen from the field test.

5. The site is not conducive to aspen seed germination and establishment as the planting area lacks cool, moist, bare mineral soil devoid of competition.

6. Aspen seeds lack dormancy. If seeds were to be produced, they will germinate immediately after dispersal or will die. Seeds are viable for only a few days unless given special storage conditions.

7. The supplemental permit conditions stipulate that an annual report be submitted to APHIS that includes: a map and inventory of the plants in the test, which if any of the plants produced flowers or viable seed, which plants were removed and their disposition, and any unanticipated or adverse effects on plants, nontarget organisms and the environment. The test sites and adjacent land within 100 meters shall be monitored for any volunteer *Populus* plants every 6 months during the field test (as indicated in the permit) and for one year after completion of the field test, during which time any volunteer plants will be destroyed before they flower. During the monitoring period following completion of the field test, the site will not be planted with *Populus*, so that any volunteer seedlings that emerge can be easily identified. If volunteers or stump sprouts are still emerging at the end of the first year, a second year will be added to the monitoring period to ensure that no shoots are continuing to be produced.

8. The field test sites and the area around the two nearest sexually compatible aspens to one field site will be monitored for seedling volunteers. Any volunteers found will be devitalized with herbicide or physically removed to a contained facility. The presence and elimination of any volunteers will be reported to APHIS in an annual report.

9. All non-engineered control trees in the field test plot and any plant material removed from the field site will be treated as regulated articles.

10. APHIS has reached a determination that the proposed environmental release under permit 06–250–01r would have no effect on federally listed threatened or endangered species (TES), or species proposed for listing, and no effect on

designated critical habitat or habitat proposed for designation in the action area for the following reasons:

a. The transgenic poplars are not sexually compatible with any threatened or endangered plant species in the action area.

b. No TES plants are located in habitat that would be disturbed or otherwise affected as a result of the conduct of the trial and no critical habitat is present in the location of the trial.

c. None of the TES animal species utilize poplars in the action area for food, cover, or nesting.

d. With the exception of the diphtheria toxin A–chain, the Cry3A toxins, the transgenic modifications are not intended to result in the production, or increase the production of a toxin, natural toxicant, allelochemical, pheromone, or hormone that could directly or indirectly result in killing or interfering with the normal growth, development, or behavior of a TES or species proposed for listing in the action area. The DTA toxin is only produced in very few cells in immature flowers and would be at miniscule levels in the flower tissues. Intercellularly expressed DTA cannot be taken up by adjacent plant cells or by organisms feeding on the plant tissue. The Cry3A toxin is intended to kill Chrysomelid beetles which are a serious pest in Poplar plantations. None of the TES species are coleopteran species. Thus no exposure to these toxins at deleterious levels to TES or proposed species should occur.

11. APHIS has determined that there are no past, present, or reasonably foreseeable actions that would aggregate with effects of the proposed action to create cumulative impacts or in any way reduce the long-term productivity or

sustainability of any of the resources (soil, water, ecosystem quality, biodiversity, etc.) associated with the release site or the ecosystem in which it is situated.

Because APHIS has reached a finding of no significant impact resulting from this field release of transgenic *Populus* species and hybrids, no Environmental Impact Statement will be prepared regarding this proposed field release.

For the reasons enumerated above, and those presented in the EA, which are consistent with regulations implementing the Plant Protection Act, the field test of transgenic *Populus* species and hybrids is hereby authorized.

Michael C. Gregoire

Michael Gregoire
Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
U. S. Department of Agriculture
Date: FEB 4 2008

Attachment
Finding of No Significant Impact
Response to Comments
APHIS 06-250-01r

On July 18, 2007, APHIS published a notice in the *Federal Register* (72 FR 39378–39379, Docket No. APHIS–2007–0018) announcing the availability of an Environmental Assessment for public comment prepared in response to permit application 06–250–01r for a controlled release of *Populus* species and hybrids. During the 30-day comment period, which ended on August 17, 2007, APHIS received 5 comments. Comments opposed to APHIS granting the permit were submitted by two individuals and a public interest group. Comments that supported APHIS granting the permit were submitted by the permit applicant and a limited liability company. The pertinent issues that were raised during the comment period and APHIS' responses to those issues follow:

Issue 1. Two commenters submitted comments reiterating the findings of the EA and also outlined the benefits of using forest trees for cellulose feedstock for production of ethanol in the development of a renewables-based energy and materials economy. The commenters also listed the benefits of the study which included, among others, that the study has the potential to provide substantial benefits to the nation by providing information enabling improved yields in plantations, reduce the risk of spread of transgenic trees, and reducing many of the biological and legal issues that have plagued GE crops.

Response: While the results of the field test might aid in meeting the demands of the nation's biofuel industry and aid in addressing research questions, these comments do not deal with questions addressing plant pest risk and are irrelevant to APHIS's decision-making process. APHIS does not judge the merits of a field test or the necessity of the research being conducted. APHIS evaluates the environmental impacts of a field test, regardless of the merits of the field test.

Issue 2. One commenter expressed a concern about the presence of barnase in the trees since it is a toxin aimed at inducing sterility. The commenter was concerned that barnase will be present in the leaves, stems, and roots of trees and this will adversely affect not only the transgenic plant, but also the fauna and flora of the forest ecosystem. The commenter indicates that "The toxicity of barnase to mammals is well known" and gives citations from the journal Science and Society in support of these claims.

Response: As pointed out in the EA, these genes have been engineered to be expressed during flower development. In this case barnase (a ribonuclease) is expressed primarily in the cells of developing flowers. Barnase is expressed in a small number of cells in developing flowers which last only a short time in the environment. There may be a very low level expression in vegetative tissues since some of the promoters that drive the genes are not exclusively

expressed in floral tissues. However the levels expressed in vegetative tissues are significantly lower than that expressed in developing flower parts. Barstar, a specific inhibitor of barnase, is also being expressed in the vegetative tissues to counteract the low level of barnase activity that might inhibit vegetative growth (1).

Barnase is not toxic when ingested. Ribonucleases, such as barnase, are naturally expressed in all plant tissues and therefore are already part of human and animal diets. (see FDA consultations BNF No. 000031 http://www.cfsan.fda.gov/~rdb/bnfm031.html, BNF No. 000032 http://www.cfsan.fda.gov/~rdb/bnfm032.html BNF No. 000057 http://www.cfsan.fda.gov/~rdb/bnfm057.html and BNF No. 000066 http://www.cfsan.fda.gov/~rdb/bnfM066.html.). Barnase and barstar have also been in products previously deregulated by APHIS and which have gone through a full FDA food and feed safety consultation, for example corn (Petitions 95-228-01p and 98-349-01p), *Cichorium intybus* (Petition 97-148-01p) and Rapeseed (Petitions 98-278-01r and 01-206-01p).

APHIS disputes the claim that the toxicity of barnase to mammals is well known. The articles the commenter cites are not from a credible science source but from a journal of Marxist thought and analysis. As stated above, no adverse effects to wildlife or humans are expected from expressing the barnase in the developing flowers. Because the hazard of the protein is extremely low and has been consumed by animals and humans with no adverse effects, APHIS reasonably concludes that there should not be a significant adverse effect on wildlife from the expression of barnase and barstar in this GE field release.

Issue 3. One commenter was concerned about the use of the diphtheria toxin A-chain to induce sterile flowers and the lack of any published studies on the safety in animals from eating transgenic plants modified with the diphtheria A-chain.

Response: As explained in the EA, there should be very limited exposure of animals to the A-chain component of *DTA.* That is because the A-chain is primarily expressed in the cells of developing flowers. During flower development there would be very few cells expressing the protein and for only a short period of time. There could be a very low level of expression in vegetative tissues based on the promoter being used to drive the gene. The promoter has been shown to impart vegetative expression, but at a level approximately 100-fold below that in floral tissues (2).

The diphtheria toxin A-chain is not expected to present a hazard to animals consuming it because the toxin activity is dependent on two components, an A-chain and a B-chain. The B-chain component allows movement of the holotoxin into cells while the A-chain component disrupts protein synthesis in eukaryotic organisms by inhibiting translocase, the enzyme involved in the elongation phase of protein synthesis. As the GE trees lack the B-chain, the

inhibition of protein synthesis is restricted to a few cells in the developing flowers. In studies conducted by the applicant, no adverse effect on vegetative growth was observed indicating that the expression of the DTA protein was too low to be active in vegetative cells (2). The *DTA* is disarmed from entry into other cells and is expected to only cause rapid death of the cells in which it is expressed. For animals consuming the tissues that contain the A-chain component, A-toxin is not expected to be absorbed into the cells of the GI tract in the absence of the B-chain. Therefore there should be no toxic effect to animals consuming the flowers of plant parts. Together with the low exposure, APHIS reasonably concludes that there should not be a significant adverse effect on wildlife from the expression of diphtheria toxin A in this GE field release.

Issue 4. One commenter was concerned about MADS-box genes being present in the transgenic trees and indicated that "MADS-box transgenes should not be presumed safe, as they are related to the extensively studied animal homeotic genes that regulate development and may well be active in animals."

Response: APHIS does not agree that the introduction of an additional MADS-box gene into poplar trees should be presumed unsafe because they are related to animal homeotic genes and may be active in animals. First, there is no scientific reason to believe that plant MADS proteins could have a significant impact on animals. APHIS is unaware of and the commenter has not provided any evidence that plant MADS box proteins have any activity on animals. Second, animals are unlikely to get the plant MADS box genes through horizontal gene transfer from plants to animals as this process does not occur except perhaps on an evolutionary time scale. Third, animals are unlikely to be exposed to active plant MADS box proteins as these will be digested upon consumption and are unlikely to remain active in the GI tract of animals. Finally, plants are estimated to have about 100 MADS box genes (3) so animals are naturally and continuously exposed to plant MADS box genes and proteins. The addition of one more plant derived MADS box gene into Poplar trees through genetic engineering should have no incremental impact on activity in animals even in the unlikely case that such proteins may be active in animals.

Issue 5. One comment points out the use of RNA interference (RNAi) gene therapy and its potential for adverse health effects based on studies with mice. RNAi involves the use of a small interfering double stranded RNA of approximately 21-15 nucleotides that is complimentary to a known messenger RNA that is used to block its expression. In this case Populus RNAi genes have been used in an attempt to induce sterile flowers.

Response: The safety of nucleic acids is widely accepted. Both RNA and DNA are part of all food products that we consume. Gene therapy is a technique for correcting defective genes responsible for disease development

(http://www.ornl.gov/sci/techresources/Human_Genome/medicine/genetherapy.shtml). It is still in the early experimental stages and has not proven successful in clinical trials. Researchers may use one of several approaches for correcting faulty genes including RNAi. In the current case, RNAi is being used to inactivate a gene involved in flower development and thereby induce sterile flowers in a plant and not to correct a defective gene in a patient (or mammalian model organism) for gene therapy. The potential adverse health effects of gene therapy are not relevant to this field test as the situations are so entirely different that a comparison is without meaning.

Issue 6. One commenter was concerned about the fact that the Poplars were engineered with genes involved with light response and gibberellin metabolism genes and that the transgenes in these releases have not been studied regarding any potential untoward effects.

Response: The gibberellin gene family and phytochrome receptor genes have been studied in many other plant species and include some of the most extensively studied genes in plant science due to their effects on growth and development. Natural and induced mutations in gibberellin biosynthesis have been isolated and have been exploited in conventional plant breeding to produce dwarf varieties in many crops including fruit trees. Similarly, gibberellins are routinely applied as growth regulators to many food and feed crops and ornamentals to increase the yield and quality of crops. As with any field trial, the responsible party is required to report any unusual effects to APHIS should they occur, and the field trial is confined to prevent the establishment of the regulated material outside the test site. Thus while there is no reason to believe that the light response and gibberellin genes will have any untoward effects, the experiment is conducted in a way that even if they did, the material will be confined to the test site.

Issue 7. One commenter was concerned about the use of the 4CL1 gene from *Populus tremuloides* inserted to alter lignin levels. The commenter is concerned that low lignin trees are likely to be more susceptible to pests and to be prone to wind damage because they lack mechanical strength.

Response: One of the purposes of these field studies is designed to answer the above question. To date the permittee has observed no changes in the incidence of pests, beneficial insects or pathogens in the existing field tests. However, the test will be used to gather data to answer the above question, and would be important information to gather to determine if indeed low lignin leads to a greater incidence of plant pests on these transgenic trees. Since there are very few trees, and the test is a confined field trial, there should be no impact on the environment should these few trees be found to be more susceptible to pests or wind. The trees in the field test will be monitored for any increased disease or pest susceptibility at least once a year and any unusual occurrence must be reported to APHIS.

Issue 8. One commenter was concerned about the use of activation tagging. Activation tagging is insertional mutagenesis using insertion vectors that contain a strong transcription enhancer to up-regulate a gene near the insertion site. The insertions appear randomly in the genome, resulting in gain of function dominant mutations. The commenter does not believe that it is safe for field test releases because "it is likely to cause unintended insertional mutagenesis in a range of microorganisms and animals that interact with the transgenic plants."

Response: APHIS disagrees with this comment. The commenter did not provide any refereed citations that would substantiate their assumption. The inserted DNA used for the activation tagging is stably integrated into the Poplar genome. It is no more likely to cause unintended insertional mutagenesis in another organism than any other DNA within the Poplar genome.

Issue 9. One commenter is concerned about the ability of the applicant to monitor the field sites, indicates that mechanical pruning to prevent flowering seems risky in a large complex array of experimental trees, and believes that it will be inevitable that the transgenes will be dispersed.

Response: APHIS disagrees with this comment. Pruning of poplar trees on a large scale is very easy and effective. The trees are all planted in clonal blocks and are easily maintained to prevent flowering. It is a common and well-established practice to maintain poplar clones at close spacing with severe pruning to prevent flowering and to maintain material for research purposes. In addition, APHIS will be inspecting the field trial to verify compliance to permit conditions.

Issue 10. One commenter is concerned about horizontal gene transfer and indicates that it is a distinct possibility because "the extensive root system of trees is a hotbed for horizontal gene transfer and recombination."

Response: APHIS disagrees with this comment. The commenter provides no evidence to support the claim that the root system of trees is a hotbed for horizontal gene transfer and recombination. APHIS has reviewed the scientific literature on horizontal gene transfer and concluded that it is very unlikely to occur from trees to any other organisms (discussed in the EA on page 12).

Issue 11. One commenter indicated that the proposal made no attempt to present the genetic modifications of the many transgenic lines in a rational and coherent manner, with diagrams detailing the transgenic constructs in each line being tested along with an explanation of the function of each gene.

Response: A list of all the genes and donors that are in the field test are listed in Table 1 of the EA. In addition, a description of each of the genes is covered in Appendix III of the EA.

Issue 12. One commenter indicated that there are so many separate lines being tested in one big 320 acre site that transgene escape form the site is bound to happen due to human error.

Response: The commenter is mistaken in that the field tests are on three different sites totaling 30 acres and not 320. This permittee has an excellent compliance record, and has been inspected a number of times with no compliance issues. APHIS has confirmed that the applicant has the appropriate resources to maintain the test and monitor for volunteers. In addition, numerous redundant confinement measures are employed to minimize the likelihood of escape such as isolation distances, use of male sterility, removal of flowers, inhospitable environment (others)

References

1. Wei, H., R. Meilan, et al. (2007). Field trial detects incomplete barstar attenuation of vegetative cytotoxicity in Populus trees containing a poplar LEAFY promoter:barnase sterility transgene. Molecular Breeding **19**: 69-85.

2. Skinner, J. S., R. Meilan, et al. (2003). The *Populus PTD* promoter imparts floral-predominant expression and enables high levels of floral-organ ablation in *Populus*, *Nicotiana* and *Arabidopsis*. Molecular Breeding **12**: 119-132.

3. Nam, J., J. Kim, et al. (2004). Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. PNAS 101: 1910-1915.

USDA/APHIS Environmental Assessment

In response to permit application (06-250-01r) received from
Oregon State University for a field-test of genetically engineered
Populus alba and *Populus hybrids*

U.S. Department of Agriculture
Animal and Plant Health Inspection Service
Biotechnology Regulatory Services

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I. INTRODUCTION

A. Summary

USDA/APHIS has prepared an environmental assessment in response to a permit application (APHIS Number 06-250-01R) received from Oregon State University, to conduct a field test with genetically engineered (transgenic) clones of *Populus* species and hybrids. These plants have been genetically engineered with a number of different constructs. The primary purpose of the test is to examine the effects of the genetic constructs on the intended traits - reproductive sterility, reduced stature, reduced light response, and modified lignin content. In addition, trials are planned to examine the functions of various genes in poplar through a transgenic process that results in the hyper-activation of native genes. Some of the trees have been engineered to express tolerance to phosphinothricin herbicides.

A previous EA was prepared for a subset of trees in this test under Permit 95-031-01R. Under this permit, trees engineered with sterility constructs were allowed to flower. Since the researcher intends to add more trees to the permit and allow these additional trees to flower, a new EA has been prepared that updates the previous EA.

This environmental assessment was prepared in accordance with: (1) The National Environmental Policy Act of 1969 (NEPA), as amended (42 U.S.C. 4321 et seq.); (2) regulations of the Council on Environmental Quality for implementing the procedural provisions of NEPA (40 CFR parts 1500-1508); (3) USDA regulations implementing NEPA (7 CFR part 1b); and (4) APHIS NEPA Implementing Procedures (7 CFR part 372).

The field tests are in progress on three sites located in Benton County, Oregon, following the earlier Permit 95-031-01R. The additional tests are proposed to continue for up to nine years in order to evaluate the expression of male sterility traits, modified tree chemistry and domestication genes.

Confinement of these field trials is achieved through a combination of factors that limit the potential for pollination of nearby native or planted trees and the dissemination of viable seed that could establish and persist in the environment. The chance of asexual spread is also low. Monitoring the area of the test site should be sufficient to detect seedlings or suckers and destroy them before they could establish.

The proposed field test is a controlled release of the regulated article into the environment. Procedures outlined in the permit application for termination of the field test should be sufficient to ensure that none of the transgenic poplar plants persist in the environment. The remote distance from any sexually compatible species and the inhospitable environment for seedling germination in the field test area make it unlikely that the introduced genes will move from the transgenic test plants and persist in native populations of *Populus*. The proposed field test should not significantly impact plant or animal populations, including any species that are Federally listed as threatened or endangered in the test site county.

The APHIS review and analysis of the data indicate that the proposed field test should not present a risk of introduction and dissemination of a plant pest and should not have a significant impact on the quality of the human environment. Therefore, APHIS concludes that it is proper to issue a three year permit, with supplemental permit conditions that include additional monitoring and reporting.

B. Regulatory Authority

The authorities for regulation of genetically engineered *Populus* are the Plant Protection Act, 7 U.S.C. 7701-7772, and USDA, APHIS regulations under 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests." A genetically engineered organism is considered a regulated article if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxonomic groups listed in the regulation and is also a plant pest, or if there is a reason to believe it is a plant pest. In this submission, the plants have been genetically engineered using disarmed *Agrobacterium tumefaciens*, which is one of the listed taxa in 7 CFR 340.2. The DNA sequences introduced into the transgenic plants also contain regulatory sequences from the plant pests cauliflower mosaic virus, tobacco mosaic virus, *Aspergillus nidulans* and *Agrobacterium tumefaciens*.

This environmental assessment (EA) was conducted under the authority of the National Environmental Policy Act (NEPA), 42 U.S.C. 4321 and 7 CFR part 372, NEPA Implementing Procedures. Generally issuance of a permit for confined field releases of regulated articles is categorically excluded from requirements for an environmental assessment (EA) under APHIS NEPA implementing procedures (7 CFR 372.5(c)(3)(ii)). However, when APHIS determines that a confined field release of genetically engineered organisms has the potential to affect significantly the quality of the human environment, as those terms are defined in 40 CFR 1508.14 and 1508.27, an environmental assessment or environmental impact statement will be prepared, pursuant to 7 CFR 372.5(d). This EA was prepared because the permittee intends to allow the trees to grow under permit for a number of years and intends to let some of the trees flower. The actions described in the application for permit 06-250-01r involve the release of transgenic *Populus tremula* (European aspen) *x Populus alba* (White poplar), *Populus tremula* x *Populus tremuloides* (Quaking aspen), and *Populus alba* into the environment and allowing them to flower. In addition the permit includes four clones derived from a cross of *Populus trichocarpa* (Black Cottonwood) *x Populus deltoides* (Eastern Cottonwood) that will not be allowed to flower. A previous EA was prepared for a subset of trees in this test under Permit 95-031-01r. Under permit 95-031-01r, subsequently renewed under 00-151-01r, trees were engineered with sterility constructs and were allowed to flower. Since the researcher wishes to add additional trees to the permit and to allow these trees to flower, APHIS is preparing an Environmental Assessment to address issues raised by the release of trees transformed with these new constructs.

II. NEED FOR THE PROPOSED ACTION

A. Proposed Action

The proposed action is for APHIS, Biotechnology Regulatory Services (BRS), to issue a permit for field-testing clones of *Populus* species and hybrids (see Appendix I) engineered to express a number of different genes (see Appendix III). There are 95 constructs in the field test. These can be categorized into reproductive sterility genes, genes affecting stature or light response, genes aimed to modify tree chemistry, and activation tagging mutants (see Appendix III for details) aimed at the development of "experimental domesticates." With the exception of a subset of trees (8 events in 1 clone of *Populus tremula x Populus alba*, 1 clone of *Populus tremula* x *Populus tremuloides* and 4 clones of *Populus trichocarpa x Populus deltoides*) held in a clone bank, flowering will be allowed to examine the efficacy of the introduced constructs.

B. Purpose of this Environmental Assessment

The purpose of this EA is to assess any potential adverse environmental effects of a field research study in Benton County, Oregon. The permit application was received by APHIS, BRS on September 7, 2006. It was submitted by Dr. Steven Strauss, Oregon State University, Oregon. The application number is 06-250-01r.

C. Need for This Action

Under APHIS regulations, the receipt of a permit application to introduce a genetically engineered organism requires a response from the Administrator:

Administrative action on application. After the receipt and review by APHIS of the application and the data submitted pursuant to paragraph (a) of this section, including any additional information requested by APHIS, a permit shall be granted or denied. 7 CFR 340.4(e).

D. Purpose and Description of the Research

The focus of the research under this permit is on field evaluation of genes that can promote the biosafety and economic value of trees used for intensive forestry, for bioenergy production, and as woody ornamentals. Part of the research is aimed to reduce or eliminate the risks from gene flow and spread of transgenic trees (Strauss et al. 1995). The aim is to introduce genes that have "domestication" effects that will reduce fitness in competition with non-transgenic or wild plants. Four categories of traits are being examined in these field trials:

1. Reproductive sterility – intended to make transgenic trees less able to produce viable pollen and/or seeds.
2. Reduced stature/light response – intended to make transgenic trees and their progeny much less able to compete with non-transgenic trees.
3. Modified tree chemistry – intended to reduce compounds such as lignin.
4. Activation tagging mutants - aimed at the development of "experimental domesticates," where genes are incorporated that reduce fitness of the engineered plants when growing in competition with non-transgenic plants.

For further details on the species and hybrids in the field test, anticipated phenotypes, and which trees will be allowed to flower, see Appendices I and III.

III. ALTERNATIVES

A. No Action

Under APHIS/BRS regulations, the Administrator must either grant or deny permits properly submitted under 7 CFR part 340. For the purposes of this Environmental Assessment, the No Action alternative would be the denial of permit application 06-250-01r.

A subset of these transgenic *Populus* hybrids was previously approved for planting under permit 95-031-01r. An EA was prepared for this permit and the trees were allowed to flower. Permit 95-031-01r expired and was subsequently renewed under permit 00-151-01r. Under permit 00-151-01r additional constructs were added to the field test. These additional trees were not allowed to flower. The permittee has requested that plants added under permit 00-151-01r and plants planted under Notifications (04-096-04n, 05-236-03n, 05-236-04n, 06-069-05n, 06-096-04n, 06-109-08n, 06-125-104n) be incorporated under this new permit (06-250-01r) and also be allowed to flower. This new permit covers all of the current and planned field trials by the applicant with *Populus*. Under the No Action Alternative, if this permit is denied, the trees under the existing permit and notifications will either continue to be allowed to be tested under a new permit that does not allow flowering or will be removed from the existing field tests.

B. Issue the Permit as Received

Issuing this permit would allow the research to proceed at locations in Benton County, Oregon under the conditions provided by the permittee and the standard permit conditions under 7 CFR 340.4(f)1-11 (http://www.access.gpo.gov/nara/cfr/waisidx_05/7cfr340_05.html). Under this alternative, the field release of the genetically engineered *Populus* plants would be authorized at the specified location for the duration requested by the applicant (up to September 2016 for some of the trees) with no additional conditions imposed by APHIS/BRS.

The following redundant mitigation measures/factors are incorporated into the field test design by the permittee to promote a confined field release and to ensure the least amount of harm to the environment:

a. The test site is on land controlled by Oregon State University and is expected to provide adequate physical security.
b. There are no sexually compatible wild trees within 20 miles. Sexually compatible trees are located in the Cascade mountains at 3,000 – 4,000 feet above sea level.
c. Flowering of the trees in the field test will be earlier than for the native aspens in the Cascades due to the effect of the difference in elevation on flowering time.

d. There are two ornamental trees that are sexually compatible located in an area within 2000 feet of one of the field sites. However, these are in a landscape setting where grass is mowed beneath the trees thereby making it unlikely that progeny could establish in the vicinity. The trees will be monitored for the production of seedlings from the unlikely event of crossing with trees in the field test, to ensure that no progeny establish if these two trees are fertilized from pollen from the field test.
e. The site is not conducive to aspen seed germination and establishment as the planting area lacks cool, moist, bare mineral soil devoid of competition.
f. Aspen seeds lack dormancy. If seeds were to be produced, they will germinate immediately after dispersal or will die. Seeds are viable for only a few days unless given special storage conditions.

C. Issue Permit with Supplemental Conditions

The APHIS-preferred alternative is to issue the permit with supplemental permit conditions and for only three years, a shorter duration than requested. The permit will need to be renewed to allow the transgenic plants to remain in the ground beyond this time period. Under this alternative, APHIS would issue the permit to allow the research to proceed at field test sites in Benton County Oregon where supplemental permit conditions, based on APHIS scientific analysis of the permit application, input from the State of Oregon, and public comment from this environmental assessment, would be required. If warranted, based on environmental risk of escape of the engineered organism, APHIS will require further mitigating measures and monitoring to prevent spread of the organism outside the field production area.

Of those trees requested to be allowed to flower in the field test, APHIS proposes to allow all of them to flower. APHIS proposes to include the following measures specific to this permit to promote a confined field release and to ensure no significant harm to the environment:

a. The field test sites will be monitored for flowering and seed formation. Data will be provided to APHIS in an annual report documenting which trees produced flowers and which if any produced viable seeds.
b. The field test sites and the area around the two nearest sexually compatible aspens to one field site will be monitored for seedling volunteers. Any volunteers found will be devitalized with herbicide or physically removed to a contained facility. The presence and elimination of any volunteers will be reported to APHIS in an annual report.
c. All non-engineered control trees in the field test plot and any plant material removed from the field site will be treated as regulated articles.

The full proposed supplemental permit conditions are included as Appendix VI to this EA.

IV. ENVIRONMENTAL CONSEQUENCES OF THE PROPOSED ACTION AND ALTERNATIVES

A. Deny the Permit

To deny the permit application would have no expected potential adverse environmental impact, would prevent the field research from proceeding, and prevent any benefits associated with the knowledge gained from this research study.

B. Issuance of the Permit as Received

Under this alternative APHIS would issue the permit as received and would impose no supplemental permit conditions. No adverse consequences to non-target organisms or environmental quality are expected from the field release of these transgenic *Populus* for the reasons stated below:

- With the exception of the Bt construct which is expected to have insecticidal properties against certain insects that feed on the transgenic plant, the proteins produced by genes introduced into these *Populus* lines are not expected to have toxicological or allergenic affects. The trees engineered with the Bt construct will be maintained in the clone bank as managed hedges and will not be allowed to flower.

- With the exception of the trees engineered with the Bt construct, none of the introduced genes are intended to provide the engineered *Populus* trees with any selective advantage over non-engineered *Populus* in their ability to be disseminated or to become established in the environment. To eliminate the possibility of pollen gene flow to trees outside the field trial, trees engineered with the Bt construct will not be allowed to flower.

C. Issuance of the Permit with Additional Conditions

Under this APHIS-preferred alternative, APHIS will authorize the permit for only three years and impose additional measures and monitoring included in the proposed supplemental permit conditions summarized in III. C. above and in detail in Appendix VI to further ensure that the field test remains confined and there will be no significant harm to the environment. This alternative is not expected to have any adverse environmental impacts for the same biological and physical reasons as indicated above for issuance of the permit as received. The proposed monitoring and annual reporting of a) flowering, b) seed formation, and c) the presence and devitalization of volunteers will allow APHIS to assess whether additional monitoring is required, whether the monitoring area should be extended, and whether devitalization methods should be modified in the event that the permit is renewed for a longer duration. This should address any concerns that may arise due to unanticipated effects or phenotypes since many of the new transgenic trees have genetic mutations for altered flowering or sterility or insertion or activation-tagged mutations whose phenotypes are not yet fully characterized. In addition to monitoring required by the applicant, all field tests are subject to inspection by APHIS as a standard permit condition.

A person who is issued a permit and his/her employees or agents shall comply with standard permit conditions under 7 CFR 340.4(f)1-11 http://www.access.gpo.gov/nara/cfr/waisidx_05/7cfr340_05.html and any supplemental conditions (Appendix VI) which shall be listed on the permit, as deemed by the Deputy Administrator to be necessary to prevent the dissemination and establishment of plant pests 7 CFR 340.4(f)

D. Potential Environmental Impact of the Research using Transgenic Populus.

The potential environmental impact of this research was also covered in the EA for permit 95-031-01r located at: http://www.isb.vt.edu/biomon/releapdf/9503101r.ea.pdf. A subset of the trees in the pending permit 06-250-01r was covered in the previous EA and has been flowering for some time as requested under the original permit. Parts of the previous EA are incorporated for reference.

1. Possibility of Gene Flow Outside of the Field Test:

To understand gene flow in *Populus* it is important to know that the genus *Populus* is made up of five sections. Hybrids between some sections are common while hybrids between other sections rarely, if ever, occur (see Figure 1 in Appendix II). For a detailed discussion of the biology of *Populus*, and potential sexual crosses that can occur, see Appendix II.

The poplar clones used in this permit that would be allowed to flower are *Populus* hybrids or *Populus alba*. Three clones were used in the study and are the subject of this EA. These are 717-1B4, a female clone derived from a cross between *Populus tremula* x *Populus alba*; 353-38, a male clone derived from a cross between *Populus tremula* x *Populus tremuloides*; and 6K10, a female clone of *Populus alba*. All are from the section Populus (Leuce). These are commonly known as aspens and white poplar.

In addition, four clones of the hybrid *Populus trichocarpa* x *Populus deltoides* of the sections Aigeiros and Tacamahaca that were transformed are proposed to be grown under this permit. These are commonly known as cottonwoods. This case is a cross between black cottonwood and Eastern cottonwood. These four clones will not be allowed to flower and will be maintained in a clone bank with frequent pruning. A clone bank is a collection of trees typically grown at narrow spacing where the trees are not allowed to grow tall and are frequently pruned to keep them short and manageable. Cuttings can be collected from these trees to vegetatively propagate the clone for plantation establishment or to re-establish new clone banks.

Native black cottonwood, *Populus trichocarpa*, is found in the vicinity of the field test site and occurs widely in the Willamette valley in Oregon. *P. trichocarpa* is in the section Tacamahaca. Also in the sections Tacamahaca and Aigeiros are cottonwoods and poplars planted in landscape settings. Such trees may be sexually compatible with the four clones of the hybrid *Populus trichocarpa* x *Populus deltoides* which are also in sections Aigeiros and Tacamahaca. However these clones are only being grown in the clone bank and will not be allowed to flower. The trees that are in the field tests that will be allowed to flower are clones from hybrid aspen and white poplar. These are in the section Populus (Leuce) and are not sexually compatible with the native cottonwoods and poplars in the area (sections Tacamahaca and Aigeiros). Furthermore, pollen

formation is expected to be minimal as most of the transgenic poplars have been engineered with traits which prevent the trees from producing viable pollen. The test sites are at least 20 miles distant from the nearest native aspens that could hybridize with trees from the test site. As these sexually compatible native trees are located at a high elevation in the Cascade mountains, the two populations are unlikely to be flowering at the same time. The incidence of planted aspens in Corvallis is rare. The permittee has identified two nearby aspen trees, in a landscaped setting, that could theoretically cross with pollen from the male clone 353-38 in the field test. Similarly, there is a possibility that the female clones within the field test can cross with the male clones to produce viable offspring.

In the unlikely event that pollination occurs, the conditions surrounding the trees are not conducive to seedling establishment. For establishment, seedlings require moist sunny sites free from competing vegetation. Such sites are not found in close proximity to the test site. Furthermore, because aspen seeds lack dormancy, they are not going to accumulate in a seed bank that would contribute to a germinating population at a future time should the environment change to favor aspen establishment. All areas within the test site and in the vicinity of sexually compatible trees are regularly monitored for progeny. Should any be found, such progeny would be destroyed. If hybrids were to occur, they would be easily recognizable due to their distinct leaf and shoot morphology compared to wild cottonwoods. To date the permittee has not found any seedlings produced from the trees currently flowering under permit despite the fact that the 717-1B4 female hybrid of *P. tremula* x *P. alba* has produced seed for several years under APHIS permit.

The engineered traits are unlikely to significantly increase the ability of poplars to survive and reproduce. The tolerance to the herbicide phosphinothricin (glufosinate) will not affect the sensitivity of these trees to a range of other herbicides that are registered for use on poplars. Therefore in the unlikely event that any seedlings were produced from the trees containing the *bar* gene, these seedlings could be treated and destroyed with other herbicides. Triclopyr, imazapyr, 2,4-D, and hexazinone are effective options.

For these reasons, APHIS concludes that gene flow outside the test site is highly unlikely.

2. Possibility of Vegetative Propagation / Persistence Outside of the field test.

Poplars (cottonwoods), particularly of section Tacamahaca but also of Aigeiros, can be easily propagated vegetatively by rooting of cuttings from trees of any age. This is a common method for the propagation of elite clones and plantation establishment. Rooting of branches broken from trees can be a means for occasional vegetative spread along river corridors. Natural abscissions of branches, or cladoptosis, may also play a role in vegetative propagation (Galloway and Worrall 1979).

However, rooting of shoots is very difficult among most species of the section Populus. These are very difficult to propagate by rooted stem cuttings. Therefore it is highly unlikely that any shoots that fall or that are removed from the trees would propagate themselves in the wild.

Suckering (production of shoots from subterranean roots) is very common and prolific in *Populus* (Baker 1918) in both natural and commercial plantings. However, these shoots are always in the

vicinity of existing trees and are easily removed by hand pruning. Since these trees are being maintained in a plantation setting any suckers that are produced will be eliminated by hand pruning. When the planting is to be devitalized the repeated use of registered systemic herbicides or contact herbicides will aid in the termination of the field test.

3. Horizontal Gene Transfer to Other Organisms

Horizontal gene transfer of the genetic constructs engineered into the transgenic trees to bacteria and subsequent expression of the DNA is unlikely to occur. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Kaneko et al. 2000);(Wood et al. 2001, Kaneko et al. 2002). There is no evidence that these organisms contain genes derived from plants. Second, in cases where review of sequence data implied that horizontal gene transfer occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Koonin et al. 2001, Brown 2003). Third, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus even if horizontal gene transfer occurred, proteins corresponding to the transgenes are not likely to be produced. Fourth, the FDA has evaluated horizontal gene transfer from the use of antibiotic resistance marker genes and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is remote (http://vm.cfsan.fda.gov/~dms/opa-armg.html). Therefore APHIS concludes that horizontal gene transfer poses no significant environmental risk.

4. Fate of Transgenic DNA in Humans and Animals

The permittee has taken steps to reduce animal access to the transgenic and recipient plots, and there is no intention to use the transgenic *Populus* for animal feed. Therefore the information presented in this section is for the unlikely event of accidental consumption by browsing animals.

Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production and microbial fermentation (cheese and yogurt) since the late 1970's. More than 1 billion cumulative acres of engineered food and feed crops have been grown and consumed world wide in the past seven years (International Service for the Acquisition of Agri-biotech Applications, (ISAAA) at: http://www.isaaa.org/kc/CBTNews/press_release/briefs30/es_b30.pdf. The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (66 FR 37817-37830).

There have been several studies in humans and animals following the fate of DNA once consumed (Mercer et al. 1999, Beever and Kemp 2000, Duggan et al. 2000, Einspanier et al. 2001, Chambers et al. 2002, Netherwood et al. 2002, Duggan et al. 2003). The majority of DNA consumed is degraded in the gastro-intestinal tract although the degradation is not 100% efficient. There is evidence that DNA from consumed food can move from the GI tract lumen to other areas of the

body and that this is a normal occurrence. No risks have been identified as a result of this movement.

5. Risk of the Gene products on the Environment

Genes used as selectable markers

All of the introduced constructs contain the *nptII* gene from *Escherichia coli* or the *bar* gene from *Streptomyces hygroscopicus* as a selectable marker gene to facilitate the selection of transformed plant tissue in the laboratory. Some constructs also use the β-glucuronidase gene (*gus*) from *E. coli* as another marker in the transgenic plants.

The selectable marker gene *nptII*, encodes for neomycin phosphotransferase NPTII, which confers tolerance to the antibiotic kanamycin. Neomycin phosphotransferase is an enzyme that inactivates the antibiotic kanamycin thereby allowing cells containing this gene to grow on medium containing kanamycin. The *nptII* gene has been given GRAS (Generally Recognized as Safe) status since 1993 and is devoid of inherent plant pest characteristics (Fuchs et al. 1993). Therefore, APHIS has determined there would be no significant environmental impacts from this trait as a result of the proposed trial.

The *bar* gene encodes phosphinothricin acetyltransferase, an enzyme that confers tolerance to the phosphinothricin (glufosinate) class of herbicides. This was also used in the selection of transformants in the laboratory. The EPA reviewed the safety of phosphinothricin acetyl transferase (40 CFR part 180) and found the *bar* enzyme protein to be non-toxic to mammals. An existing tolerance exemption, 40 CFR 180.1151, exists for phosphinothricin acetyltransferase and the genetic material necessary for its production in all plants. The phosphinothricin class of herbicides is not widely used to control trees, therefore tolerance to this herbicide would not affect control strategies for poplars, even if the trait were to become established in the native population. APHIS therefore concludes there would be no significant environmental impacts from this trait as a result of the proposed trial.

The gene for β-glucuronidase expression has been evaluated in four transgenic crops that have been approved for deregulation by USDA/APHIS (petitions # 96-068-01p, # 97-008-01p, # 98-173-01p, and # 00-342-01p), and deemed not to pose a threat to agriculture or the environment. In its review, the EPA concluded that there is a lack of similarity between the enzyme β-glucuronidase and known mammalian toxins or human allergens, thus the EPA deemed the enzyme β-glucuronidase exempt from a tolerance requirement under the FFDCA (66 FR 42957-42962). Therefore APHIS concludes there would be no significant impact on the environment from this trait as a result of the proposed trial.

Genes conferring reproductive sterility

Some of the transgenic plants have been engineered with the barstar and barnase genes derived from the bacterium *Bacillus amyloliquefaciens*. These genes have been engineered to be expressed in the tapetum cells of the pollen sac during pollen development. Barnase is the common name for a specific extracellular ribonuclease secreted by the bacterium *Bacillus amyloliquefaciens*.

Ribonuclease enzymes are naturally occurring and found in many types of organisms, including bacteria and eukaryotes. Barstar is the name for a specific inhibitor of the barnase enzyme. The inhibition of the barnase enzyme by barstar is highly specific. Barnase and barstar show no significant sequence homology to known allergens, and ribonucleases and ribonuclease inhibitors (such as barnase and barstar) are naturally expressed in various plant tissues and therefore are already part of human and animal diets (see FDA consultations BNF No. 000031 http://www.cfsan.fda.gov/~rdb/bnfm031.html, BNF No. 000032 http://www.cfsan.fda.gov/~rdb/bnfm032.html BNF No. 000057 http://www.cfsan.fda.gov/~rdb/bnfm057.html and BNF No. 000066 http://www.cfsan.fda.gov/~rdb/bnfM066.html.) Barnase and barstar have been in products previously deregulated by APHIS, for example corn (Petitions 95-228-01p and 98-349-01p), *Cichorium intybus* (Petition 97-148-01p) and Rapeseed (Petitions 98-278-01r and 01-206-01p).

Similarly some of the transgenic poplars have been engineered with the *DTA* gene derived from the bacterium *Corynebacterium diptheriae*, the causal agent of diptheria. Like the barnase/barstar construct, the preferential expression of the *DTA* genes in floral tissues results in reproductive sterility. The *DTA* gene encodes the A-chain portion of the diptheria toxin. Diptheria toxin is comprised of the B-chain component that allows movement of the holotoxin into cells and the A-chain component that disrupts protein synthesis in eukaryotic organisms by inhibiting translocase, the enzyme involved in the elongation phase of protein synthesis. In this case the A-chain is expressed without the B-chain component and therefore the inhibition of protein synthesis is restricted to the cell in which the gene is expressed. Thus the *DTA* is disarmed from entry into other cells and is expected to only cause rapid death of the cells in which it is expressed. The DTA protein is expected to be expressed only in very few living cells of surviving, developing flowers, will be present in only miniscule concentrations, and should not pose a danger to humans or other eukaryotes.

Other genes resulting in reproductive sterility, reduced stature, altered light response, modified tree chemistry, and activation tagging mutants

Many of the other genes being introduced into *Populus* are derived from other plants. In some cases the native gene is being expressed and in other cases the expression of the native gene or protein is modified. These modifications are aimed to produce sterile plants, plants with modified growth or altered levels of lignin. In most cases the genes of interest are *Populus* genes being engineered back into *Populus*. Other genes are derived from *Arabidopsis thaliana* and *Phaseolus coccineus* (see Table 1 in Appendix III for details). These genes are intended to alter pathways that already exist in plants, and primarily those involved in flower morphology. An antisense copy of the *4CL1* gene from *Populus tremuloides* was inserted to reduce lignin levels and affects lignin levels and other enzyme levels in the lignin synthesis pathway. The constructs were designed to reduce the expression of native poplar genes and not to express any additional proteins. Therefore there is no reason to believe that the expression of these genes will result in the development of toxic proteins. Therefore APHIS concludes there would be no significant impact on the environment by the introduction of these genes into the field test.

Genes in plants in the clone bank:

In three cases the marker gene *Gfp* from *Aequorea Victoria*, which codes for Green Fluorescent Protein (GFP) was used for selection and gene expression studies. In addition there is one clone that was transformed with the *Cry3A* gene from *Bacillus thuringiensis*, which codes for the Cry3A protein toxic to coleopteran insects. All of the transgenic plants containing GFP and Cry3A will be confined by keeping them in the clone bank, which will be pruned, and will not be allowed to flower. *Gfp* does not code for a toxic protein so should not pose a danger to humans or other eukaryotes. The Cry3A gene was engineered into the trees to control Chrysomelid beetles. Any insects that feed on the leaves of *Populus* containing the *Cry3A* gene that might be killed by the presence of the Cry3A protein are considered plant pests. These trees are only a small percentage of trees in the field test and will be regularly pruned, so the amount of leaf fall would be minimal and would be unlikely to pose a significant risk to non-target beetles since the protein is expected to be degraded as the leaves dry down and decompose.

Non-coding sequences.

The transgenic *Populus* also contain non-coding regulatory sequences derived from plants (*Nicotiana, Solanum,* and *Arabidopsis*) and plant pathogens (CMV, TMV, *Aspergillus nidulans*, and *A. tumefaciens).* The non-coding regions of the plant pathogens do not result in disease in the plants into which they have been inserted. None of these sequences are expected to pose a plant pest risk.

6. Alteration in Weediness characteristics

None of the genes introduced into *Populus* code for traits that would be expected to make the plants more weedy or invasive. Furthermore, the preferred alternative includes additional conditions and monitoring to ensure that these transgenic *Populus* do not persist in the environment as a result of the proposed field tests. The introduced genes that might change plant morphology are expected to affect flowering and are aimed to make the trees produce less pollen; and are therefore likely to decrease fitness. The genes introduced to affect stature or response to light are expected to produce plants that are dwarf or have other forms of growth inhibition.

7. Alteration in Susceptibility to Disease or Insects

With the exception of the Bt Cry 3A gene discussed above, there has been no intentional genetic change in these plants to affect their susceptibility to disease or insect damage. Reduced lignin could potentially result in changes in fitness or susceptibility to bark beetles in some species or environments (Wainhouse et al. 1990, Pedersen et al. 2005). However, in this study, the permittee has observed no changes in the incidence of pests, beneficial insects or pathogens in the existing field tests. None of the other genes being engineered into the *Populus* plants are expected to alter the susceptibility of the transgenic *Populus* plants to disease or insect damage.

Execution of the prescribed periodic monitoring of the field plots will allow the detection of any unexpected infestation by plant disease organisms or animal pests and the application of remedial measures, including removal of the trees if necessary. The permittee is required to report any such unanticipated effects to APHIS under the terms of the permit. See 7 CFR 340.4(f)(10)(ii).

8. Effects on Native Floral and Faunal Communities

a. Native Floral Communities

The field sites in the permit application are located in the Willamette Valley in Oregon. It is a mixture of croplands and forested areas, primarily consisting of conifers. These areas are unsuitable for the establishment of the species of *Populus* in this permit. Aspens need high sunlight conditions and high moisture to germinate and establish. Aspens live and proliferate in very different environments than exist in the Willamette Valley. Serious stresses are placed on them because of the long summer drought, moist winters that favor fungal attack, and the extremely fast growth of competing plants. Because aspens are adapted to considerably cooler climates with short growing seasons, it is unlikely they could compete with local vegetation. The lands nearby are frequently tilled and cultivated. The plantations themselves will be cultivated and weeds controlled by herbicides.

The inhospitable climate, in combination with the confinement conditions imposed by the permittee and APHIS, will successfully limit the establishment of any of these species in the surrounding area. Therefore APHIS concludes there would be no significant effect on any native floral species.

b. Terrestrial Animals

The most likely animals to encounter the transgenic *Populus* trees in this field experiment would be browsing mammals (e.g. deer) burrowing animals (such as rodents) and leaf consuming insects (considered plant pests). The browsing by deer should be eliminated since the test sites are fenced to exclude deer. In the unlikely event of accidental consumption of plant material or seeds by other animals, the gene products produced by the selectable marker genes and genes of interest do not produce any toxin or have any similarity to known toxins with the exception of the toxins produced by the *DTA* gene and the Bt *Cry3A* gene. The DTA is produced in very few cells of developing flowers and is therefore at very low concentration in floral tissues. Intercelluarly expressed DTA cannot be taken up by adjacent plant cells or by organisms feeding on the plant tissue (Skinner et al. 2000) so it would be unlikely to adversely affect organisms that might feed on immature flower parts. Therefore APHIS concludes there would be no significant effect on any native vertebrate or invertebrate animal species.

d. Aquatic Organisms

The Willamette River is within about a quarter mile from one of the field sites and a reservoir is approximately a third of a mile from another field site. As stated above, there is no expectation of toxicological effects on any organism due to the ingestion of the transgenic plant material in this study. Furthermore, even if transgenic trees were to escape from the field test site, they would be unlikely to become established or competitive in the environment since surveys of the area have turned up very few trees that are the same or compatible with those that are being allowed to flower under the proposed field test. APHIS therefore concludes there would be no significant effect on any aquatic species.

9. Risks to Threatened and Endangered Species

BRS has reviewed the data in accordance with a process mutually agreed upon with the U.S. Fish and Wildlife Service to determine when a consultation, as required under Section 7 of the Endangered Species Act, is needed. APHIS has reached a determination that the proposed environmental release will have no effect on federally listed threatened or endangered species or species proposed for listing, and no effect on designated critical habitat or habitat proposed for designation in the action area. Consequently, consultation under Section 7 of the Endangered Species Act with the United States Fish and Wildlife Service is not required for the action described in the preferred alternative of this EA.

10. Cumulative impacts

The applicant has grown these trees under Permit and multiple Notifications since 1995 and wishes to grow these for up to an additional 9 years under permit. Prior to the establishment of this field test the sites have been used as experimental farms for agricultural crops and forest trees from 20 to 50 years, depending on the location. Therefore if a 3-year permit is granted, it is reasonably foreseeable that the applicant may request to further extend the permit for this environmental release for additional years to observe the growth of these trees to maturity. The temporary change from agricultural crops to a tree crop may result in a temporary change in resident animal and plant species, but after harvest it is reasonably foreseeable that the land will return to agriculture or be replanted to tree research. The only past, present, and reasonably foreseeable actions associated with the locations for the proposed releases are those related to agricultural production. Because the proposed field test will have no significant effects on the human environment, APHIS has determined that there are no past, present, or reasonably foreseeable actions that would aggregate with effects of the proposed action to create cumulative impacts or reduce the long-term productivity or sustainability of any of the resources (soil, water, ecosystem quality, biodiversity, etc.) associated with the release site or the ecosystem in which it is situated. No resources will be significantly impacted due to cumulative impacts resulting from the proposed action.

Considering the organism and traits introduced, the limited duration of the trial, and the manner in which the trial must be conducted, the size and location of the proposed field releases are unlikely to impact the capacity of the release to significantly affect the quality of the human environment.

11. Impact on Existing Agricultural Practices

This small field test will not have any significant impact on existing agricultural practices because this test is solely for research purposes. It is located in an area specifically allocated and designed for field testing forest trees. Conduct of the field test is not expected to adversely impact ongoing or future field testing of forest trees at this site.

12. Potential Impacts on Humans, Including Minorities, Low Income Populations, and Children

Because the field test is on an isolated property controlled by Oregon State University, the public will not be exposed to these transgenic plants. The trees will be isolated from the public. When the test is terminated the trees will be cut down and chipped on site. Herbicide applications will be used to control volunteers from root sprouts. None of the regulated material will leave the test site other than as samples taken to the laboratory for analysis. All the harvested material will be stored in dedicated storage containers on site and transferred to a laboratory setting for analysis.

Consideration of these potential impacts are specified in Executive Orders 13045 and 12898 and address the identification of health or safety risks that might disproportionately affect children or have adverse impacts on minorities and low-income populations. The proposed actions are not expected to adversely affect any of these groups.

13. Consistency of proposal with other environmental requirements

The proposal is believed to be consistent with other environmental requirements. This environmental assessment was prepared in accordance with: (1) The National Environmental Policy Act of 1969 (NEPA), as amended (42 U.S.C. 4321 et seq.); (2) regulations of the Council on Environmental Quality for implementing the procedural provisions of NEPA (40 CFR parts 1500-1508); (3) USDA regulations implementing NEPA (7 CFR part 1b); and (4) APHIS' NEPA Implementing Procedures (7 CFR part 372).

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APPENDIX I: Description of the Field Experiments

Each of the regulated trials is organized into distinct field-blocks. Transgenic trials planted at each field site are generally spaced at least 8 ft apart. Space between any transgenic plants or trials is kept clear of any suckers or volunteers from *Populus* species. To avoid border effects on experimental trees, every field trial is surrounded by non-transgenic or transgenic border trees (produced from the same constructs as the experimental material). Both transgenic and non transgenic border trees will also be monitored and treated as regulated material. The total planting size of all three locations combined is approximately 30 acres. The field trials are proposed to be terminated at different times, the longest being September 2016.

Location *A*
This trial includes reproductive sterility, gibberellin (GA) metabolism, reporter gene constructs and activation tagging mutants. This location also includes the clone bank containing trees that will not be allowed to flower. Two pairs of ramets (vegetative clones) per event are planted in a completely randomized design. These trials have trees spaced at a distance of 6 to 10 ft. For certain trials, at least two pairs of ramets per event will be planted in a completely randomized design and will be spaced at 10 X 10 ft or 7 X 7 ft spacing. Trees in the clone bank are at 3 X 3 ft spacing and are trimmed every year or two, and never allowed to flower.

Location B.
This trial is planted in two blocks, with a goal of measuring competition and yield of GA metabolism-modified trees. Each plot within respective blocks has 25 trees arranged in a completely randomized design.

Location C
This trial contains the trees engineered with the lignin modification gene. Two pairs of ramets per event are planted in a completely randomized design. Trees are spaced at a 10 X 10 ft distance.

Isolation of field tests from possible sexually compatible trees

In August 2006 the permittee surveyed the broad vicinity around all three field sites to find the nearest possible cross-compatible aspens (section *Populus*). None were found near site A, a few were found in a commercial nursery near site B (which are to be sold) and two very unhealthy trees were found at approximately 2,000 ft from site C. These trees are planted in a landscape setting. The permittee has also surveyed the sites and their vicinities many times over the years as part of routine maintenance and never observed a plant that appeared to be a seedling from female clone 717-1B4, the most frequently used clone to produce transgenics. Because this tree is a female, it is highly unlikely to produce pollen that could pollinate any aspens in the area.

At all of these field sites, the permittee will continue to monitor for seedling establishment to ensure that any established aspen-like trees within or near to sites that are not experimental trees, are destroyed via mechanical and/or chemical means. Particular attention will be paid to any trees that are not identical in morphology and within a distance to be root sprouts, as they may be seedlings. This will be done by yearly surveys of the entire field sites. Because of the large

abundance of root sprouts from these clones, no data will be taken on the surveys, except in the case that seedlings are identified based on their location away from trees, and with distinct morphology from parent and wild trees.

APPENDIX II: Biology of *Populus*

Characteristics of *Populus*

Taxonomy

The genus *Populus* (hereinafter called poplars) and the genus *Salix* (willows) are the two major genera of the willow family, *Salicaceae*. Most species in the family require moist, high light environments for growth; they are usually considered pioneer and/or riparian species. Poplars have an extensive distribution in the northern hemisphere; the well known quaking aspen *(Populus tremuloides)* has the widest distribution of any tree in North America. The number of species recognized in *Populus* has ranged from about 22 to about 85; these are divided into six taxonomic sections - Populus (synonym Leuce, aspens and white poplar), Aigeiros (cottonwoods or black poplars), Tacamahaca (balsam poplars and cottonwoods), Leucoides (swamp poplar), and Abaso and Turanga (subtropical poplars) (Eckenwalder 1996).

Populus sections related to this permit

Poplar clones used in this permit are hybrids between species of section Populus, and a *P. alba* clone from the same section. The permittee is retaining a few transgenic clones from earlier work in a clone bank that are species or hybrids between *Populus trichocarpa* x *Populus deltoides* of the sections Aigeiros and Tacamahaca. These are no longer the subject of research, and are pruned frequently to prevent any flowers from forming. These will be maintained in the clone bank under this permit, but will not be allowed to flower.

These field trials are being conducted in Benton County, Oregon, located in the Willamette Valley The Willamette Valley has native populations of black cottonwood (*P. trichocarpa)* of section Tacamahaca. Large populations of this species occur in the vicinity of the field test. There are also various cottonwood hybrids, and planted black poplars, including the Lombardy Poplar (derived from *P. nigra* of section Aigeiros) and *P. trichocarpa x deltoides* hybrids, in the general area.

Reproductive biology

The following is based on reviews in (Peterson 1992);(Dickmann and Stuart 1983);(Pryor and Willing 1982);(OECD 2001);(Vanden Broeck et al. 2005): The *Salicaceae* family is composed predominantly of dioecious species (individuals produce either male or female flowers) whose inflorescences (catkins) bear numerous tiny flowers lacking a significant perianth (sepals and petals). Poplar pollen is dispersed mainly by wind, though insects may visit flowers. Breeding experiments have shown that pollen germinates rapidly and has a very limited period of viability unless stored carefully (Stanton and Villar 1996). In the Willamette Valley, catkins are produced before trees leaf-out in spring, and most seeds are released several weeks later. The seeds are approximately 1 mm in length (lacking significant endosperm), and are widely dispersed by wind and flowing water with the aid of their "cotton," which reduces their effective density and promotes movement. The lack of seed reserves, intolerance of shade and other physiological characteristics of poplars, limits establishment to moist sites free of most competing vegetation. The seeds lack dormancy, thus there is no seed bank accumulation. Poplars remain extremely sensitive to

competition during their lifespan, mainly a result of their intolerance of shade (Baker 1949);(Fowells 1965);(OECD 2001).

Vegetative propagation

Poplars are well known for their ability to reproduce vegetatively. Species of the section Populus spread effectively by vegetative propagation. The principle means is through suckering (production of shoots from subterranean roots (Baker 1918). Spread through suckering is considered a primary means for spread of aspens in montane regions. While effective, this type of propagation proceeds extremely slow compared to spread of sexual propagules. Consequently, isolation or mechanical treatments in field trials helps to ensure confinement. Because of its persistent suckering, efficient devitalization of established clones is greatly aided by the repeated use of systemic herbicides.

Cottonwoods, of section Tacamahaca and Aigeiros, can be vegetatively propagated by rooting of cuttings from trees of nearly any age. Vegetative spread along river corridors occasionally occurs from rooting of branches broken from trees. This type of vegetative spread is unlikely to occur for species in the section Populus and consequently is not expected to occur with the transgenic hybrids.

Crossability of species

Natural intersectional hybrids with section Populus

Intrasectional hybrids are common in all poplar sections. Intersectional hybrids between Tacamahaca and Aigeiros species have been widely used in forestry, and natural hybrid swarms are also known (Ronald et al. 1973). Section Populus, however, is reproductively isolated from most other sections, including Tacamahaca and Aigeiros. (see Figure 1).

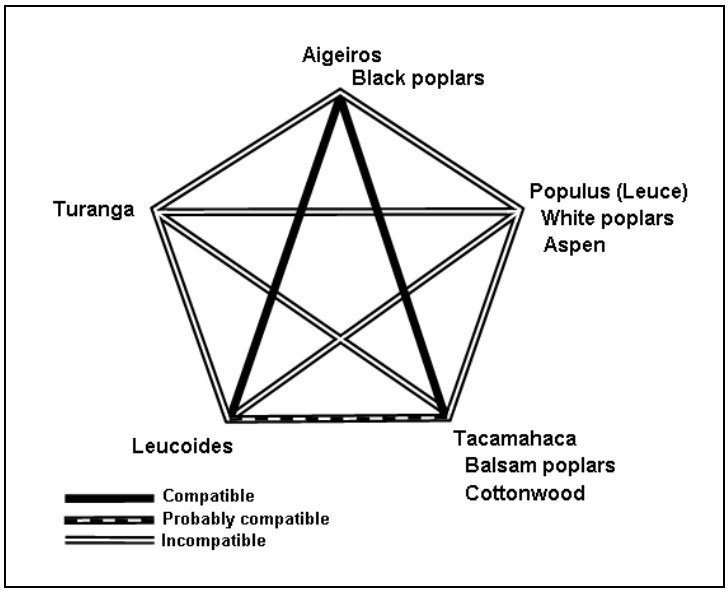

Figure 1. Known compatibility relationships between the sections of genus *Populus*. Species from section Leuce (Populus) are incompatible with the species of other Sections. Figure adapted from (Willing and Pryor 1976).

Despite several casual suggestions of natural hybridization in Canada (reviewed in Peterson 1992), hybrids between section Populus and other sections have never been verified. Moreover, when careful studies were conducted in zones of extensive sympatry (occupying the same geographical location) among Populus and Tacamahaca or Aigeiros species in Canada, they have failed to uncover hybrids (Ronald et al. 1973), nor have any natural hybrids been identified in the United States (Harper et al. 1985). No verified hybrids are known between *P. tremuloides* and either *P. trichocarpa* or *P. balsamifera* despite considerable zones of overlap in Eastern Washington and Alaska, respectively (R. Stettler, pers. comm. with the permittee, 2006; letter attached Appendix V). Dr. J. Eckenwalder, who has studied taxonomy and natural hybridization of *Populus* for many years (Eckenwalder 1977), knows of no instances of natural hybridization between *P. tremuloides* and other poplars in North America (pers. comm. with the permittee). In a recent review of natural hybridization among poplar species that included a description of a large number of known hybrid swarms (Vanden Broeck et al. 2005), none were of intersectional hybrids that involved section Populus.

Intersectional hybrids with section Populus produced via controlled crosses.
Controlled crossing experiments have demonstrated that intersectional hybrids with Populus are extremely difficult to produce; they usually require special treatments such as use of irradiated pollen (mentor pollen) or application of organic solvents to the stigma or pollen (see Figure 1), (Knox et al. 1972);(Willing and Pryor 1976); (Stettler et al. 1980);(Pryor and Willing 1982). Barriers to crossability appear in several stages, including pollen germination, subsequent pollen tube growth, micropyle penetration, and embryo/fruit development (Guries and Stettler 1976);(OECD 2001). Because of the extensive efforts to produce poplar hybrids in several laboratories worldwide over the last century, however, a small number of successful crosses have been reported using standard breeding methods (reviewed in Paule 1949 and Muhle Larsen 1970). However, many of the germinable seedlings produced are often weak and develop abnormally (Muhle Larsen 1970), showing slow growth, chlorophyll deficiency, lack of apical dominance, and other defects (Heimburger 1940), (Stettler 1968);(Willing and Pryor 1976);(Stettler and Guries 1976). In extensive studies at the Korean Institute of Forest Genetics, intersectional hybrids with Populus were reported several fold more difficult to make than other kinds of hybrids, and all progeny were classed as "weak." The only cross type in this category (cited in Muhle Larsen 1970 and Stout and Schreiner 1934) produced 178 seedlings of a *Populus balsamifera x grandidentata* cross, but none of the progeny were found with promising growth compared to the parents or other hybrids. Of several *P. deltoides x alba* and *P. alba x nigra* hybrids produced in Australia and still alive at age 20, their slow growth compared to other clones and hybrids has precluded commercial interest (Pryor and Willing 1982).

To further confirm observations on hybridization between species of sections Populus and Tacamahaca, and Populus and Aigeiros, the permittee recently conducted a survey among poplar breeders and geneticists confirming and extending the above observations with respect to the rarity and low vigor of the majority of controlled-cross hybrids involving section Populus. No sources of concern were uncovered with respect to the specific kinds of hybrids that might be produced at the Corvallis test site. This general conclusion was also supported by letters to the applicant from two leading poplar breeders and geneticists, Drs. R. Stettler and B. Stanton (Appendix V).

The permittee has surveyed the site where the transgenic poplars have been grown under permit over the years that the field trials have been allowed to flower, and has never observed a plant that appeared to be a seedling from flowering, transgenic female parent of hybrid parentage (717-1B4, *Populus tremula x alba*). They have also not seen any plants that appeared to be *P. trichocarpa x tremula/tremuloides/alba* (or reciprocal) hybrids. However, seedlings of wild cottonwoods in the area are very common, and 717-1B4 has produced seeds for several years under APHIS permit. Were seedlings from this clone found to occur, they should be visible and strikingly different from the wild *P. trichocarpa* volunteers in the area due to its distinct leaf and shoot morphology compared to wild cottonwoods. There would also be much variation in form in these traits for the segregating progeny of this interspecific hybrid when compared to the common root sprouts of the parent clone near to the planted trees. Weed control and irrigation in the test site provide a more open and moist habitat which favors seedling establishment, yet no hybrid seedlings have been found. This provides strong evidence that establishment of transgenic progeny is extremely rare, even within sites where weed control and irrigation in many areas provide improved habitat for initial establishment compared to nearby undisturbed plant communities. This is likely the combined result of low hybrid fertility, maladaptation of progeny of the parent species to the local environment, and poor phenological overlap with interfertile male poplars leading to low seed yields and low seed viability.

APPENDIX III: Description of the Regulated Articles

1. Trees that will be allowed to flower

The transgenic poplar lines to be tested and that will be allowed to flower, have been developed from three recipient clones. The original clones transformed were:
717-1B4 - a female hybrid of *Populus tremula* x *Populus alba* (Sect. *Populus*)
353-38 - a male hybrid of *Populus tremula* x *Populus tremuloides* (Sect. *Populus*)
6K10 - a female clone of *Populus alba* (Sect. *Populus*)

All of these *Populus* hybrids and species are member of the section Populus (also known as Leuce). These hybrid poplar clones were chosen for the development of the transgenic poplars of this test for the following reasons: (1) They are readily transformed compared to most cottonwoods. (2) They may flower relatively early in their lifespan compared to cottonwoods, facilitating tests of flowering and sterility genes. (3) They are not adapted to or invasive in the region. (4) They are expected to be reproductively isolated from the common cottonwood species and cottonwood hybrids native and planted in the region.

Genes engineered into the three poplar clones:

The poplar plants have been modified with a variety of genetic constructs which are described and referenced thoroughly in the permit application and summarized briefly below. All are listed in Table 1.

The genetic constructs introduced into the three original clones fall into four phenotypic categories:
1. Reproductive sterility – intended to make transgenic trees less able to produce viable pollen and/or seeds.
2. Reduced stature/light response – intended to make transgenic trees and their progeny much less able to compete with non-transgenic trees.
3. Modified tree chemistry – intended to reduce the compound lignin.
4. Activation tagging mutants - aimed at the development of "experimental domesticates."

In addition, all of the introduced constructs contain the *nptII* gene from *Escherichia coli* or the *bar* gene from *Streptomyces hygroscopicus* as a selectable marker gene to facilitate the selection of transformed plant tissue in the laboratory. The *bar* gene encodes phosphinothricin acetyltransferase, an enzyme that confers tolerance to the phosphinothricin (glufosinate) class of herbicides. In addition, some constructs also use the β-glucuronidase gene (*gus*) from *E. coli* as another marker in the transgenic plants.

Genes conferring reproductive sterility. Some of the transgenic plants have been engineered with the barstar and barnase genes. The barstar and barnase genes were derived from the bacterium *Bacillus amyloliquifaciens*. This genetic construct is designed to confer male sterility. This approach utilizes a ribonuclease (trivial name -barnase) that is expressed only in the tapetum cells of the anther's pollen sac during pollen development. Expression of barnase in these cells apparently results in degradation of

host RNAs and arrests cell development. Therefore, expression of barnase in this tissue blocks pollen development and results in a male sterile plant.

Because barnase can inhibit *Agrobacterium* viability (the vector organism used for the transformations) and the regeneration of transgenic cells, the barnase gene construct is accompanied by the barstar gene, whose protein product acts as a specific inhibitor of barnase. Therefore, expression of the barstar gene in the *Agrobacterium* host cell protects the bacterium from the ribonuclease effects of the barnase gene.

Some of the transgenic poplars have been engineered with the *DTA* gene derived from the bacterium *Corynebacterium diptheriae*, the causal agent of diptheria. The *DTA* gene encodes the A-chain portion of the diptheria toxin. Diptheria toxin is comprised of the B-chain component that allows movement of the holotoxin into cells and the A-chain component that disrupts protein synthesis in eukaryotic organisms by inhibiting translocase, the enzyme involved in the elongation phase of protein synthesis. When the A-chain is expressed without the B-chain component, the inhibition of protein synthesis is restricted to the cell in which the gene is expressed. Like the barnase/barstar construct, the preferential expression of the *DTA* genes in floral tissues results in reproductive sterility.

Some of the transgenic poplars have been engineered with other reproductive sterility genes. These include *PTLF*, *PTD*, *PTAG*, *PTAP*, *PMFT*, *PCENL*, *PSV*, *PFCL*, and *PAGL24,* all from *Populus trichocarpa*. In addition trees have been engineered with *AG* and *AP1*from *Arabidopsis thaliana* (See Table 1). These genes all affect flowering and flower formation and have been engineered to reduce or eliminate flowering, pollen production, or seed formation.

Genes conferring reduced stature/light response

Some of the poplars were engineered with gibberellin (GA) metabolism genes. These include *GAI* and *rgl-1* from *Arabidopsis thaliana, PcGA2 OXI1* from *Phaseolus coccineus* and *PtaGA2 OXI1* from *Populus tremula x alba.* Phytochrome receptor genes *PHYB1* and *PHYB2* from the poplar species *Populus trichocarpa* were inserted to affect light response (see Table 1). These genes have been engineered to affect the stature of the trees in order to produce plants with reduced growth (dwarfed) or other growth inhibition characteristics.

Gene to modify tree chemistry

The *4CL1* gene from *Populus tremuloides* was inserted to alter lignin levels (see Table 1). This is an antisense copy of the 4-Coumarate CoA ligase (*4CL*) gene which the applicant has shown results in a reduction in the messenger RNA of the target *4CL* gene. The 4 coumarate:coenzyme A (CoA) ligase (4CL) leads to a major branch point in phenylpropanoid metabolism in the lignin biosynthetic pathway. The product 4- coumaroyl:CoA is a precursor for lignin and flavonoids. Lignin, cellulose, and hemicellulose form the cell walls of xylem, which transports water and supports the tree. Studies have shown that when expression of the gene encoding 4CL is downregulated in trembling aspen (*Populus tremuloides*), lignin content is decreased, cellulose is increased, and growth is stimulated (Hu et al. 1999).

Activation tagging

Populus clone 717-1B4 were engineered with a putative transcriptional regulator gene from *Populus tremula x alba* and a putative AP2 domain-containing transcription factor from *Populus tremula x alba.* These trees have been randomly hyperactivated (activation tagging) with genetic mutations in an attempt to develop "experimental domesticates" (See Table 1). Other activation tagging mutant constructs contain only non-coding CaMV 35 S promoter regions and a selectable marker gene which are expected to create random mutations via native gene disruption or up-regulation.

Non-coding sequences

Some of the noncoding regulatory sequences introduced as part of the genetic constructs in these poplars were derived from plants (*Nicotiana, Solanum, Arabidopsis*) and the plant pathogens cauliflower mosaic virus, tobacco mosaic virus, *Aspergillus nidulans*, and *A. tumefaciens*.

2. Trees that will not be allowed to flower (maintained in the clone bank)

The permittee is retaining a few transgenic clones from earlier work in a clone bank that are species or hybrids between *Populus trichocarpa* x *Populus deltoides* of the sections Aigeiros and Tacamahaca. These will be maintained in the clone bank under this permit, will be pruned frequently, and will not be allowed to flower. These are as follows:

- 24-305 Triploid male hybrid of *Populus trichocarpa* x *Populus deltoides* (Sect. Tacamahaca and Aigeiros)
- 50-197 Diploid hybrid of *Populus trichocarpa* x *Populus deltoides* (Sect. Tacamahaca and Aigeiros)
- 189-434 Triploid hybrid of *Populus trichocarpa* x *Populus deltoides* (Sect. Tacamahaca and Aigeiros)
- OP-367 Diploid hybrid of *Populus trichocarpa* x *Populus deltoides* (Sect. Tacamahaca and Aigeiros)

These trees contain the *Cry3A* gene encoding a coleopteran-active insecticidal protein from *Bacillus thuringiensis*, the *Gfp* gene from *Aequorea victoria*, the *nptII* gene from *Escherichia coli*, the *bar* gene from *Streptomyces hygroscopicus*, and the *PTLF* gene from *Populus trichocarpa* in various combinations.

Transformation techniques.

The genes were transferred to *Populus* via well characterized laboratory techniques that utilize DNA sequences from *Agrobacterium tumefaciens* to transfer introduced genes into the chromosome of the recipient plant (see reviews by Klee and Rogers 1989 and Zambryski 1988). *A. tumefaciens* is a bacterial plant pathogen that can cause crown gall disease on a wide range of dicotyledonous plant species. Although some of the DNA sequences used in the transformation process were derived from *A. tumefaciens*, the genes that cause crown gall disease are first removed, and therefore the recipient plant does not have crown gall disease. Following

transformation, the agrobacteria are eliminated from the transformed plant tissue, and the DNA sequences introduced into the plant are maintained and inherited as any other genes of the plant cell.

Table 1. List of genes and donors in permit 06-250-01r

Gene	**Donor**
Selectable Marker	
Npt II	*Esherichia coli*
Bar	*Streptomyces hgroscopicus*
*Gfp**	*Aequorea victoria*
Gus / Gus-INT	*Esherichia coli*
Gene of interest	
PTLF	*Populus trichocarpa*
Barnase	*Bacillus amyloliquefaciens*
DTA	*Corynebacterium diphtheriae*
PTLF	*Populus trichocarpa*
PTD	*Populus trichocarpa*
PTAG	*Populus trichocarpa*
PTAP	*Populus trichocarpa*
*Bt (Cry3a)**	*Bacillus thuringiensis*
PMFT	*Populus trichocarpa*
PCENL	*Populus trichocarpa*
Barstar	*Bacillus amyloliquefaciens*
PHYB1	*Arabidopsis thaliana*
PHYB2	*Arabidopsis thaliana*
GAI	*Arabidopsis thaliana*
Gai	*Arabidopsis thaliana*
rgl-1	*Arabidopsis thaliana*
PcGA2 OXI1	*Phaseolus coccineus*
PtaGA2 OXI1	*Populus tremula x alba*
as-4CL1	*Populus tremuloides*
AG	*Arabidopsis thaliana*
AP1	*Arabidopsis thaliana*
PSVP	*Populus trichocarpa*
PFCL	*Populus trichocarpa*
PAGL24	*Populus trichocarpa*
Unknown transcription regulator	*Populus tremula x alba*
Putative transcription factor	*Populus tremula x alba*

* The applicant proposes to maintain plants containing these genes in such a way so that they will not be allowed to flower.

APPENDIX IV: Threatened and Endangered Species Analysis

Action Area

The proposed field trial of the transgenic poplar is located in Benton County, Oregon, in the Willamette Valley. This site is estimated to be about 1/4 mile from the Willamette River, 1/3 mile from a reservoir, and 4 miles from the city of Corvallis. The total area of all field trials is about 30 acres, and the transgenic organism is *Populus*. The poplar clones used for the transgenic research are hybrids between species section *Populus* and a *P. alba* clone from the same section. According to the permit application, the Willamette Valley has native populations of black cottonwood (*P. trichocarpa*) of section Tacamahaca, various cottonwood hybrids and planted black poplars that occur in the vicinity of the field test. However, these trees are unlikely to produce viable, competitive hybrids with the section *Populus* transgenic hybrids because of their very low compatibility in the absence of artificial breeding, and the poor viability of the hybrid progeny in this environment.

The USFWS website[1] was accessed on 12/11/06 to analyze the TES for Benton County, Oregon. The following is the list of the 34 animal species and 15 plant species identified as threatened and endangered in Oregon:

Animals

- Fender's blue butterfly (*Icaricia icarioides fenderi*)
- Oregon silverspot butterfly (*Speyeria zerene hippolyta*) *
- Canada Lynx (*Lynx canadensis*) *
- Northern spotted Owl (*Strix occidentalis caurina*) *
- Brown pelican (*Pelecanus occidentalis*)
- Short-tailed albatross (*Phoebastria albatrus*)
- Marbled murrelet (*Brachyramphus marmoratus marmoratus*) *
- Western snowy plover (*Charadrius alexandrinus nivosus*) *
- Columbian white-tailed deer (*Odocoileus virginianus leucurus*)
- Bald eagle (*Haliaeetus leucocephalus*)†
- Salmon, chinook fall Snake R. (*Oncorhynchus tshawytscha*) *
- Salmon, chinook (*Oncorhynchus tshawytscha*) *
- Salmon, chinook spring/summer Snake R. (*Oncorhynchus tshawytscha*) *
- Salmon, chinook upper Willamette R. (*Oncorhynchus tshawytscha*) *
- Salmon, chum (*Oncorhynchus keta*) *
- Salmon, coho (*Oncorhynchus kisutch*) *
- Sea turtle (*Chelonia mydas*) *
- Sea turtle, leatherback (*Dermochelys coriacea*) *
- Sea turtle, loggerhead (*Caretta caretta*)

† Since this analysis was conducted the Bald eagle has since been delisted.

- Sea-lion, Steller (*Eumetopias jubatus*) *
- Steelhead Snake R. Basin (*Oncorhynchus mykiss*) *
- Steelhead middle Columbia R. (*Oncorhynchus mykiss*) *
- Steelhead upper Willamette R. (*Oncorhynchus mykiss*) *
- Sucker, Lost River (*Deltistes luxatus*)
- Sucker, shortnose (*Chasmistes brevirostris*)
- Sucker, Warner (*Catostomus warnerensis*) *
- Trout, bull (*Salvelinus confluentus*) *
- Trout, Lahontan cutthroat (*Oncorhynchus clarki henshawi*)
- Whale, humpback (*Megaptera novaeangliae*)
- Chub, Borax Lake (*Gila boraxobius*) *
- Chub, Hutton tui Hutton (*Gila bicolor ssp.*)
- Chub, Oregon (*Oregonichthys crameri*)
- Dace, Foskett speckled Foskett (*Rhinichthys osculus ssp.*)
- Fairy shrimp, vernal pool (*Branchinecta lynchi*) *

Plants

- Catchfly, Spalding's (*Silene spaldingii*)
- Checker-mallow, Nelson's (*Sidalcea nelsoniana*)
- Daisy, Willamette (*Erigeron decumbens var. decumbens*)
- Desert-parsley, Bradshaw's (*Lomatium bradshawii*)
- Four-o'clock, MacFarlane's (*Mirabilis macfarlanei*)
- Fritillary, Gentner's (*Fritillaria gentneri*)
- Howellia, water (*Howellia aquatilis*)
- Lily, Western (*Lilium occidentale*)
- Lomatium, Cook's (*Lomatium cookii*)
- Lupine, Kincaid's (*Lupinus sulphureus (=oreganus) ssp. kincaidii (=var. kincaidii)*)
- Meadowfoam, large-flowered woolly (*Limnanthes floccosa ssp. grandiflora*)
- Milk-vetch, Applegate's (*Astragalus applegatei*)
- Popcornflower, rough (*Plagiobothrys hirtus*)
- Thelypody, Howell's spectacular (*Thelypodium howellii spectabilis*)
- Wire-lettuce, Malheur (*Stephanomeria malheurensis*) *

Analysis of the TES animals

About the species

Both butterfly species (*Icaricia icarioides fenderi* and *Speyeria zerene hippolyta*) are nectarivores. They generally use grassland and herbaceous systems as natural habitats. The Canada lynx lives in boreal and conifer forest while the spotted northern owl utilizes old-growth forest for survival. The threatened and endangered birds (brown pelican, short-tailed albatross, marbled murrelet, and western snowy plover) are seabirds or shorebirds. The endangered Columbian white-tailed deer whose habitats include tidal marsh and cottonwood according to USFWS[2], and the threatened bald

eagle might visit the poplar plantings proposed in the permit application[‡]. However in the state of Oregon, the endangered deer occurs in Columbia River and Douglas County according to USFWS[3]. When flocks of migratory waterfowl arrive in the winter, the Klamath Basin is used as the major stopover. The remaining animals on the USFWS TES list above are animals (fish, turtle, and shrimp) whose habitats (aquatic systems) do not overlap with the three field sites.

About species critical habitats

Twenty-one out of 34 of the above-mentioned threatened and endangered animal species have critical habitat designated by USFWS[5]. Those species are marked with an asterisk (*). Note that Coho salmon's critical habitat is still proposed.

According to USFWS[5], the only TES that have designated critical habitat in Benton county are the Marbled Murrelet and Northern Spotted Owl. However these locations are about 10 miles away from the action area, and do not interfere with the proposed test site. There is no habitat proposed for designation as critical habitat in this county

Analysis of the TES plants

About the species

Of the 15 TE plant species listed above, only Bradshaw's Desert-parsley resides in Benton County. However this species primary prefers moist meadows and remnant prairie patches at low elevations[4] and is not present in the field test locations.

Summary and Conclusion

The analysis for this OSU permit indicates that of all TES identified in the state of Oregon, very few reside in Benton County, and only 2 have designated critical habitats in that jurisdiction according to USFWS[6]. The proposed action area does not include or interfere with habitats or designated critical habitats used by the TES of concern. APHIS has reached a determination of no effect on TES and no adverse modification of designated critical habitat for the proposed field trials in this permit for the following reasons:

1) The transgenic poplars are not sexually compatible with any threatened or endangered plant species in the action area.

2) No TES plants are located in habitat that would be disturbed or otherwise affected as a result of the conduct of the trial and no critical habitat is present in the location of the trial.

3) None of the TES animal species utilize poplars in the action area for food, cover, or nesting.

4) With the exception of the DTA the Cry3A toxins, the transgenic modifications are not intended to result in the production, or increase the production of a toxin, natural toxicant, allelochemical, pheromone, or hormone that could directly or indirectly result in killing or

[‡] Since this analysis was conducted the Bald eagle has since been delisted.

interfering with the normal growth, development, or behavior of a TES or species proposed for listing in the action area. The DTA toxin is only produced in very few cells in immature flowers and would be at miniscule levels in the flower tissues. Intercelluarly expressed DTA cannot be taken up by adjacent plant cells or by organisms feeding on the plant tissue. The Cry3A toxin is intended to kill Chrysomelid beetles which are a serious pest in Poplar plantations. None of the TES species are coleopteran species. Thus no exposure to these toxins at deleterious levels to TES or proposed species should occur.

Sources (accessed on 12/11/06)

1. USFWS: http://ecos.fws.gov/tess_public/StateListingAndOccurrence.do?state=OR
2. USFWS: http://www.fws.gov/refuges/archives/columbianWhite.html
3. USFWS: http://ecos.fws.gov/speciesProfile/SpeciesReport.do?spcode=A002
4. CPC Nat'l. Collection http://www.centerforplantconservation.org/ASP/CPC_ViewProfile.asp?CPCNum=2658
5. USFWS: http://crithab.fws.gov

APPENDIX V: Letters to permittee

APPENDIX VI

Letter from Dr. Reinhard F. Stettler (sent via email)

Dr. Steven H. Strauss, Department of Forest Science
Oregon State University, Corvallis, OR 97331-5752

Seattle, 25 April, 2006

Re. Negligible likelihood of natural hybrids between Sections *Populus, Aigeiros, and Tacamahaca* in the Genus *Populus*.

Dear Steve,

This is a letter in support of your submission for a new permit from APHIS.

The message of my letter is captured in the title above and is based on substantive evidence from (1) crossability studies conducted by myself, co-workers, and collaborating scientists; (2) the scientific literature; and (3) continued observations in the field to this day.

(1) Crossability studies have shown that multiple ecological, temporal, pre-zygotic, post-zygotic, and inviability barriers prevent a successful completion of sexual reproduction under natural conditions between the three taxonomic sections above. Manipulated crosses with some elaborate techniques have occasionally given rise to rare hybrid seedlings, which however have shown low viability because of incongruity *sensu* Hogenboom (1984). A systematic mating matrix with 28 experimental cross combinations, conducted by Guries (1976) has confirmed that crossing success in poplars – as in many other tree genera – is proportionate to the degree of taxonomic relatedness. With Section *Populus* being distant from *Tacamahaca* and even *Aigeiros*, the above results are not surprising.

(2) Scientific literature: A summary of crossability studies, including those above and related publications by other researchers is given in Chapter 4 of *Biology of Populus and its Implications for Management and Conservation* (R. F. Stettler et al., eds. Ottawa: Can. National Research Council Press, 1996).

(3) Field observations: I can say that I probably have spent more time in natural populations of *Populus* than most other poplar researchers, and still do. Many of my field trips take me to the areas on the east slope of the Cascades where *P. trichocarpa* and *P. tremuloides* co-exist, often in close proximity. Being well versed in the study of natural hybrid zones of other poplar species, I have yet to spot my first hybrid between aspen and black cottonwood. I don't rule out the possibility but rate it infinitesimally small.

All in all, I consider your research material ideally suited in this region to further explore the promising prospects of genetic engineering in *Populus,* and to now take full advantage of the sequenced genome to shed light on the genetic underpinnings of growth and adaptation in this genus. Best wishes for continued productivity!

Reinhard F. Stettler
Emeritus Professor of Forest Resources, University of Washington, Seattle, WA 98195

Letter from Dr. Brian Stanton

One World Trade Center
121 SW Salmon St. Suite 1020
Portland, Oregon 97204
Tel: (503) 274-0438
Fax: (503) 478-0751

May 12, 2006

Dr. Steven H. Strauss
Department of Forest Science
College of Forestry
321 Richardson Hall 97331-5752
Corvallis, OR

Dear Steve,

While I have not directly studied the opportunity for intersectional hybridization between species of section *Populus* and sections *Aigeiros* and *Tacamahaca*, my impression from conversations with poplar ecologists and breeders is that the probability of cross pollinations leading to the establishment of viable hybrid offspring under natural conditions is extremely small. The barrier seems to be multifaceted, including problematic fertilization and abnormal seedling development.

I do not believe that the potential for introgression between section *Populus* and sections *Aigeiros* and *Tacamahaca* is real. Nor should it preclude the granting of necessary permits for your research application.

Sincerely,

Brian J Stanton

Brian J. Stanton, Ph. D.
Managing Director, Resource Management Group

APPENDIX VI: Supplemental Permit Conditions – Permit 06-250-01r

SUPPLEMENTAL PERMIT CONDITIONS
For Release of *Populus* Species and Hybrids
USDA-APHIS-BRS Permit 06-250-01r

Compliance with Regulations

1. Any regulated article introduced not in compliance with the requirements of Title 7, Code of Federal Regulations, Part 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act (7 U.S.C. 7701-7772).

2. This Permit (APHIS form 2000) does not eliminate the permittee's legal responsibility to obtain all necessary Federal and State approvals, including: (A) for the use of any non-genetically engineered plant pest or pathogens as challenge inoculum; (B) plants, plant parts or seeds which are under existing Federal or State quarantine or restricted use; (C) experimental use of unregistered chemicals; and (D) food, feed, pharmacological, biologic, or industrial use of regulated articles or their products and co-mingled plant material. In the latter case, depending on the use, reviews by APHIS, the U.S. Food and Drug Administration, or the U.S. Environmental Protection Agency may be necessary.

3. Please note that transportation of all test and plant materials to and from the field test site must be done in accordance with APHIS/USDA regulations outlined in "Container requirements for the movement of regulated articles", 7CFR 340.8(b)(I & ii) unless a shipping container variance has been approved by APHIS-BRS.

4. BRS should be notified in writing of any proposed changes to the permit application (or approved permit) including for example confinement protocols, transgenic lines or constructs, release locations, acreage, etc. Changes usually require amendments to the permit and must be pre-approved by BRS. Requests should be directed to Regulatory Permit Specialist, USDA APHIS BRS, Biotechnology Permit Services, 4700 River Road, Unit 147, Riverdale, Maryland 20737.

5. APHIS/BRS and/or an APHIS/PPQ personnel may conduct inspections of the test locations, facilities, and/or records at any time.

6. Harvested plant material may not be used for food or animal feed unless it is first devitalized and approved for such use by the U.S. Food and Drug Administration; and for plant-incorporated protectants, a tolerance for the pesticide must first be established by the U.S. Environmental Protection Agency.

7. The test sites and adjacent land within 100 meters shall be monitored for any volunteer *Poplulus* plants every 6 months during the field test and for two years after completion of the field test, during which time any volunteer plants will be destroyed before they flower. During the monitoring period following completion of the test, the site will not be planted with poplars, so that any volunteer seedlings that emerge can be easily identified. If volunteers (sucker shoots) are still emerging during the second year, a third year will be added to the monitoring period to ensure no that no shoots are continuing to be produced.

8. All non-engineered control trees in the field test plot and any plant material removed from the field site will be treated as regulated articles.

9. Reporting Unauthorized Releases:

According to the regulation in 7 CFR 340.4(f)(10)(i), APHIS shall be notified orally immediately upon discovery and notified in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.

- For immediate oral notification, contact APHIS/BRS Compliance Staff at (301) 734-5690 and ask to speak to a Compliance and Inspection staff member.
- In the event of an emergency and you are unable to reach the BRS Compliance Staff at the above number, you may call:

The APHIS/BRS Regional Biotechnologist:
For Western Region, contact the Western Region Biotechnologist by phone at (970) 494-7573 or e-mail BRSWRBT@aphis.usda.gov

Or

The APHIS/PPQ Regional Biotechnology Coordinator assigned to the state where the field test occurs:
For Western Region, contact Stacy Scott by phone at 970-494-7577 or e-mail Stacy.E.Scott@aphis.usda.gov

Or

The APHIS State Plant Health Director for the state where the field test occurs. The list of APHIS State Plant Health Director is available at http://ceris.purdue.edu/napis/names/sphdXstate.html.

10. Reporting Unintended Effects:

According to the regulation in 7 CFR 340.4(f)(10)(ii), APHIS shall be notified in writing as soon as possible but within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the permit application or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms).

Written notification should be sent by one of the following means:

By e-mail:
BRSCompliance@aphis.usda.gov

By mail:
Biotechnology Regulatory Services (BRS)
Compliance and Inspection Branch
USDA/APHIS
4700 River Rd. Unit 147
Riverdale, MD 20737

11. Reports and Notices:

Send notices and all reports (CBI and CBI-deleted or non-CBI copies) to BRS by e-mail, mail, or fax.

BRS E-mail:
BRSCompliance@aphis.usda.gov

BRS Mail:
Animal and Plant Health Inspection Service (APHIS)
Biotechnology Regulatory Services (BRS)
Compliance and Inspection Branch
4700 River Rd. Unit 147
Riverdale, MD 20737

BRS Fax:
Compliance and Inspection Branch
(301) 734-8669

The following reports are required:

a. Planting Report

Within 28 calendar days after planting, submit a report, in paper format or electronically, that includes the following information for each field test location:
i. Permit number;
ii. Regulated article;
iii. Release location [provide state, county, internal identification number (if available), and either a single GPS coordinate as a reference point (center of plot or specify corner) or specific address];
iv. Approximate number of seeds or plants or acres planted per transformed line (event) for each construct (transformation code);
v. Total acreage of regulated articles planted and border rows;
vi. The actual planting date

If multiple plantings occur that are separated in time by more than a month, then an activity report is required within 28 days of each planting.

b. Annual Report

Within 30 days after the anniversary date (one year increments from the effective date) an Annual Report must be submitted to APHIS. FAILURE TO SUBMIT ANNUAL REPORTS MAY RESULT IN REVOCATION OF THE PERMIT. The Annual Report shall reflect the current status and observations to date for each location. It shall include the information submitted in the Activity Report, plus the following:

i. An accounting of the acreage or number of plants per line (event) for each construct that remain in the ground
ii. A detailed map of the plantings.
iii. Total remaining acreage.
iv. The methods of observation;
v. The resulting data and analysis regarding all deleterious effects on plants, non-target organisms, or the environment. This should include, but not be limited to, data on insect damage, disease susceptibility, gross morphology and any indications of weediness.
vi. If any material was harvested, removed, or terminated or otherwise destroyed, a disposition table with the following information for each line (event) released should be provided:
date(s) of harvest, removal, and/or termination; a formal record of how the regulated material was removed from the environment; what material and how much was harvested or removed and where it was transported, stored and further processed up to the time it is or was to be taken to a contained facility; and what was done to devitalize residual and/or harvested material at the location.

In this report also provide data documenting which trees produced flowers and which if any produced viable seeds. Also document seedling volunteer monitoring, including the dates and locations monitored; the location, number, and type of any volunteers found; and the method of devitalization.

c. Final Field Test Report

Within 6 months after the expiration date of the permit, the permittee is required to submit a Field Test Report. NOTE: If a new application is approved to continue the field test past its scheduled expiration date, an annual report should continue to be submitted until the final expiration date, at which point the Field Test Report will be due after 6 months. Field Test Reports provide the final status and observations at each location and shall include:
i. Total constructs and specific transformed lines (event) planted;
ii. Planting date(s);
iii. Total acreage of the test;
iv. The methods of observation;
v. The resulting data and analysis regarding all deleterious effects on plants, non-target organisms, or the environment. This should include, but not be limited to, data on insect damage, disease susceptibility, gross morphology and any indications of weediness.

vi. A final disposition table with the following information for each line (event) released should be provided:

Date(s) of harvest, removal, and/or termination; a formal record of how the regulated material was removed from the environment; what material and how much was harvested or removed and where it was transported, stored and further processed up to the time it was taken to a contained facility; and what was done to devitalize residual and/or harvested material at the location.

We encourage the inclusion of other types of data if the applicant anticipates submission of a petition for determination of non-regulated status for their regulated article. APHIS considers these data reports as critical to our assessment of plant pest risk and development of regulatory policies based on the best scientific evidence. Failure by an applicant to provide data reports in a timely manner for a field trial may result in the withholding of permission by APHIS for future field trials.

c. Final Monitoring Report

The final monitoring report is due no later than 2 months from the end of the volunteer monitoring period.

The report must include:
i. Dates when the field location and perimeter fallow zone were inspected for volunteer plants;
ii. Number of volunteers observed;
iii. Any actions taken to remove or destroy volunteers.