Finding of No Significant Impact and Decision Notice

Animal and Plant Health Inspection Service

Issuance of a permit for a Field Study of Genetically Modified Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

The Animal and Plant Health Inspection service (APHIS) of the United States Department of Agriculture (USDA) received a permit application (APHIS number 05-098-01r) from USDA APHIS Center for Plant Health Science and Technology (CPHST) to conduct field trials of pink bollworms genetically engineered to contain the enhanced green fluorescent protein (EGFP) as a marker gene for identification of radiation sterilized pink bollworm released in areas of Arizona in which they are already endemic. An Environmental Assessment (EA) was prepared pursuant to the APHIS regulations (7 CFR 372) promulgated under the National Environmental Policy Act. This Assessment included a description of the field trial.

APHIS received a request to amend the permit application after issuing a Finding of No Significant Impact for the action of issuing the original permit in Pima County, AZ. The requested changes to the permit are:

- The field trial location is changed from Pima County, AZ to Yuma County, AZ.
- The requested size of the field trial has been reduced from a total of 12 acres to 4.6 acres.
- The density of moths per acre would increase, but the overall number of moths would decrease from a maximum of 150,000 moths per day to a maximum of 5000 moths per day.
- The field trial will be conducted on Bt-cotton instead of non-Bt-cotton.

A draft EA was prepared for the original permit request and submitted for public comment for 30 days. Two comments were received and were addressed in the Federal Register on April 19, 2006 (Docket No. APHIS–2006–0015, 71 F.R. 20068-20069) in a notice that announced the availability of the FONSI and final EA for the original permit application.

APHIS prepared an addendum to the EA that addresses potential new issues that may have arisen as a result of changing the size, location, and experimental design as described in the amended permit application.

APHIS proposed two different actions to take in response to the amended permit application: the denial of the permit (Alternative A) and the granting of the permit as amended with Supplemental Permit Conditions containing additional safety requirements and a requirement of filing field test reports as described in appendix 1 (Alternative B).

Based on the analysis documented in the Environmental Assessment and its addendum APHIS has determined that the action proposed in Alternative B will not have a significant impact on the quality of the human environment because:

- No adverse consequences to non-target organisms or environmental quality are expected from incorporation of this marker into the pink bollworm.
- Green fluorescent protein is a naturally occurring protein, not known to cause adverse effects (Appendix V). The normal digestive process of predators would preclude transfer of functional genetic material to the predator so such transfer is not expected with eating this insect. In addition, there have been no adverse effects to birds that are naturally exposed to the GFP through feeding directly on jellyfish.
- Released pink bollworms will be sterile adults that are not expected to produce fertile offspring. No adverse consequences are expected to beneficial insects (e.g., natural enemies, decomposers, pollinators) from release of transgenic PBW adults expressing GFP.
- The moths will be released into a Bt-cotton field; therefore any offspring resulting from the release are not expected to survive.

Because APHIS has reached a finding of no significant impact of this field release of transgenic pink bollworm, no Environmental Impact Statement will be prepared regarding this decision.

Pursuant to its regulations (7 CFR 340) promulgated under the Plant Protection Act of 2000, APHIS has determined that this field trial will not pose a risk of the introduction nor dissemination of a plant pest for the following reasons:

EGFP transgenic insects will not persist in the environment. They will be sterilized by irradiation (Tothova and Merec, 2001) The EGFP PBW line to be released has significantly lower fecundity than wild-type PBW. Redundant mitigation measures are incorporated into the experimental procedures to insure that genetically modified EGFP PBW will not become established in the environment. These measures are as follows:

- The cotton in the proposed release site expresses *Bacillus thuringinesis* toxin that kills PBW larvae.

- There are no sexually compatible relatives of the pink bollworm in the United States so the transgene cannot spread via hybridization with other species.

- The *piggyBac*-derived transposable element used to make the transforming construct has no functional transposase gene, eliminating its ability to mobilize itself. (Appendix IV).

- The release area will be monitored intensively with pheromone traps that attract and collect PBW male moths. Traps will be set will be up to 5 miles away from the site.

- If adverse persistence is observed, unwanted bollworms will be killed with insecticides. Larvae from eggs oviposited on Bt cotton will not survive.

- PBW populations can be suppressed by flooding the area with a high ratio of sterilized bollworms to field insects.

- All moths will be securely managed and contained in production and transport (Appendix VI) using SOPs with extremely high reliability developed for a long running Sterile Insect Technique program.

- All living bollworms reared for this field trial that are not used as part of the environmental release will be killed.

For the reasons enumerated above, which are consistent with regulations implementing the Plant Protection Act, the field trial of EGFP transgenic Pink Bollworms is hereby authorized.

Cindy Smith
Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
U. S. Department of Agriculture
Date

[illegible] 07 2006

Addendum to Environmental Assessment
Addressing amendments to permit number APHIS No. 05-098-1r

Introduction:
On April 10, 2006, APHIS reached a FONSI for an EA that was developed in response to a request for APHIS to issue a permit for a field study of genetically engineered Pink Bollworm containing enhanced green fluorescent protein as a marker gene for identification of radiation sterilized pink bollworm. That EA is available at http://www.aphis.usda.gov/brs/aphisdocs/05_09801r_ea.pdf.
After reaching a FONSI, APHIS received a request to amend the permit application to change the size, confinement conditions, and the location of the field trial. This document is an addendum that supplements the original EA to address the requested changes.

Purpose and Need: APHIS has received a request to amend APHIS permit 05-098-01r. When APHIS receives a request to amend a permit application, APHIS must consider whether or not to amend the permit. Because the amendment to the permit application changes the location, size and confinement conditions of the original request, APHIS is assessing potential impacts that may result from these changes in this supplement. This supplement does not replace the original EA. It only addresses new potential impacts associate with the changes to the permit application.

Description of alternatives:
A. No Action
For the purpose of this supplemental EA, the no action alternative is to not issue the permit as requested in the amended permit application.
B. Issue an Amended Permit
Issuing this permit would allow the following research to proceed at a cotton field site in Yuma County, AZ.

Potential Impacts on the Human Environment.

1. Potential impacts on endangered species
Changing the site of the field trial from Pima County Arizona to Yuma County Arizona reduces the total number of Threatened and Endangered listed species from 23 in Pima County to 9 in Yuma County. These species are all vertebrate animals. Of these 9 species (7 listed, one candidate, and one proposed species), three are not on the Pima County list and were not evaluated in the completed Environmental Assessment (EA) and Finding of No Significant Impact (FONSI). They are:
Razorback sucker, *Xyrauchen texanus*, Endangered
Yuma clapper rail, *Rallus longirostris yumanensis*, Endangered
Flat-tailed horned lizard, *Phrynosoma mcallii*, Proposed.

The razorback sucker is a fish found in rivers and lakes, the Yuma clapper rail is a water bird living in marshes, and the flat-tailed horned lizard lives in pristine desert areas. In contrast, the release area is a highly disturbed agricultural cotton field area. As none of these sensitive ecosystems are present in the areas near the release field and none of these threatened and endangered species are expected to frequent agricultural fields where cotton is grown, there are not expected to be any direct or indirect interactions between transgenic EGFP pink bollworm moths and any of these species not previously considered in the EA. Thus none of the threatened and endangered species of Yuma County are expected to incur any risk or jeopardy by the proposed field release under alternative B. There would also be no impact on threatened or endangered species under the no action alternative.

2. Potential impacts from this field trial on cotton production in Yuma County.

Change in the release site from non-Bt cotton to Bt-cotton. The field trial is designed to restrict the reproductive potential of the transgenic moths. Appendix I describes the original experimental design. In the amended permit, moths will be released into Bt cotton. Should the released male moths reproduce, their offspring would not survive in the field because pink bollworm larvae are killed by feeding on Bt cotton. This is a change from the original field design which was on non-Bt cotton surrounded by Bt cotton, which may have allowed an F1 generation of transgenic moths to survive locally, if the transgenic males were fertile (see description of F1 sterility in Appendix II). Because no offspring are expected to survive, the effects associated with release of irradiated transgenic moths in this area would be minimal. There would also be few effects should the moths not be released. Pink bollworm is currently present throughout Arizona. This project will not change the distribution of Pink Bollworm

Change in the minimum distance from conventional cotton. The original permit application proposed a minimum distance of 7.5 miles from the nearest non-Bt cotton. In the modified application, the proposed minimum isolation distance is changed to at least one mile from conventional cotton. The one mile isolation distance should be more than sufficient to confine the moths to Bt-cotton, because research has shown that the majority of irradiated male moths disperse less than 1000 m (Tabashnik, 1999). Additionally, other safeguards are incorporated into the experiment to prevent a free-living population from establishing. For example, the EGFP moths to be released will be sterilized with exposure to 10 kilorads (KR) radiation. This should significantly reduce their fertility, and is predicted to make any offspring infertile as well. Therefore in the unlikely event that a released moth does disperse to conventional cotton, it is unlikely to produce fertile offspring. If the permit is not granted, there would be no change in the pink bollworm population in Arizona.

Change in the number of moths to be released and the size of the experimental area. Both the physical size of the field trial and the number of moths to be released has been reduced in the amended permit application. The original permit requested that up to four 3 acre plots for a total of 12 acres of conventional cotton be used in the release. The 12 acres were to be within a 240 acre field, the remainder of which

would be planted with BT cotton. The current proposal is for a 4.6 acre release site of BT cotton surrounded by another 40-80 acres of BT cotton.
The number of EGFP moths to be released has been reduced 30 fold from the original application. Like the original proposal, these moths and any offspring resulting from them are not likely to persist in the environment. These moths are irradiated to reduce fertility and generate F1 sterile offspring. Reducing the number of moths further reduces the already minute likelihood of establishment of a population of GE-pink bollworm. If the permit is not granted there would be no change in the pink bollworm distribution in Yuma County, Arizona.

References

Tabashnik, B.E., A.L. Patin, T.J. Dennehy, Y-B. Liu, E. Miller, R.T. Staten. 1999. Dispersal of pink bollworm (Lepidoptera: Gelechiidae) males in transgenic cotton that produces a *Bacillus thuringiensis* toxin. J Econ Entomol. 92(4): 772-780.

Finding of No Significant Impact and Decision Notice

Animal and Plant Health Inspection Service

Issuance of a permit for a Field Study of Genetically Modified Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

The Animal and Plant Health Inspection service (APHIS) of the United States Department of Agriculture (USDA) has received a permit application (APHIS number 05-098-01r) from USDA APHIS Center for Plant Health Science and Technology (CPHST) to conduct field trials of pink bollworms genetically engineered to contain the enhanced green fluorescent protein (EGFP) as a marker gene for identification of radiation sterilized pink bollworm released in areas of Arizona in which they are already endemic. A description of the field tests may be found in the attached Environmental Assessment (EA), which was prepared pursuant to the APHIS regulations (7 CFR 372) promulgated under the National Environmental Policy Act. The field tests are scheduled to begin in 2006 in Pima County of Arizona.

A draft EA was prepared and submitted for public comment for 30 days. Two comments were received and are addressed in the Federal Register notice that announces the availability of this document.

APHIS proposed two different actions to take in response to the permit application: the denial of the permit (Alternative A) and the granting of the permit with Supplemental Permit Conditions containing additional safety requirements and a requirement of filing field test reports as described in appendix 1 (Alternative B).

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

No adverse consequences to non-target organisms or environmental quality are expected from incorporation of this marker into the pink bollworm
Green fluorescent protein is a naturally occurring protein, not known to cause adverse effects (Appendix V). The normal digestive process of predators would preclude transfer of functional genetic material to the predator so such transfer is not expected with eating this insect. In addition, there have been no adverse effects to birds that are naturally exposed to the GFP through feeding directly on jellyfish. Released pink bollworms will be sterile adults that are not expected to produce offspring. No adverse consequences are expected to beneficial insects (e.g., natural enemies, decomposers, pollinators) from release of transgenic PBW adults expressing GFP.

Because APHIS has reached a finding of no significant impact of this field release of transgenic pink bollworm, no Environmental Impact Statement will be prepared regarding this decision.

Pursuant to its regulations (7 CFR 340) promulgated under the Plant Protection Act of 2000, APHIS has determined that this field trial will not pose a risk of the introduction nor dissemination of a plant pest for the following reasons:

EGFP transgenic insects will not persist in the environment. They will be sterilized by irradiation (Tothova and Merec, 2001) The EGFP PBW line to be released has significantly lower fecundity than wild-type PBW. Redundant mitigation measures are incorporated into the experimental procedures to insure that genetically modified EGFP PBW will not become established in the environment. These measures are as follows:

1. All the surrounding cotton expresses *Bacillus thuringinesis* toxin that kills PBW larvae.

2. There are no sexually compatible relatives of the pink bollworm in the United States so the transgene cannot spread via hybridization with other species.

3. The *piggyBac*-derived transposable element used to make the transforming construct has no functional transposase gene, eliminating its ability to mobilize itself. (Appendix IV).

4. The release area will be monitored intensively with pheromone traps that attract and collect PBW male moths. Traps will be set will be up to 5 miles away from the site.

5. The area of release is less than 12 acres with no more than 3 acres per plot.

6. If adverse persistence is monitored, unwanted bollworms will be killed with insecticides. Larvae from eggs oviposited on Bt cotton will not survive.

7. PBW populations can be suppressed by flooding the area with a high ratio of sterilized bollworms to field insects.

8. All moths will be securely managed and contained in production and transport (Appendix VI) using SOPs with extremely high reliability developed for a long running Sterile Insect Technique program.

9. All living bollworms reared for this field trial that are not used as part of the environmental release will be killed.

For the reasons enumerated above, which are consistent with regulations implementing the Plant Protection Act, the field trial of EGFP transgenic Pink Bollworms is hereby authorized.

Cindy Smith
Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
U. S. Department of Agriculture
Date

APR 10 2006

Field Study of Genetically Modified Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

Environmental Assessment

(Sept 13, 2005)

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I. PURPOSE AND NEED

A. Introduction and Spread in USA.

The pink bollworm (PBW), *Pectinophora gossypiella* (Saunders), was described from larvae recovered from infested cotton bolls in India in 1843 (Noble 1969). It has since become one of the most destructive pests of cotton in many of the major cotton-growing regions of the world. The first reported cotton infestation in North America occurred in 1911 in Mexico, presumably from Egyptian cotton seed shipments (Glick 1967). In the United States, PBW was detected first in Robertson County, Texas in 1917 (Scholl 1919). By 1926, the pest had spread from Texas through New Mexico and into eastern Arizona and became a major economic pest of cotton in Arizona and southern California by 1965 (Burrows *et al*. 1982). Preliminary analysis of a 2000-2001 survey indicated no PBW were present in Arkansas, Louisiana, Oklahoma, and most of Texas. PBW populations exist in west Texas and south central New Mexico and surveys continue to indicate wide distributions of PBW throughout Arizona and in southern California.

The San Joaquin Valley of California remains the last cotton growing area in the Southwest that is not infested with PBW. Prevention of its establishment is attributed primarily to the ongoing Sterile-Insect Technique (SIT) program established jointly in 1968 by USDA, APHIS, California Department of Food and Agriculture, and the California cotton growers. The SIT program for the PBW is described in Appendix III on the Pink Bollworm Eradication Plan.

B. Economic Importance

Control costs for PBW in Southern California and Arizona were estimated to exceed $1.2 billion over the past thirty years (Roberson *et al*. 1998, Antilla *et al*.1999). Yield losses caused by PBW ranged from $85-$170 per acre (Antilla *et al*. 1999). Most recently, the National Cotton Council estimated that U.S. cotton producers' annual losses to pink bollworm are about $21 million due to prevention, control costs and lower yields caused by plant damage (NCC, 2001). In Egypt, China, and Brazil, it commonly causes cotton losses of up to 20 %, although losses can be much higher.

C. Control of Pink Bollworm

Conventional insecticides have not provided a long-term solution to the pink bollworm problem (Henneberry 1986). Considerable amounts of basic biological and ecological information have been accumulated and applied in developing PBW control programs. No single control method is completely satisfactory. The possibility of combining a number of methods into a single control system appears the most promising approach (Henneberry *et al*. 1980). United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) assists states in controlling the pest and preventing its spread. APHIS enforces a quarantine in infested areas, requiring certification for the interstate movement of regulated articles. (7 C.F.R. 301.52)

A 2002 Environmental Assessment (EA) on Southwest Pink Bollworm Eradication Program was conducted and the FONSI for this EA stated that the eradication program was the preferred alternative because it could achieve the eradication objectives in a way that reduces the scale of potential environmental consequences. The 2002 EA provides a good review of integrated pest

management of the PBW and two of the insecticides used to control PBW and their risks. It is located at this URL: http://www.aphis.usda.gov/ppq/enviro_docs/pdf_files/swpbwea.pdf

D. Biology of Pink Bollworm

Pink bollworm larvae feed inside the growing cotton boll and destroy the cotton. It prefers cotton, but will feed on okra, kenaf, and hibiscus. It has four stages of development: egg, larva, pupa, and adult. In early June, female moths lay 100 to 200 eggs on young cotton bolls. The eggs hatch in about five days producing larvae. Hatched larvae bore into the cotton bolls and feed from 10 to 14 days on the seed. One larva eats a whole seed or parts of several seeds. First and second instar larvae are smaller, ivory in color, and have dark heads. Late instar larvae are larger and have bodies with pink bands. Fully-grown larvae are 7 to 10 mm long (1/4 to 3/8 inch). When larvae mature, they either drop to the ground or remain in the boll to pupate. Pupation can also take place under ground trash. Pupae emerge as moths in 8 to 10 days. In warmer areas, most larvae overwinter in cotton bolls left in the field after harvest. In colder climates, larvae may form cocoons in soil crevices to overwinter. Larvae can also remain in cotton seed after the cotton is ginned, and if the seed is not fumigated, larvae can emerge from stored seed the next spring.

Egg-to-adult development takes 26–32 days during the cotton-growing season. Adult moths are grayish brown and about the same length as fully grown larvae. Their wingspan is 15 to 20 mm (5/8 to 7/8 inch). The females start laying eggs 1 to 3 days later after mating. Adults are active only at night and live about 10 days. PBW is well adapted to the long growing seasons in the desert valleys of the southwest where 5 to 6 generations develop each year.

E. Regulatory Authority

APHIS has authority for regulation of genetically modified PBW under the Plant Protection Act of 2000, 7 U.S.C. 7701-7772, and USDA, APHIS regulations under 7 CFR § 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. The pink bollworm, *Pectinophora gossypiella* (Saunders), is the recipient organism and is a plant pest. The source of the *piggyBac* transposon used to make the transforming vector was isolated from a cell culture of the cabbage looper, *Trichoplusia ni* (Huebner), which is also a plant pest. A transposon or transposable element is nucleic acid able to move from one chromosome site to another site and may carry other genetic material with it when it moves. A description of the transposable element in transforming the pink bollworms is describe in Appendix IV. Modified transposons are frequently used in molecular biology to insert new genes into organisms.

This EA was conducted under the authority of the National Environmental Policy Act (NEPA), 42 U.S.C. 4321 and 7 CFR § 372, NEPA Implementing Procedures. Except for actions that are categorically excluded, approvals and issuance of permits for proposals involving genetically

engineered or nonindigenous species normally require environmental assessments, but not necessarily environmental impact statements (7 CFR § 372.5(b)(4)). The actions described in the application for permit 05-098-01r involve the release of a transgenic insect employing a novel combination of confinement methods, and APHIS' NEPA implementation rules include an exception for categorically excluded actions (7 CFR 372.5(d)(4)):

> When a confined field release of genetically engineered organisms or products involves new species or organisms or novel modifications that raise new issues.

APHIS believes that the combination of confinement methods described in this permit application raise new issues which justify the preparation of an Environmental Assessment.

APHIS has previously published a notice of intent in the Federal Register to prepare an Environmental Impact Statement (EIS) for release into the environment of transgenic pink bollworms containing an autocidal gene combined with the green fluorescent gene. (Docket No. 01-124-1, 2/4/2002, V. 67, No. 23, p 5086), Environmental Impact Statement, "Genetically Engineered Pink Bollworm." The URL is: http://frwebgate.access.gpo.gov/cgi-bin/getdoc.cgi?dbname=2002_registeranddocid=02-2604-filed

This FR Notice states the following: "The objective of such a release would be to provide an opportunity for evaluating the use of genetically engineered pink bollworm in an autocidal biological control system for area-wide management of pink bollworm. APHIS plans to prepare an environmental impact statement (EIS) that examines potential environmental effects associated with the field release of genetically engineered pink bollworm containing the enhanced green fluorescent protein marker gene and a temperature-sensitive lethal gene, and other alternatives." The APHIS intention to do an EIS remains in effect when the autocidal genetic technology reaches an appropriate stage of development. However, the permit application that is the subject of this EA is not for release of insects transformed with an autocidal gene, but only with a green fluorescent protein marker gene. The intent of work detailed in this EA is to improve SIT by better optimizing the Sterile Insect Technique program that uses irradiation to control pink bollworms. This is distinct from autocidal genetic techniques.

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 of pink bollworms genetically modified to express green fluorescent protein (GFP) or enhanced green fluorescent protein (EGFP) (Appendix V). The study will use genetically modified PBW that are also reproductively sterilized with 10 kilorad (kr) radiation to determine if the fluorescent marker is suitable for monitoring and tracking both released and any

first generation (F_1) offspring.

B. Purpose and Need for this EA

The need for this EA is to assess any potential adverse environmental effects of a field research study in Pima County, Arizona. The application for a permit was received by APHIS, BRS April 8, 2005. It was submitted by the USDA, APHIS, Plant Protection and Quarantine (PPQ), Center for Plant Health Science and Technology (CPHST), Decision Support and Pest Management Systems Laboratory, Phoenix, Arizona. The application number is 05-098-01r and the body of the application information is in Appendix I.

C. Similar EAs

An EA has already been published for confined studies of genetically engineered pink bollworms bearing the EGFP gene: "Confined Study of a Transgenic Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)" dated October 1, 2001. The EA, Finding of No Significant Impact (FONSI), and Response to Comments are at the following URL: http://www.aphis.usda.gov/brs/arthropod_assess.html

The present EA also addresses genetically modified PBW that express EGFP. Technical aspects of this modification are described in detail in Appendix IV. EGFP-modified pink bollworms are to be treated with 10 kr $cobalt_{60}$ radiation to achieve F_1 sterility. F_1 sterility is described in detail in Appendix II.

D. 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 applications*. After 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 340.5(e)

III. ALTERNATIVES

A. No Action

Under APHIS/BRS regulations, the Administrator must either grant or deny permits properly submitted under 7 CFR 340. For the purposes of this Environmental Assessment, the No Action alternative would be the denial of permit application 05-098-01r.

B. Issue a Permit

Issuing this permit would allow the following research to proceed at a cotton field site in Pima

County, AZ. Appendix I contains details of the research plan from the permit application.

1. Purpose of the Research

The purpose of EGFP genetically marked PBWs is for monitoring the effectiveness of first generation (F_1) sterility for PBW eradication program use. F_1 sterility with PBW is obtained through the use of 10 kr $cobalt_{60}$ irradiation to obtain high insect sterility while preserving sexual competitiveness. See Appendix II for detailed description of F_1 sterility. With higher doses of radiation, the insects sustain significant injury and are not competitive in mating. At a lower 10 kr dose, a few of the irradiated insects mate and produce progeny, but these offspring are sterile, thus sterility is passed to the F_1 generation. The genetic marker provides a practical and useful way to differentiate F_1 progeny from other PBW that reproduced in the field because they glow green under fluorescent light. This allows more accurate estimates of the numbers of sterile insects needed for release. Without genetic markers, all PBW insects found are considered to have come from the field. Based on this assumption, many more irradiated sterile insects need to be produced and released according to the Pink Bollworm Eradication Program plan (Appendix III).

2. Description of the Research

The EGFP pink bollworm will be released in no more than four, 3 acre or less, test plots of conventional cotton adjacent to Bt cotton. Bt cotton has been genetically engineered to produce *Bacillus thuringinesis* toxins that kill or inhibit growth of lepidopteran larvae. Conventional cotton planted near Bt cotton provides a refuge for the purpose of preventing or slowing Bt resistance development by cotton pests in the insect order Lepidoptera. The closest adjacent cotton is one planting 7.5 miles from this farm in Pima County, AZ. All surrounding cotton except the release plots will be Bt cotton, which prevents PBW larval development.

All PBW used in the study will be irradiated with 10 kr of ^{60}cobalt prior to field testing. The treatment groups consist of the following:

1. Conventional APHIS PBW irradiated at 10 kr.

2. APHIS genetically modified EGFP PBW irradiated at 10 kr.

Each treatment will be applied from first square through a 90-day period by hand or mechanized release in small two acre refuge strips of conventional cotton adjacent to Bt cotton. These fields will have no other contiguous non-Bt cotton capable of supporting PBW. Final field selection will be based on actual field distribution of the 2006 crop.

Each field will be monitored for the following:

1. F_1 EGFP PBW and native larval production in blooms during the first generation and in bolls during the 2nd and 3rd generation of insects.

2. Adult PBW moth populations will be monitored using Delta^{Tm} pheromone insect traps spread over the entire farm and checked 3 times per week. Traps will also be placed along

roadways for 5 miles in each accessible direction. The moths from the traps will be identified and sampled using techniques to preserve them for genetic analysis.

3. Mating sequences of native female moths, APHIS female moths, and the EGFP moths will be tested with the same general protocols as used in previous field cage studies described in permit number 01-029-01R. The URL for the EA, FONSI and Response to Comments for 01-029-01R is cited above. PBW will be reared in containment according to procedures in Appendix VII. All sampled PBW will be killed by freezing at minus (-) 20^0C for 24 hours. This destroys all life stages of this insect.

IV. ENVIRONMENTAL CONSEQUENCES OF THE PROPOSED ACTION AND ALTERNATIVE

The test site in Pima County, AZ is within the geographic area that has already been infested by pink bollworms. There are no ornamental hibiscus known within 10 miles of the testing site and the permit applicants have found PBW will not complete its biological development on the contemporary ornamental cultivars locally available from nurseries and other area retailers. Okra, another host of PBW, is predominantly grown in southern parts of the U.S. including Texas, Florida, Georgia and California and not expected to occur near the field test sites in Arizona.

A. Summary of Consequences

Issues	No Action	Issue Permit
Possibility of unanticipated change to the 10 kr irradiated EGFP PBW resulting in risk to the environment	No effect	No effect
Risk of EGFP to the environment	No effect	No effect
Persistence of 10 kr EGFP PBW compared to the field grown wild-type	No effect	No effect
Redundant physical and/or biological confinement of the EGFP BPW	No effect	No effect
Gene transfer to offspring or related species	No effect	F_1 Sterility
Horizontal gene transfer to other organisms, such as predators, saprophytes, or parasites	No effect	No effect
Potential impacts on humans, including minorities, low income populations, and children	No effect	No effect
Effects on chemical (pesticide, herbicide, fungicide) load on the environment	No effect	Potential benefit

Issues	No Action	Issue Permit
Risks to nontarget plants and animals including threatened and endangered species	No effect	No effect

B. Deny the Permit Application.
To deny the permit application would have no expected potential adverse environmental impacts and would prevent this confined field research from proceeding and prevent any benefits derived from it being realized in the future.

C. Issuance of the Permit.
The proposed action is not expected to have any adverse environmental impacts for the following biological and physical reasons:

No adverse consequences to nontarget organisms or environmental quality are expected from incorporation of this marker into the pink bollworm. The unmodified pink bollworm has no *EGFP* gene; therefore, it does not glow a characteristic fluorescent green when illuminated with light of proper wavelength for excitation of EGFP. Neither *piggyBac* transposase replication activity, nor any antibiotic resistance is conferred to the transgenic PBW by the introduced genetic material because genetic material encoding these proteins was not integrated into the PBW genome (Appendix IV).

D. Analysis of Issues, Consequences, and Theoretical Risks of Field Research on EGFP PBW

1. Possibility of some unanticipated change to the 10 kr irradiated EGFP PBW resulting in risk to the environment
The possibility of the genetically modified organism reverting to or undergoing some form of unanticipated genetic transformation are low based on 30-generations of observation by the petitioner. Additionally, the insects will be irradiated to make them sterile. An analysis was also conducted using a green fluorescent protein-specific antibody to establish that the EGFP protein produced was the expected size showing that no additional sequence was being translated into protein fused to the EGFP (Appendix IV).

2. Risk of EGFP to the environment

GFP and EGFP have been used in the genetic modification of at least 100 different organisms from plants to arthropods to mammals and birds. Its primary use has been as a marker gene to verify insertion presence of other linked genes of physiological interest. Several of these GFP or EGFP modified organisms have been exposed to the environment under field conditions over more than a decade with no adverse environmental, nontarget organism, or adverse human health

effects seen or published. GFP and derivatives are not significantly toxic administered orally to mammals nor are they notably cytotoxic when expressed in a transgenic organism (Richards, *et al.* 2003). Consequently, the use of EGFP in PBW is also not expected to cause any adverse effects. The proposed field research has monitoring components (e.g., traps placed along roadways for 5 miles in each accessible direction) that include observations for adverse effects and redundant mitigation measures to eliminate any adverse effects should any be seen. Appendix V contains a review of safe uses of GFP and EGFP.

3. Persistence of 10 kr EGFP PBW compared to the field grown wild-type

It is highly unlikely that the EGFP transgene described here would persist in the environment, first because the EGFP transgenic insects would have been sterilized by irradiation (Tothova and Merec, 2001) and second, because the EGFP transgene provides no fitness advantage to the PBW- in fact, fecundity in the EGFP PBW to be released is significantly lower than non-EGFP insects. In over 30 generations, EGFP PBW consistently showed lowered reproductive fitness due to lower fecundity. Egg survival was also reduced. Even if the EGFP gene were somehow to be introduced into a field population of the pink bollworm, it would not confer any selective advantage but in fact would be negatively selected. There were no differences between EGFP PBW and the parental strain in length of time spent and mortality in larval instars. Mortality in pupal EGFP PBW was comparable to non-transformed PBW. However, EGFP female moths produced 19.8 % fewer eggs than non-transformed PBW and their successful egg hatch rate was 26% lower for a total fitness reduction of at least 40% per generation as compared to the untransformed colonized parental strains (Miller *et al*. 2001).

4. Conditions for permit: Redundant physical and/or biological confinement of EGFP transgenic PBW

The following redundant mitigation measures are incorporated into the experimental procedures to insure that 10 KR genetically modified EGFP PBW will not become established in the environment:

4.a. The experimental site is isolated. The nearest susceptible non-Bt cotton is 12.5 km (7.5 miles) away.

4.b. All the released EGFP moths will be irradiated with 10 kr to sexually sterilize them. The F_1 sterility mechanism also sterilizes offspring (Appendix II).

4.c. The EGFP transgene has fitness cost to the pink bollworm due to lower fecundity. This negative selection pressure would drive loss of this gene in the population.

4.d. 10 kr irradiation has a fitness cost to the PBW due to radiation injury to the insect.

4.e. All the surrounding cotton expresses *Bacillus thuringinesis* toxin that kills PBW larvae.

4.f. There are no sexually compatible relatives of the pink bollworm in the United States so the transgene cannot spread via hybridization with other species.

4.g. The *piggyBac*-derived transposable element used to make the transforming construct has no functional transposase gene eliminating its ability to mobilize itself. (Appendix IV).

4.h. The release area will be monitored intensively with pheromone traps that attract and collect PBW male moths. Traps will be set will be up to 5 miles away from the site.

4.i. The area of release is less than 12 acres with no more than 3 acres per plot.

4.j. If adverse persistence is monitored, unwanted bollworms will be killed with insecticides. Larvae from eggs oviposited on Bt cotton will not survive.

4.k. PBW populations can be suppressed by flooding the area with a high ratio of sterilized bollworms to field insects.

4.l. All moths will be securely managed and contained in production and transport (Appendix VI) using SOPs with extremely high reliability developed for a long running SIT program.

4.m. All living bollworms left over from the testing will be killed.

5. Gene transfer to offspring or related species

The PBW is not native to the United States and there are no known sexually compatible species in North America. The nearest relatives to Pink Bollworm are in Australia.

6. Horizontal gene transfer to other organisms, such as predators, saprophytes, or parasites.

6.a. Predators and parasites. Pink bollworms may be eaten by predatory insects, birds, or mammals that venture into cotton fields in spite of pesticide use. Green fluorescent protein is a naturally occurring protein, not known to cause adverse effects (Appendix V). The gene has been found in nature only in the jellyfish (*Aequora Victoria)* from which it is derived. Jellyfish have been prey or subject to saprophytic digestion by other organisms since their ancient origins in the Precambrian period. There is no current evidence that this gene has been ever been transferred through predation, natural decay, or parasitism. The normal digestive process of predators would preclude transfer of functional genetic material to the predator so such transfer is not expected with eating this insect. In addition, there have been no adverse effects to birds that are naturally exposed to the GFP through feeding directly on jellyfish.

Pink bollworms may also serve as hosts for parasitic insects, nematodes, and various microorganisms. These parasitic and infectious organisms are unrelated to the PBW and would not be expected to assimilate functional DNA from their hosts leading to modification of the parasite or microorganism. Organisms transgenic for GFP and related proteins have been released to the environment previously with no reported environmental effects (Anon. 2005). Pink bollworms will be released in the field as sterile adults that are not expected to produce offspring. Natural enemies including predators and parasitoids have only been reported for the egg, larvae and pupae stages of the PBW (Hagler and Naranjo 1994a and 1994b, Naranjo and Hagler 1998, Henneberry and Naranjo 1998, http://faculty.ucr.edu/~legneref/biotact/ch-86.htm). There have been no reports of predators or parasitoids attacking PBW adults. Therefore, no adverse consequences are expected on the beneficial insect community from release of transgenic PBW adults containing the GFP. In addition, these parasitic and infectious organisms are unrelated to the PBW and would not be

expected to assimilate functional DNA from their hosts leading to modification of the parasite or microorganism.

6.b. Transposon ("jumping gene") immobilization. The transposase gene of the *piggyBac* transposon used to transform PBW was destroyed by deleting a portion of that gene. This procedure destroys the ability of the transformation construct to move or transfer horizontally on its own (Appendix IV). Based on inverse PCR, the *piggyBac* integration is a singular event that occurred in a transposase-dependent manner with no plasmid sequences flanking the transposon ends (Peloquin *et al.* 2000).

The potential for instability and unwanted mobilization of *piggyBac*-derived transforming constructs was further addressed in respect of the EGFP transgenic line. Stability of the *EGFP* transgene was examined in total of ~50 individuals from the 58th generation of the EGFP transgenic strain. Analysis indicated that the original insertion has been stable and no evidence of mobilization during the 58 generations was detected (Park, unpublished data; See Appendix IV).

6.c. Stability of construct. A back cross of EGFP heterozygotes to wild type insects results in a 1:1 ratio of EGFP positive to wild type progeny. This supports the hypothesis that EGFP in this strain is transmitted as a single-locus, dominant gene. This observation has been confirmed by genomic Southern hybridization in the 58th generation that revealed only one 2.4 kb band for the insertion that was described for the original EGFP genetically modified line (Appendix IV).

7. Potential impacts on humans, including minorities, low income populations, and children. 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. Because the field trial release of the genetically modified PBW is a controlled release within a specified location without public access, the effects of this proposed action are not6 expected to directly nor casually affect children, minorities or low income populations, therefore are not expected to adversely impact any of these groups.

8. **US Environmental Protection Agency registered pesticides, primarily insecticides, are used more intensively on cotton than most other crops. The purpose behind these field trials is to depress, and eventually eradicate PBW populations in the US, a pest most commonly found in cotton crops. The success of these efforts should allow a decrease in the use of pesticides on these crops. The pesticides that may be used associated with this proposed field study would be limited in amount and such uses will strictly adhere to EPA labeling restrictions. The proposed research is not expected to result in a significant additional pesticide load on the environment and therefore is not expected to have any significant impact on the environment.**

9. APHIS has determined that the proposed test with have no effects on listed threatened or endangered species. There are 18 threatened or endangered plants and animals with no insect species listed for Pima County (http://www.fws.gov/arizonaes/Documents/CountyLists/Pima.pdf). Most of the species are endangered because their habitat has been destroyed. Upon review of the nature of the species, their habitats, and other comments concerning their distribution, none of the threatened and endangered species of Pima County are expected to incur any risk or jeopardy by EGFP or other cnidarian derived fluorescent proteins in PBW that have been treated with 10 kr radiation. In addition, the listed endangered species do not occur in agricultural fields where cotton is grown (Appendix VII).

10. Likelihood of effects on environmental quality
The effects on environmental quality are expected to be nonexistent or negligible because the *EGFP* marker gene is not known or expected to negatively impact the environment.

The work described in permit application 05-098-01r as this experiment was specifically suggested by a working group sponsored by the Food and Agriculture Organization of the United Nations as a first experiment using transgenic arthropods (Ashburner *et al*. 1998).

11. Degree of uncertainty of effects on the environment and unique or unknown risks
The *EGFP* gene is not known nor expected to affect the environment, therefore, the degree of uncertainty is low. There have been many applications of the *EGFP* gene in biology, agriculture and medicine with no reports of adverse effects (See Appendix V for details). Furthermore, Miller *et al.* (2001) have shown that the *EGFP* gene provides no selective advantage to the PBW.

12. Consistency of proposal with other environmental requirements
The proposal is believed to be consistent with other environmental requirements.

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APPENDIX I

Description of Proposed EGFP Plus F_1 Sterility Field Research Project (from Permit Application)

From Application for Permit under 7 CFR 340
Date submitted: April 8, 2005
Permit application No: 05-098-01r
Submitter:
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ENCLOSURE B

A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non modified

parental organism.

The additional genetic material in the pink bollworm results in the expression of a modified version of the Green Fluorescent Protein (GFP) derived from the jellyfish *Aequora victoria*. The GFP transgenic pink bollworm strain developed by Peloquin (*et al*. 2000) fluoresces strongly green when viewed in the 3rd instar larval stage at 510 nm with 460 to 500 nm illumination. GFP confers no competitive advantage to the recipient, as demonstrated in laboratory experiments over 30 generations in our facility. (E. Miller, USDA Plant Protection Center in house report, 2000). No ecological or other consequences resulting from incorporation of this marker into the transgenic pink bollworm can be envisioned. The non modified pink bollworm has no GFP gene, therefore, it does not fluoresce strongly green when illuminated under the same light frequency. Neither *piggyBac* transposase activity nor any antibiotic resistance is conferred to the transgenic pink bollworm by the introduced genetic material.

ENCLOSURE C

A detailed description of the molecular biology of the system that was used to produce the regulated article.

The *piggyBac* element is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (Inverted Terminal Repeats) are intact, is capable of integrating DNA flanking by element specific DNA into other DNA through mediation of a transposase encoded by an ORF (Open Reading Frame) within the element. In the construct used for transformation of the pink bollworm, the transposase gene of the *piggyBac* element was irreversibly destroyed by insertion of the GFP gene. Transformation was effected by introducing, with the transforming construct, a helper plasmid which supplied transposase activity but was itself unable to transpose into other DNA. This transposition defective helper plasmid has an ORF (Open Reading Frame) encoding *piggyBac* transposase under the control of the *Drosophila melanogaster* hsp70 promoter. One of the inverted terminal repeats that flank the wild type *piggyBac* transposase in *piggyBac* has been removed in the helper plasmid so that the helper plasmid cannot, itself, integrate even though it encodes for active *piggyBac* transposase.

The potential for instability and unwanted mobilization of *piggyBac* derived transforming constructs must be addressed as follows. It could be argued that if there were endogenous *piggyBac* like elements in pink bollworm, they might provide a source of transposase that could mobilize transgenes flanked by *piggyBac* derived ITRS. Demonstration of elements homologous to *piggyBac* in the recipient organism, pink bollworm, might then suggest caution regarding stability of the transgene. However, the DNA mediated element, *Hermes*, has been used to successfully transform *Aedes aegypti* with little or no evidence of instability of the transgenes over at least 10 generations, even though there are in *Aedes aegypti* endogenous elements (presumably hAt-like as is *Hermes*) with close enough homology to *Hermes* so that these endogenous hAt and *Hermes* like elements are detected even in higher stringency Southern blots with a *Hermes* probe (Jasinskiene *et al*. 1998, PNAS 95:3743–3747).

In the case of pink bollworm, low stringency Southern blot experiments on pink bollworm DNA with radio labeled DNA probes derived from *piggyBac*, which would be even more likely to

detect elements with low homology to *piggyBac* than the higher stringency methods used in Jasinskiene (*et al*. 1998) were unable to detect any endogenous *piggyBac* like elements. This suggests that there are no elements in pink bollworm that might reasonably be expected to mobilize a *piggyBac* derived transgene. In addition, excision and transposition assays were performed in pink bollworm embryos with *piggyBac*. This was primarily to determine if *piggyBac* could integrate into the pink bollworm genome. However, our results showed no transposition of *piggyBac* in the absence of exogenous *piggyBac* transposase in these transposition assays, strongly suggesting there were no unknown elements in the pink bollworm genome. We can thus be reasonably certain there would not be unexpected interactions between the components of the pink bollworm genome and the transforming construct that would result in instability of the transgenes. Further demonstration of the stability of the transgene is demonstrated by the current rearing at the Phoenix Quarantine Facility of 10 generations of GFP strain PBW with no evidence of instability of the GFP transgene.

ENCLOSURE D

Country and locality where the donor organism, recipient organism, and vector or agent were collected, developed and produced.

All final engineering of the transforming constructs were performed at the University of California, Riverside, California, Riverside County, United States. The genes used from the donor organism and the *piggyBac* derived portions of the vectors used to build the transforming construct were cloned off site. Specifically, *E. coli* was the immediate host for the plasmids carrying the cloned genes used to make the transforming constructs.

The *piggyBac* transposable element was discovered in *Trichoplusia ni* cell culture by Malcolm Fraser at the University of Notre Dame. The *Bombexi mori* actin A3 promoter was cloned by Steve Thibault at the University of California, Riverside, California using PCR from the embryos of *Bombexi mori*, purchased from Carolina Biological. The EGFP gene is a modified version of GFP which was cloned by Douglas Prasher (USDA/APHIS Otis AFGB, MA) from the jellyfish *Aequora victoria*. The plasmid source of EGFP was purchased from Clontech, Inc.

The recipient organism, the pink bollworm (PBW), *Pectinophora gossypiella*, (Lepodoptera: Gelechiidae) is a species that is probably indigenous to India (Noble 1969). It is not a native species of the Western Hemisphere though it is now endemic to the Southwestern United States and Mexico and associated with commercial cotton production. Introduction of the pink bollworm into the United States appears to have been via infected cottonseed. The pink bollworm appeared in Hearne, Texas, in 1917 and within a decade had spread across Western Texas, New Mexico, and into Arizona by 1929. It has since become an established major pest of cotton in the Southwest.

The colony transformed at University of California in Riverside originated from the mass reared stock of the Pink Bollworm Rearing Facility, (PBWRF) in Phoenix, Arizona. The origin of the PBWRF stock is from commercial cotton fields located in the Colorado River basin of California and Arizona. The PBW strains maintained in the PBWRF have been in existence since at least

1970. However, the gene stock of the colony maintained in the PBWRF is periodically infused with gene stock from endemic field populations of PBW. The strain selected for transformation was last infused with wild type genes in 1996.

One of the premises of a program employing the Sterile Insect Technique (SIT) is that the mass reared and sterilized insects can compete successfully for mates with their native counterparts. Van Steenwyk (*et al*. 1979) reported that mass reared irradiated PBW males were less competitive than their native counterparts and that mass reared and irradiated PBW females were equal to or more competitive than native females. However, he also indicated that the combined release of both male and female PBW provided a sterile population that was as competitive as native males and females in mating ability. The current PBW-SIT program releases both sexes. Miller *et al*. (1994) reported that native and sterile mating with native males were confined in field stations. The authors also indicated that sterile male PBW entered commercial pheromone traps during the same time interval as native PBW males. Male and female PBW both mate more than once requiring the PBW-SIT program maintain relatively high ratios of sterile to native insects. The dynamics of sterile insect release strategies and its correlation to an insect's mating preferences is discussed by Davidson (1974). The release strategy used in the PBW-SIT program is to release moths season long (average of 160 release day per year) on approximately 25,000 hectares of the 450,000 hectares of cotton planted yearly in the San Joaquin Valley of CA. The objective of the program is to prevent the establishment of PBW moths blown into the San Joaquin Valley on storm systems originating in Mexico and the Southern California cotton growing regions (Staten *et al*. 1992).

The pink bollworm is well adapted to the long growing seasons prevalent in the desert valleys of the Southwest where 5-6 generations are developed each year. Egg to adult development takes 26-32 days during the cotton growing season. PBW diapause (over winter) in the larvae stage in cotton bolls or other field debris or at gins and seed storage facilities. A more detailed description of the life stages of this insect can be found in Noble (1969).

ENCLOSURE E

A detailed description of the purpose for the introduction of the regulated article including a detailed description of the proposed experimental design.

The most important use of a genetically marked pink bollworm will be in evaluation of F1 sterility systems in program use. When irradiation levels are reduced (to 10 KR from 20 KR) limited numbers of sterile progeny reach adulthood and can be expected to increase efficacy of sterile release. More importantly we have a more competitive release moth if irradiation is reduced. By using a genetic marker practical evaluations of F1 progeny can be made from insects which have reproduced in the release fields. This enables more accurate estimates of the numbers of sterile insects needed in a given field. Without genetic markers, unmarked insects must be reacted to with additional sterile releases according to guidelines, thus an F1 moth would require the same treatment of any other unmarked insect. The use of F1 sterility has long been known and was well described by Knipling 1970.

In this test, two release strategies will be used.

1. Standard APHIS PBW irradiated at 10 KR.

2. EGFP irradiated at 10 KR.

Each treatment will be applied from first square cotton maturation stage through a 90 day period by hand release or via mechanized ground release in small 2 acre refugia strips of cotton adjacent to Bt cotton. These fields will have no other contiguous non Bt cotton capable of supporting PBW. Final field selection will be based on actual field distribution of the 2005 crop.

Each field will be monitored for

1. F1 and native larval production in blooms during the first generation and in bolls during generation 2 and 3. Samples will be returned to Phoenix laboratory in containers which prevent any escape.

2. Adult moth populations will be monitored using Delta traps serviced 3 times per week. Released moths will be identified by internal markers and sub sampled for any and all future work using techniques which will preserve them for genetic analysis. All non released moths will be counted and returned to laboratory for future work. All moths returned to the laboratory using protocols developed by Dr. T. Dennehy 2003 attached with references.

3. Mating sequences of native female moths, APHIS female moths and the EGFP moths will be tested with the same general protocols as in previous field cage studies (Staten *et al*. 2001) and (Miller *et al*. permit number 01-029-01R).

ENCLOSURE F

A detailed description of the processes and procedures and safeguards which will be used or will be used in the United States to prevent contamination, release, and dissemination of the production of the donor organism; recipient organism; vector or vector agent; constituent of each regulated article which is a product and regulated article.

This is a permit request for a targeted release of EGFP pink bollworm in substantial numbers within refugia plots in a farm separated by a minimum of 7.5 miles from all other cotton. All cotton except the release plots will be Bt cotton which will not support sustainable or normally measurable larval populations of pink bollworm. All non Bt cotton will receive releases of 10 KR irradiated sterile PBW of either the APHIS or EGFP strains.

EGFP rearing and handling procedures are as follows:

All progeny will come from adults in our Quarantine Facility as cleared in Permit 03-104-01r. All rearing through pupation will be conducted in Quarantine using the same approved procedures as in 03-104-01r. Eclosion of pupae to adults and irradiation will have to be conducted in the PBW rearing facility. Moth eclosion and collection is as follows:

Four day old pupae will be set up to preclude any eclosion prior to filling emergence trays and setting up emergence boxes used for containing the moths prior to collection.

The filling of trays will occur separately with CPHST personnel only. The room will be thoroughly cleaned of any pupae that might have spilled. No EGFP moths from the emergence room will be used for strain propagation.

Isolated dedicated moth collection lines will be used to emerge and collect EGFP moths. These lines will be clearly labeled and color coded.

Quality Control samples will be taken in the Cold Room in vials. The vials will be subjected to a minimum one hour of freezing temperatures to kill all moths before the Quality Control work of weighing and counting occurs. The weighing of bulk moths for irradiation will be done in the 2X Cold Room.

Emergence boxes will be held for nine days on the collection lines. These boxes will be spaced such that they can be easily checked for leakage and handling when the time comes to tear them down for steaming and washing. As each box is removed from a line, a fine mesh screened cap will be placed on the end of it to preclude escapes and allow the steam to freely enter.

Moths to be irradiated will be transported to the 40 degree F irradiation pass-through room in a standard cardboard canister used for irradiation. All containers used for the EGFP strain will be color coded for instant identification. Each canister will be taped shut to prevent escape should it be accidentally dropped.

Irradiation Security

The canisters for immediate irradiation will be placed in the pass-through box and irradiated in turn at 10 KR. Following irradiation it will be passed through to the Packaging Room where they will be put into a dedicated shipping box which will not contain moths from any other strain. Moths will then be transported to their destination via government vehicle.

ENCLOSURE G

A detailed description of the destinations (including final and intermediate destinations).

The EGFP pink bollworm will be released in no more than four 3 acres or less test plots of conventional cotton in a planting of 95% Bt cotton. All non Bt cotton on this farm will be used for release of either sterile APHIS or EGFP moths. The closest adjacent cotton is one planting 7.5 miles from this farm in Pima County of Arizona. (See Enclosure J.)

The entire farm will be trapped to monitor movement. Traps will also be placed along roadways for 5 miles in each accessible direction. Test insects will be transported to the field by CPHST personnel or Arizona Cotton Research and Protection Council personnel working within the framework of our cooperative agreements. All insects will be transported in color coded biomailers. All plots will be sampled for larva with samples returned to Phoenix, Arizona. Boll samples will be held in secure boll boxes in a secure location (double caged).

ENCLOSURE H

A detailed description of proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations.

All PBW sterile insect programs in California, Arizona, New Mexico and Texas will be made completely aware of systems (color coded bio mailer types, etc.) that are to be used and will be expected to act as monitors to ensure that no mixing of insect types occurs. Phoenix Plant Protection Lab personnel will be on location for all early releases.

ENCLOSURE I

A detailed description of the proposed method of final disposition.

Irradiated insects will be irradiated and released as described above in Enclosure E. Surplus insects irradiated for these trials but not released will be destroyed either by freezing or heating.

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APPENDIX II

F_1 Sterility (low dose irradiation) in Pink Bollworm Irradiated with 10 Kilorad $Cobalt_{60}$.

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Summary
The data and literature cited below show that pink bollworm (PBW) moth quality and competitiveness is significantly improved by a lower dose of irradiation [10 kilorad (kr)vs. 20 kr] based on male response to survey traps indicating improved field dispersal. The literature also indicates improved sperm competition in the males. Furthermore, a lower dose of irradiation reduces the man-hours required to irradiate the insects. In 2006, the Phoenix pink bollworm rearing facility will have a 12-14 hr irradiation time which means a two shift operation to irradiate 30 million insects per day at the 20 kr dose. A 10 kr dose reduces this to 6- 7 hours which can be handled in a single shift introducing significant reductions in manpower demands and reliability of the irradiation process. The lower irradiation dose will also lengthen the effective life of the two $cobalt_{60}$ irradiators in the facility which is a significant benefit because the cost of recharging each irradiator is about $200,000.

Inherited Sterility in Lepidoptera

Classic sterile insect technique (SIT) programs deploy sterile males and females and/or males only to reduce and control insect pest populations. The PBW SIT Program in the San Joaquin of California is a successful example of this technology. Because of the unusual chromosomal structure (holokinetic chromosomes) of Lepidoptera, an alternative novel approach to managing PBW populations is possible. This alternate approach is called Inherited Sterility or F_1 Sterility and allows the use of lower doses of irradiation as a means of improving the mating quality of the released moth.

Proverbs (1962) was the first in North America to report on the inheritance of sterility from irradiated males in studies with the codling moth, *Laspeyresia pomonella*. Later, North (1975) reported that inheritance of radiation damage had been observed in about a dozen species of

moths. Knipling (1970) discussed the theoretical advantages produced by releasing partly sterilized males rather than fully sterile males into a population. A lower dose of irradiation results in more competitive males that are better able to mate and transfer sperm while imparting sterility to any of their offspring. La Chance (1985) indicated that all theoretical models comparing this inherited sterility with the full sterility approach have demonstrated that inherited sterility is more effective than release of an equal number of totally sterile insects in reducing target populations. Furthermore, Bloem *et al*. (2001) and Reardon and Mastro (1993) demonstrated in field studies the benefits of using F_1 sterility versus full sterility with both codling moth and gypsy moth SIT programs.

Inherited Sterility in Pink Bollworm (*Pectinophora gossypiella*) (PBW)

Graham *et al*. (1972) in laboratory studies demonstrated that sterility in PBW was inherited when one sex or both of the parent generation were treated with irradiation doses as low as 5 kr. Graham's data showed that a 10 kr dose to both parents resulted in the production of 2.5 normal appearing adult moths per female parent with a normal sex ratio of ca 1:1. When mated as adults (self-crossed), these F_1 moths produced eggs, but none hatched. Based on his laboratory results, he proposed further work in field cages using irradiation doses of 5 to 10 kr to suppress PBW populations.

Flint *et al*. (1977) using greater numbers of moths than Graham in a laboratory study of self-cross mating of PBW moths irradiated with doses ranging from 10 to 17.5 kr found a recovery of 4 F_1 adults per 1,000 irradiated parent females with 20 % containing deformities compared to 2% for the control. Self-crosses of these F_1 adults (parents irradiated with 17.5 kr) produced no viable F_2 eggs. His results with moths irradiated at 10 kr differed from that of Graham. Where Graham reported no egg hatch from F_1 x F_1 mating of parents irradiated with 10 kr, Flint reported a hatch rate of 12.6 %. He did not continue the study beyond the F_1 egg stage. His data also differed from Graham's as he reported a skewed sex ratio favoring males 2:1. Flint attributed the differences in results to strain differences and greater numbers of F_1 moths used in his study.
Cheng and North's (1972) study on the effects of sub-sterilizing doses of irradiation on male PBW moths and on the inheritance of radiation damage by the F_1 using irradiation doses of 15 and 20 kr showed that the distortion of the sex ratio favoring males in the F_1 generation would be an advantage if F_1 sterility were used in the field for control of PBW. Their results also showed that moths irradiated with 15 kr would be a more effective treatment than moths irradiated with 20 kr and that smaller doses should be tested.

LaChance *et al*. (1973) study on the effects of low doses of irradiation on reproduction of male PBW and their F_1 progeny listed the following six general characterizations of Lepidopteran response to irradiation:

1. All species require high doses of irradiation to sterilize both sexes.
2. When P_1 (parental) males are treated with sub-sterilizing doses, the F_1 progeny are more sterile than the P_1 male parent regardless of the radiation dose to the male parent, and the F_1 males are usually more sterile than the F_1 females.

3. The amount of sterility inherited by progeny is far greater when the P_1 males are irradiated than when the P_1 females are irradiated.
4. Among the F_1 progeny produced by P_1 males, the sex ratio is skewed in favor of males and increases with dose. The extent of distortion varies among species.
5. At high doses of irradiation, much of the sterility of P_1 males results from lack of transfer of sperm or from failure of the sperm to fertilize the egg; at sub-sterilizing doses, the F_1 male progeny often fail to produce and transfer normal quantities of sperm.
6. When irradiated males mate with normal females, oviposition is reduced; similar results are often obtained when F_1 males mate with normal females.

One of the objectives of the La Chance study was to determine an optimal dose of irradiation to administer to males that would produce an appropriate level of sterility in the F_1 generation. The dose should also permit the production of enough viable gametes that the genotypes characterizing the released population might be incorporated into the wild population. The theory was to infuse genetic material containing a lethal mutation from the released population into a wild population. Their data suggested that irradiation doses to the P_1 generation of more than 7.5 kr would produce problems with sperm transfer and sterility in the F_1 generation that would prevent the transmission of genetic information beyond that generation. Therefore, their recommendation to accomplish the task of transferring genetic material from the released population to a field population was that the irradiation dose would have to be below 7.5 kr.

However, doses lower than 7.5 kr could create another potential problem, the production of viable eggs by the released females. This is due to the fact that F_1 progeny from irradiated females do not inherit as a high degree of sterility as do males. (LaChance 1973, North and Holt 1968). Also, the sustained season-long release of SIT moths increases the probability that female encounters with males would likely be with colony sterile males or the F_1 from crosses of colony sterile males and the wild females. In other words, most of the males that would be available to the females would be sterilized males or descendants of sterile males. Thus, the general fecundity of the population under pressure from SIT would be expected to decline.

Flint, *et al*. (1974) conducted a field test evaluating suppression of a native PBW population contained in a field cages by continuous release of irradiated PBW treated with either 10 or 20 kr doses of cobalt $_{60}$. His results indicated that moths irradiated with 10 kr were more effective than moths irradiated with 20 kr in suppressing native PBW populations. His results were similar to other studies that showed a 10 kr dose appears to be more effective than 20 kr in controlling PBW populations. A significant improvement in moth quality, i.e improved field fitness (improved mobility and more competitive sperm) of the released insects would reduce the overall costs of PBW SIT programs by reducing the numbers required to get a high ratio of sterile to wild insects (60:1) or reduce the ratio that is needed to acquire a high level of sterility in the field. This could increase the number of acres a given number of sterile moths will protect by reducing the numbers released per acre without impacting the effectiveness of the treatment.

This research with PBW demonstrates that F_1 progeny from irradiated parents are more sterile

than their irradiated parents, a characteristic of Lepidoptera. Although laboratory mating studies indicate higher numbers of adult F_1 progeny are produced by parents irradiated with 10 kr than would be expected from a 20 kr treatment, the 10 kr dose is a viable option for controlling the pest through inherited sterility. The higher the dose of irradiation, the more negative the impact is on the competitiveness of the released insect which in turn reduces the overall effectiveness of the program. The possibility of F_1 offspring from parents irradiated with 10 kr producing a self-sustaining population from self-crosses or out-crosses between F_1 males and native females is highly unlikely.

The mating scenario that is the most promising for producing a self-sustaining population are F_1 females out-crossed to native males, though the probability of this occurring in the field is lower than it would be in a laboratory study for the following reasons:

1. The moths are widely dispersed in the field and in a sterile release environment there is an overwhelming ratio of release moths to F_1 populations and/or wild insects throughout the cotton-growing season.

2. There is a behavioral difference in a mass-reared female's selection of oviposition sites on a cotton plant versus that of the wild female. Native females generally lay their eggs between the calyx and the carpel wall of the cotton boll where they are protected from parasites, predators, and other environmental hazards. But laboratory reared females deposit the bulk of their eggs in the vein junctions of leaves, the terminal end of the plants, and on the stems resulting in higher mortality from parasites, predators and insecticides.

3. Field environmental conditions found in the Southwest are much harsher than those maintained in a laboratory-rearing environment, thus drastically lowering the biologic potential of the released population.

Progeny of irradiated moths would not be common due to the high degree of sterility achieved by this treatment. These "native" catches (F_1 progeny of release insects) would not require increased releases of sterile moths because of the high degree of inherited sterility of such crosses (Miller *et al*. 1984).

An example of reduced biologic potential in the field was illustrated in a study by Miller *et al*. (1984) in which 2.25 million PBW irradiated with 20 kr were put in field cages in a cotton field over a three week period. Subsequent monitoring of the site using pheromone traps and cotton boll examinations produced no evidence of the presence of F_1 progeny as the result of self-cross mating by release moths. Therefore, assuming a 1:1 sex ratio, 1.125 million females produced no detectable F_1 progeny in a field study. The authors had projected a capture of 30 F_1 males in pheromone traps in the cage study based on their laboratory data. In the same report 720,000 PBW moths reared under laboratory conditions and irradiated with 20 kr produced 963 moths of which 59.1 % were visually deformed while other non-visual physiologic damage must also be expected.

Inherited Sterility in Genetically Modified Pink Bollworm (*Pectinophora gossypiella*)

The available information about non-modified PBW strain response to irradiation from field and laboratory studies and theoretical projections indicates that partial sterilization may be a more effective approach to managing PBW pest populations than full sterilization in SIT programs. However, there is the issue of whether the genetic transformation of a PBW strain with a genetic marker changes the insect's radiation sensitivity, thus requiring higher or lower doses of irradiation to produce the same degree of sterility. A study by Miller *et al*. (2002) addressed this issue though they reported only on a full sterilizing dose. The authors reported on the effects of a 20 kr dose of irradiation on a strain of PBW containing an enhanced green fluorescent protein (*EGFP*) marker gene and a normal strain of PBW under laboratory conditions. The results indicated that radio-sensitivity to 20 kr of gamma irradiation of the transformed strain of PBW containing the EGFP genetic marker was similar to its wild type ancestors, the APHIS strain of PBW. However, the data indicated that the reproductive potential of EGFP strain parents treated with a 20 kr dose of irradiation or even untreated was lower that that of the non-modified APHIS strain of PBW due to lower fecundity rates and male and female mating frequencies.

Inherited Sterility vs. Full sterility in a PBW SIT-Eradication Program

There are two major concerns involved in implementing an F_1 sterility strategy in PBW SIT/Eradication. First, will the released PBW population irradiated with a 10 kr dose produce a self-sustaining population, thus adding additional economic damage to the cotton crop? Secondly, how will the program personnel be able to differentiate F_1 of the released population from native insects, since these progeny would not be labeled with the fat soluble red dye used in the released population? This is important to the program because sterile moth distribution over cotton areas under program management is based on sterile to native moth ratios in survey traps.

The Phoenix, USDA, APHIS, Center for Plant Health Science and Technology (CPHST) Laboratory conducted two years of preliminary field studies (Miller, E., unpublished data) where they compared the release of 10 kr moths in commercial cotton fields versus moths irradiated with 20 kr. In 2003, a test was conducted in commercial *Bacillus thuringiensis* (Bt) genetically modified cotton fields with an infield refuge. Fields receiving aerially released moths irradiated with 10 kr were isolated by at least 1/2 mile from fields receiving 20 kr moths. A total of ca. 414,000 (release rate of 250 moths/acre) moths per irradiation treatment were released over a three-week period. Moths receiving the 10 kr treatment carried a genetic marker (orange eye, acquired through conventional means and not genetically engineered), while those irradiated with 20 kr had normal eye color. All released moths were internally marked with a red dye. Since the 10kr moths carried a recessive genetic marker, any F_1 progeny produced by a self-cross mating of the 10 kr release population could be readily identified by their eye color and the absence of the internal dye marker. Following the first release, a PBW heat unit model was used to project the emergence of any adult F_1 progeny from the released population. During the time frame when anticipated eclosion of adult F_1 progeny was expected, survey trap monitoring was increased to five times a week. Random boll samples of 100 bolls per field were collected according to model projections for the presence of 3rd instar larvae in the field. Similar field studies were conducted

in 2004. Changes in the test protocol included ground releases instead of aerial releases of PBW moths and the cotton fields selected for the test were planted with Pima cotton, a non-Bt variety. Instead of the three-week release period, moths were released season long. Total moth releases were also lower with 173,141 (release rate of 250 moths/acre) orange eye PBW moths irradiated with 10 kr released and 224,469 normal eye moths irradiated with 20 kr and released.

Field test results in 2003 showed recapture rates of moths irradiated with the 10 kr treatment were 50 % higher than moths irradiated with 20kr. Furthermore, no F_1 moths were captured in the intensive trapping schedule coordinated in time to detect eclosion of potential F_1 adult progeny produced by self-cross mating of the 10 kr released population. Boll samples also resulted in no detection of larvae carrying the genetic marker. The native PBW population in the test area exploded ca. two weeks following initial releases. During releases, the sterile to native ratio was 7.8:1. However, at the time, the heat unit model projected adult F_1 progeny in the field from released moths, the traps were overloading on a nightly basis with a native:sterile ratio of 47:1. The high number of native females in the field also reduced chances of capturing F_1 by increasing pheromone competition to the traps.

In 2004, males irradiated with 10 kr showed recapture rates 25.8% higher than males irradiated with 20 kr of $cobalt_{60}$. In the 2004 study, two F_1 male moths were captured in our season long trapping survey of the test fields. However, no F_1 larvae were detected in weekly boll samples.

Based on the review of the literature cited herein and coupled with the data from two years of field studies it appears that: 1) Concerns of PBW irradiated with a 10 kr dose establishing a self-sustaining population or producing F_1 progeny numbers that would impart significant damage to the cotton crop are un-founded and 2) the identity issue of F_1 progeny of a released population irradiated with 10 kr of $cobalt_{60}$ would not be factor of concern in a SIT program where detectable native populations exist, as is the case with the Texas, New Mexico, and Mexico PBW Eradication Program. However, before F_1 sterility could be used in the San Joaquin Valley, a dominant genetic marker would have to be incorporated into the program. This is because the CA PBW SIT program is viewed as a preventive program where no established PBW populations reside but the threat of establishment is ever present from the southern desert valleys of California.

The current availability of PBW strains with dominant genetic marker genes, (EGFP and DsRed) would solve the need for a dominant genetic marker. It would allow the San Joaquin SIT program to use the 10kr irradiation dose and thus, benefit from a more sexually competitive insect. It would also provide onsite program managers with a rapid method of discriminating between wild and F_1 insects. This would allow for the most efficient dispersal of released insects in the areas targeted for control of the pest. Genetically marked insects can be distinguished from a native pink bollworm by screening with a fluorescent microscope and/or with polymerase chain reaction (PCR) (Peloquin and Miller 2000).

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APPENDIX III

Pink Bollworm Eradication Plan in the U.S.

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Abstract
An area-wide pink bollworm eradication program, involving growers and state and federal cooperators, has been proposed by the National Cotton Council's Pink Bollworm Action Committee. The program's objective is to eradicate the pink bollworm (PBW) from the infested areas of the U.S., essentially the southwestern portion of the Cotton Belt. Through the coordinated efforts of cotton producer communities, and federal, state, and local entities in the U.S. and Mexico, the plan is to implement the eradication program in three phases. Phase I began in 2001/2002, and consists of the El Paso/Trans Pecos region of west Texas, south-central New Mexico, and northern Chihuahua, Mexico. Phase II, to begin in 2006, consists of cotton-growing areas in southeastern and central Arizona. Phase III, proposed to start in 2008, consists of western Arizona, southern California, and the Mexicali Valley of northwest Mexico. The operational elements of the program include: 1) mapping to identify cotton field locations, acreage, and genotypes, 2) detection by trapping and visual inspection, and 3) control using cultural practices, mating disruption with pheromone, Bt transgenic cotton, sterile moth releases, and minimal insecticide applications. This report provides a summary of the strategic plan and the operational aspects of the PBW eradication program.

Introduction
The pink bollworm (PBW), *Pectinophora gossypiella* (Saunders), was described from larvae recovered from infested cotton bolls in India in 1843 (Noble 1969). It has since become one of the most destructive pests of cotton in many of the major cotton-growing regions of the world. The first reported cotton infestation in North America occurred in 1911 in Mexico, presumably from Egyptian cotton seed shipments (Glick 1967). In the United States, PBW was detected first in Robertson County, Texas, in 1917 (Scholl 1919). By 1926, the pest had spread from Texas through New Mexico and into eastern Arizona, and became a major economic pest of cotton in Arizona and southern California in 1965 (Burrows *et al*. 1982). Conventional insecticides have not provided a long-term solution to the pink bollworm problem (Henneberry 1986).
Considerable amounts of basic biological and ecological information have been accumulated and applied in developing PBW control programs. No single control method is completely

satisfactory. The possibility of combining a number of methods into a single control system appears the most promising approach (Henneberry *et al*. 1980).

Various Methods of PBW Control:
Mating Disruption with PBW Sex Pheromone (gossyplure)
Behavioral insect control by mating disruption with sex pheromone was suggested by Knipling and McGuire (1966). Hummel *et al*. (1973) identified a mixture of the Z,Z- and Z,E-isomers of 7,11-hexadecadienyl acetate as the pink bollworm sex pheromone and proposed the name "gossyplure." Shorey *et al*. (1976) initiated studies to evaluate the mating disruption method, in which the atmosphere of the cotton field was permeated with gossyplure, for PBW control.

Albany International Co., Needham, Massachusetts, developed NoMate-PBW®, a slow release formulation of gossyplure and hexane contained in 1.5 cm lengths of about 200 μ I.D. hollow fibers, sealed near one end (Brooks *et al*. 1979, Brooks and Kitterman 1978). The results of extensive testing in Arizona and southern California indicated substantial reduction in boll infestations and in the need for chemical insecticides for PBW in the NoMate-PBW treated fields (Doane and Brooks 1980). Areawide applications with PBW pheromone in the Imperial Valley of California resulted in curtailing insecticide use and significant yield increases (Staten *et al*. 1983).

Additional evaluations of the effectiveness of control of PBW using pheromones in commercial cotton conditions were made in 1981 (Butler and Henneberry 1982, Butler *et al*. 1983), and in 1982 (Butler and Henneberry 1983). The gossyplure combination used in these studies included the addition of 0.004 kg of permethrin or fenvalerate (AI) per hectare to the polybutene sticker, Bio-Tac, used to adhere fibers to leaves (NoMate-PBW Attact'n Kill). The addition of this small amount of insecticide was shown to enhance the effectiveness of the pheromone by killing male moths that encountered the fiber (Staten and Conlee, U.S. Patent No. 4671010). The small amount of insecticide, in sources that were attractive only to the pink bollworm and widely scattered (one per 2 m^2) through the top of the cotton canopy, did not appear to be a threat to insect predators (Butler and Las 1983).

Hercon Group of Herculite Products, Inc., New York, developed Disrupt®, a slow release system for gossyplure, consisting of three-layer plastic dispensers (0.05 cm^2) with gossyplure concentrated in the center reservoir and the outer layers regulating the release of the pheromone (Kydonieus 1978). The results of field tests of this product in Arizona indicated substantial reduction in boll infestations (Henneberry *et al*. 1981).

Shin-Etsu Chemical Co., Ltd, Tokyo, Japan, developed the PB-Rope®, a high rate, slow release system consisting of a wire-based, sealed polyethylene tube (8") filled with gossyplure (Flint *et al*. 1985). Extensive field trials conducted in the Imperial Valley of California and the Mexicali Valley of Mexico indicated a substantial reduction in boll infestations and insecticide applications in the PB-Rope treated fields, compared with that in conventional insecticide-treated fields (Staten *et al*. 1987). Community-wide application of the PB-Rope in the Coachella Valley of California, at the pinhead square growth stage, provided a highly effective level of control of PBW for approximately sixty days, and insecticide usage was drastically reduced or even

eliminated in some fields (Staten *et al*. 1988).

Area-wide, timely application of commercial formulations of gossyplure in the Parker Valley of Arizona, demonstrated the feasibility of suppressing PBW infestations to a near zero level in four years, and conceptualized the prospect of eradication (El-Lissy *et al*. 1993, Staten *et al*. 1995, and Antilla *et al*. 1996).

Bt Transgenic Cotton

Genes from the bacterium *Bacillus thuringiensis* (Bt) that produce the Cry1Ab or CrylAc proteins that are toxic specifically to lepidopterous insect species were inserted into cotton plants by Perlak *et al*. (1990). Several field tests of Bt transgenic cotton indicated a high degree of efficacy against lepidopterous insect pests (Wilson *et al*. 1992, Mahaffey *et al*. 1994, and Benedict *et al*. 1996). In particular, Bt cotton provided an exceptionally high level of season-long control of pink bollworm (Flint *et al*. 1995, Watson 1995, and Flint and Parks 1999). Bollgard® Cotton (Monsanto Technology LLC, St. Louis, Missouri), was the first Bt transgenic cotton, commercially released in the U.S. and other cotton-growing countries in 1996. In the first growing season of commercial Bt cotton, U.S. growers planted approximately 1.6 million acres, which represented 14 percent of the total cotton acreage (USDA, 1999). In 1997, about 25 percent of U.S. cotton acreage, approximately 3.4 million acres, was planted to Bt cotton (USDA, 1999). In Arizona, where PBW is a key pest, approximately 60 to 70 percent of the Upland cotton acreage was planted to Bt cotton in 1997 (Silvertooth, 1998), and 70 percent in 1998 (Patin *et al*. 1999).

Despite early concerns regarding potential development of PBW resistance to Bt cotton (Bartlett 1995, Watson 1995, and Patin *et al*. 1999), evaluations of Bt cotton in 1995 through 2000 indicated that this cotton continues to provide a high degree of season-long efficacy against PBW, irrespective of the suggested reduction in the amount of toxic protein in fruit tissues late in the season (Henneberry *et al*. 2001).

Sterile Insect Technique (SIT)

As early as 1937, E. F. Knipling had conceived of an approach to insect control in which the natural reproductive processes of the screwworm fly are disrupted by chemical or physical mechanisms, thus rendering the insects sterile (Knipling 1985). Sterile insects are released into the environment in very large numbers (10 to 100 times the number of native insects) in order to mate with the native insects that are present in the environment. A native female that mates with a sterile male will produce infertile eggs. Since there are 10 to 100 times more sterile insects in the population than native insects, most of the crosses become sterile. As the process is repeated, the number of native insects decreases and the ratio of sterile to native insects increases, thus driving the native population to extinction (Knipling, 1979). This unique insect control method is known as the sterile insect technique (SIT), or the sterile insect release method (SIRM).

One of the most successful SIT programs involves the pink bollworm in the San Joaquin Valley of California (Staten *et al*. 1993). This cooperative grower-state-federal effort began in 1968. Sterile pink bollworm adults, produced at the PBW rearing facility in Phoenix, Arizona, have

been released each day of the cotton-growing season on approximately one million acres of cotton. This program has proven successful in preventing the high populations of PBW occurring in the adjacent regions of southern California, Arizona, and northern Mexico, from becoming established in the San Joaquin Valley (Staten *et al*. 1993).

Cultural Control

Cultural practices affecting the survival of pink bollworm have been extensively investigated and found to have an important role in reducing overwintering populations. Adkisson *et al*. (1960) reported more than 80 percent reduction in moth emergence from fields that had been shredded and plowed. Diapausing larvae overwinter in immature cotton bolls, trash, and soil (Bariola 1984). The removal of late-season immature cotton bolls is a viable option to reduce the overwintering population (Kittock *et al*. 1973). Cultural control techniques that include shredding stalks, disking, plowing, and winter irrigation have been shown to result in high levels of mortality of diapausing larvae in bolls , trash, and soil (Watson 1980).

PBW Distributions in the United States

A two-year PBW adult detection survey was conducted in Arkansas, Louisiana, Oklahoma, Texas, and New Mexico in 2000 and 2001. PBW delta traps baited with 4.0 mg of gossyplure, were placed around cotton fields at a density of one trap per 640 acres, in the first week of August (first week of July in South Texas), and inspected weekly through the month of October. Preliminary analysis indicated that no PBW were present in Arkansas, Louisiana, Oklahoma, and most of Texas. PBW populations appear to be confined to west Texas and south central New Mexico. This was confirmed through additional trapping surveys in 2002-2004. Trapping surveys conducted in Arizona by the Arizona Cotton Research and Protection Council, and in California by Imperial Valley Commissioner of Agriculture and California Department of Food and Agriculture, continue to indicate wide distributions
of PBW in the entire state of Arizona and Southern California.

Economic Importance of PBW in the United States

Control costs for PBW in Southern California and Arizona were estimated to exceed $1.2 billion over the past thirty years (Roberson *et al*. 1998, Antilla *et al*. 1999). Yield losses caused by PBW ranged from $85-$170 per acre (Antilla *et al*. 1999). Most recently, the National Cotton Council estimated that U.S. cotton producers' annual losses to pink bollworm are about $21 million due to prevention, control costs and lower yields due to plant damage (NCC, 2001).

The Bilateral PBW Eradication Plan:

In its annual meeting on October 9-10, 2000, in El Paso, Texas, the National Cotton Council's Pink Bollworm Action Committee recommended launching a "bilateral" PBW eradication program in the United States and northern Mexico.

The plan includes coordinated efforts by cotton producer communities and federal, state, and local entities in the U.S. and Mexico to combat and eliminate the PBW from cotton-producing regions of West Texas, New Mexico, Arizona, California, and northern Mexico. Pending grower approval through scheduled referenda, adequate funding and PBW rearing capacity, the plan is to implement the PBW eradication program in three phases: Phase I in 2001/2002, Phase II in 2006,

and Phase III in 2008 (Figure 1).

Phase I
Consists of the El Paso/Trans Pecos region of West Texas, south-central New Mexico, and northern Chihuahua, Mexico. The El Paso/Trans Pecos region includes approximately 55,000 acres of cotton in Brewster, Crane, Crockett, Culberson, El Paso, Hudspeth, Jeff Davis, Loving, Pecos, Presidio, Reeves, Terrell, Ward, Winkler, and Val Verde counties. The south-central New Mexico region includes approximately 26,000 acres of cotton in Doña Ana and Luna counties. The northern Chihuahua region includes approximately 80,000 acres in Juarez, Acension, Janos, Ojinaga and the surrounding cotton-growing areas. The plan was designed to begin the program in the El Paso/Trans Pecos region in 2001, and in south-central New Mexico and northern Chihuahua in 2002. In 1999, cotton growers in the El Paso/Trans Pecos region had approved the initiation of a combined boll weevil and pink bollworm eradication program. Boll weevil eradication began in 1999, and pink bollworm operations in 2001. In 2002, producers in south central New Mexico approved the PBW referendum to start the program in the same year. Also at that time, growers in the state of Chihuahua in Mexico had approved a referendum to begin the program in 2002 as well.

Phase II
Consists of approximately 220,000 cotton acres in southeastern and central Arizona, including Cochise, Graham, Pima and Maricopa counties. The plan is to begin Phase II of the **program** in 2006.

Phase III
Consists of approximately 120,000 acres of cotton in western Arizona and Southern California. This includes Mohave, La Paz, and Yuma counties of Arizona, and Riverside and Imperial counties of California. The plan is to begin Phase III in 2008.

Materials and Methods:
Embracing the integrated pest management (IPM) concept, the operational success of the area-wide PBW eradication program hinges on three separate, yet interdependent, components including: *mapping, detection*, and *control*.

Mapping
Mapping is one of the first phases of operation implemented in the eradication program. In addition to identifying the exact location and the surrounding environment of each cotton field, another important purpose of mapping is to record and verify the cotton varieties, including Bt, non-Bt, and long-staple planted in each field. All cotton fields are mapped using the differentially corrected Global Positioning System (GPS) (El-Lissy *et al*. 1996). The program uses a numbering system that is designed to identify each cotton field in the eradication zone with a unique number.

Detection
Trapping

Pink bollworm delta traps are used as the primary tool of detection. Traps are baited with rubber septa impregnated with 4 mg of gossyplure and attached with brass fasteners to a wooden stake placed around the perimeter of each cotton field. Traps are placed at planting, or shortly thereafter, at a rate of one trap per ten acres and inspected weekly until defoliation and harvest, or a killing freeze (Leggett *et al*. 1994).

Visual Inspection (Scouting)
Beginning at the bloom stage, ten randomly selected conventional cotton (non-Bt) fields per work unit (12,000 -15,000 acres) are inspected weekly for rosetted blooms. Weekly larval surveys in bolls are conducted at the boll formation (quarter size) stage and continue through cut-out.

Control:
The control part of the eradication program consists of cultural control, mating disruption, Bt transgenic cotton, sterile moth releases, and chemical control.

Cultural Control
Uniform cotton planting and harvesting, done during timeframes recommended by the local Agricultural Extension Service, are highly encouraged, as they constitute an important strategy in providing a host-free period. Other cultural practices, including timely defoliation and stalk destruction, off-season irrigation, and burial of crop residues through normal tillage practices will continue to play an important role in reducing diapausing populations during the off-season months.

Mating Disruption (pheromones)
Aerial, ground, or hand application of pheromone is made only to conventional cotton fields (non-transgenic), or to Bt transgenic cotton fields imbedded with conventional cotton (95:5 embedded refuge), that meet the predetermined treatment threshold. A single application of NoMate-PBW®, at a rate of 15 gm/ac (1.05 gm [AI]/ac of gossyplure), mixed with
polybutene sticker (Bio-Tac) at a rate of 5.3 oz/ac and the insecticide permethrin at a rate of 0.5 fl oz/ac (0.08 lb. [AI]/ac), is made by air, each time a field meets the treatment criteria (treatment threshold). Fields meet the treatment threshold beginning at the six-node (prior to pinhead square) growth stage and when trap captures average more than zero and less than one moth per trap per night. The insecticide chlorpyrifos, at a rate of 24 fl oz/ac (0.75 lb. [AI]/ac), may be added to the pheromone application as an over-spray (doubleton application), only if the average trap capture equals or exceeds one moth per trap per night. The PB-Rope or PB-Rope* L, or equivalent formulations, may be used in the earliest planted cotton fields, in fields with a high level of moth catches, as well as in fields located near sensitive sites where aerial applications are not practical. Dispensers are hand twist-tied around the main stem of the cotton plant near
the bottom at or near six true leaf growth before the pinhead square. The PB-Rope (or equivalent) dispensers are evenly applied at a density of 400 dispensers (28 gm/ac) per acre, and the PB-Rope* L (or equivalent) dispensers are applied at a density of 200 dispensers (28 gm/ac) per acre.

Bt Transgenic Cotton
Planting of the Bt transgenic cotton varieties is highly encouraged as they provide an exceptional level of control for pink bollworm. The program maintains full compliance with the Environmental Protection Agency's (EPA) Refuge Requirements, designed as a strategy for insect resistance management (IRM).

Sterile Moth Releases
Pink bollworm sterile moths produced in the PBW rearing facility in Phoenix will be aerially released at a rate of 100 moths per acre per day, beginning at the four-leaf growth stage and until defoliation or harvest. Sterile moths will be released on all cotton fields in the **eradication** region including Bt transgenic and conventional cotton. This component is particularly important as a final control measure to achieve eradication.

Chemical Control
Aerial or ground applications of the insecticide chlorpyrifos at a rate of 24 fl oz/ac (0.75 lb. [AI]/ac), may only be made to prevent economic loss in fields that exhibit larval infestations of 5 percent or higher.

Discussion
The pink bollworm continues to seriously affect western cotton-growing regions that are critical for the export of fiber, and production of seed for the entire U.S. Cotton Belt. The **eradication** of the pink bollworm will provide significant economic gains for cotton producers through lower production costs, higher yields, and better quality of fiber. An additional benefit of eradication will be its positive effect on the environment through significant reductions in pesticide usage.
The pink bollworm eradication program utilizes a more diverse blend of control methodologies than has been used in other successful area-wide eradication programs. The incorporation of an unprecedented number of highly effective control methods, simultaneously implemented within a harmonized system, maximizes the opportunity to achieve the goal of eradicating one of the oldest and most destructive cotton pests in the world.

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APPENDIX IV

Development of a Genetically Engineered Pink Bollworm

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A. Transformation system

The transformed pink bollworm (PBW) strain produced at the University of California, Riverside (UCR), originated from the mass-reared "C" stock of the Pink Bollworm Rearing Facility, (PBWRF) in Phoenix, AZ. The origin of this PBWRF stock is from commercial cotton fields located in the Colorado River basin of California and Arizona. The PBW strains maintained in the PBWRF have been in existence since at least 1970. However, the colonies are periodically outcrossed with endemic US field populations of PBW. The parental strain that was transformed was last outcrossed with wild-type PBW in 1996. All final engineering of the transforming constructs were performed at UCR. Of the transgenic PBW strains produced by UCR scientists (Peloquin *et al.* 2000), one strain (#35) was transferred to the APHIS, Plant Protection Laboratory in Phoenix, Arizona under USDA/APHIS permit No. 98-244-02m for movement of transformed insects between laboratories in Riverside and Phoenix.

The genes used from the donor organism and the *piggyBac*-derived portions of the vectors used to build the transforming construct were cloned off site. Specifically, *Escherichia coli* was the immediate host for the plasmids carrying the cloned genes used to make the transforming constructs. Plasmids are circular, replicating DNA molecules. They are routinely propagated and amplified in weakened laboratory strains of *E. coli*. When the plasmid DNA is purified from *E. coli* cultures grown for the purpose, essentially no bacterial protein or chromosomal genetic material remains associated with the plasmid; no such material was introduced to nor presently exists in the transgenic pink bollworm strains generated using with the DNA constructs. The *piggyBac* transposable element was discovered in a cabbage looper cell culture at the University of Notre Dame (Fraser *et al*. 1995, Fraser *et al*. 1996, Wang and Fraser 1993). The *Bombyx mori* actin A3 promoter was cloned and modified by Steve Thibault at the University of California, Riverside (UCR) from the embryos of the silk moth *Bombyx mori,* purchased from Carolina Biological Supply Company. In the silk moth, this promoter controls the expression of a cytoplasmic actin gene. Cytoplasmic actin is a relatively abundant protein, present in essentially every cell. It was, therefore, anticipated that this promoter could be used to express another protein, for example green fluorescent protein (GFP, see below), at a reasonably high level in most or all cells of the silk moth and, by extension, in other moths, for example pink bollworm.

B. Green fluorescent protein and *piggyBac*:

The well-established ability of GFP and its derivatives to function as dominant, visible, nondestructive markers of insects (e.g. Brand 1995), mammalian (e.g. Pines 1995), and plant systems (e.g. Haseloff *et al*. 1997) were indicators of its potential use in PBW. The gene encoding GFP ("*GFP* gene") was cloned by Prasher, USDA, APHIS, Otis AFB, MA, from the jellyfish, *Aequora victoria* (Cubitt *et al*. 1995, Heim *et al*. 1994, Heim and Tsien 1996, Prasher and Eckenrode 1992, and Prasher 1995). The best-known derivative of GFP is a modified version with improved green fluorescence under blue light and a reduced tendency to form insoluble aggregates. One such improved version is commonly known as enhanced green fluorescent protein (EGFP). The plasmid source of EGFP was purchased from Clontech, Inc. (now BD Biosciences Clontech, a wholly owned subsidiary of Becton, Dickinson and Company). Previous plasmid-based mobility assays had shown that the mobilization of donor *piggyBac* transposon is

induced in the presence of exogenous transposase, while no mobility was seen in the absence of exogenous transposase (Thibault *et al*. 1999). Together with the previous history of successful transformation of several insect species with *piggyBac*-based genetic transformation systems, it was inferred that such a system could be a suitable for genetic transformation of pink bollworm. Therefore, a *piggyBac*-based vector was constructed containing EGFP as a marker for transformation.

The *piggyBac* element is a deoxyribonucleic acid (DNA) transposable element capable of integrating into other DNA through mediation of a transposase encoded by a transposase open reading frame (ORF) within the element, but only when its inverted terminal repeats (ITR) are intact. In the construct used for transformation of the PBW, the transposase gene of the *piggyBac* element was destroyed by insertion of an expression cassette containing EGFP ORF driven by a single copy of the *Bombyx mori*-derived BmA3 promoter. In the process, a substantial proportion of the region of the *piggyBac* element that originally encoded the transposase was deleted. This manipulation destroys the ability of the transformation construct to move on its own. Transformation was done by co-injecting a transposition and integration incompetent helper plasmid along with a donor plasmid into early stage PBW embryos. The donor plasmid contains the transforming construct flanked by *piggyBac* ITRs. The helper plasmid encodes an intact *piggyBac* transposase ORF. The gene product of this *piggyBac* transposase ORF is under the control of a promoter that directs insect cells to express *piggyBac* transposase after injection. Importantly, the helper plasmid does not have the necessary pair of *piggyBac* ITRs. These ITRs are absolutely essential for *piggyBac* transposase mediated integration. Therefore, the helper plasmid, lacking one or the other of the ITRs, cannot integrate itself into target DNA in a transposase-mediated event. This approach has been used to transform a variety of insect and other species with *piggyBac*-derived vectors, and also with equivalent vectors based on other transposable elements.

The lack of mobility of *piggyBac* elements in the absence of exogenous transposase (Thibault *et al*. 1999) suggests that *piggyBac* elements are not mobile in pink bollworm, unless provided with *piggyBac* transposase. The potential for instability and unwanted mobilization of *piggyBac*-derived transforming constructs was further addressed in respect of the EGFP transgenic line. Stability of EGFP transgene was examined in a total of ~50 individuals from the 58th generation of the EGFP transgenic strain. Genomic Southern hybridization and insert-site specific polymerase chain reactions (PCR) indicated that the original insertion described in Peloquin *et al.* (2000) has been highly (completely) stable; no evidence of mobilization during the 58 generations was detectable (Park, unpublished data). A simultaneous effort in the risk assessment was a survey of *piggyBac*-like elements (PLE) in various strains of PBW. Surprisingly, multiple copies of a PLE that is distantly related to the *T. ni piggyBac* (56% similarity and 40% identity of the encoded transposase proteins) were found in the PBW populations, including the mass-reared "C" stock of the Pink Bollworm Rearing Facility that is the strain that provided the genetic background for the EGFP line. However, as noted above, previous transposition assays indicated that mobilization of *T. ni piggyBac* in pink bollworm requires exogenous *T. ni piggyBac* transposase activity (Thibault *et al*. 1999). Therefore, these results suggest that mobilization of a

transgene based on a *T. ni piggyBac*-derived vector by the transposase of an endogenous PBW *piggyBac*-like element is highly unlikely.

C. Molecular characterization of engineered pink bollworms:

Insertion of the *piggyBac* element into genomic DNA was detected by Southern blot analysis using one of the positive lines. The presence of at least two insertions was detected in this line with the probe recognizing two bands of approximately 1.9 kb and 2.3 kb. Individuals examined contained either one of the inserts, or both. Based on inverse PCR, the *piggyBac* integration appears to have been a singular event which occurred in a transposase-dependent manner resulting in the expected TTAA target site duplication, with no plasmid sequences flanking the transposon ends. Immunoblot analysis using a green fluorescent protein-specific antibody was also used to differentiate expression of EGFP from autofluorescence in wild-type animals and establish that the EGFP protein produced was the expected size showing that no additional sequence was being translated into protein fused to the EGFP.

The helper plasmid contained a *piggyBac* transposase gene driven by the *Drosophila* hsp70 heat-shock promoter instead of the endogenous *piggyBac* promoter. The endogenous *piggyBac* promoter was removed, along with one of the ITRs, and replaced by the *Drosophila* hsp70 promoter. The complete *EGFP*-containing element is 2.6 kb in length. Construction of the vector resulted in deletion of approximately 1 kb within the original *piggyBac* transposase open reading frame, resulting in inactivation.

D. Stability of genetic integration

The enhanced green fluorescent protein (EFGP) positive lines were maintained as heterozygotes by serial backcrosses to the wild-type strain. At the time of backcross analysis, the lines had been backcrossed for four generations. This would likely separate any transformed loci that were not tightly linked. Thus, the EGFP-positive parental insects used in the diagnostic backcrosses were expected to be heterozygous for a single copy of the gene. At the time of backcross analysis of the heterozygote lines, the first line produced 191 positive and 207 negative progeny and the second line produced 555 positive and 616 negative progeny. These were not significantly different from the expected 1:1 ratio by χ^2 statistical analysis. Therefore, a relatively close 1:1 ratio of EGFP versus wild-type supports the hypothesis that EGFP was transmitted as a single-locus, dominant gene. This observation has been also confirmed by genomic Southern hybridization in the 58th generation that revealed only one 2.4 kb band for the insertion that was described for the original EGFP transgenic line (Peloquin *et al*. 2000, Park, unpublished data).

E. Fitness compared to wild-type

The stability of the gene was demonstrated further by the rearing at the Phoenix Quarantine Facility of 30 generations of EGFP strain PBW with no visual evidence of change of the EGFP gene. This study found no differences in length of time spent in larval instars, and the pupal stage in EGFP PBW compared to non-transformed PBW. However, the EGFP female moths produced 19.8 % fewer eggs than non-transformed PBW and their successful egg hatch rate was 26% lower (Miller *et al.* 2001).

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APPENDIX V

Uses of GFP in Biology and Medicine

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Cnidarian fluorescent proteins- for example GFP from *Aequorea* jellyfish, DsRed from *Discosoma* coral (Ip and Wan 2004) and Orange Fluorescent Protein from *Cerianthus* tube coral (Shaner *et al.* 2004)- have revolutionized biology by acting as specific fluorescent vital stains. Because they are relatively non-toxic, these fluorescent proteins can be expressed at sufficiently high levels within transgenic cells to be non-destructively located and act as markers. They are extremely flexible in application as they can be expressed alone or as a fusion protein thereby marking the subsequent fusion protein with a fluorescent moiety. This provides unparalleled specificity in targeting a molecule, macromolecular structures, organelles, cells, tissues or whole organisms. Hence fluorescent proteins are increasingly widely used in medicine, basic biological studies, molecular biology, biotechnology and agriculture. A recent literature search (May, 2005) of the Biosis Previews™ published biological literature database using search terms green fluorescent protein and red fluorescent protein returned over 15,000 records of published documents and books demonstrating their widespread use as biological markers in organisms from all kingdoms. This use includes bacteriophage, viruses, bacteria, algae, flowering plants, nematodes, insects, mammals and human cells. The most common categories from studies reporting use of GFP are vertebrates, mammals, humans (cell cultures), plants, bacteria, fungi, and insects. While the results of the Biosis survey do not provide a quantitative accounting of all uses of GFP and DsRed, the survey shows that the use of fluorescent proteins is well incorporated into most all branches of biology, medicine and agriculture.

Perhaps the most spectacular use of GFP is in the generation of transgenic organisms as in the stable GFP germ-line transformation of mice, frogs, nematodes, flies, bacteria, and plants. Chalfie *et al*. (1994) were the first to make GFP transgenic animals, *C. elegans*. GFP's first use in vertebrates (mammals) was in 1995 (Ikawa *et al*. 1995) when they created transgenic GFP mice using a CMV enhancer/-actin promoter. GFP-marking of transgenics has been applied to numerous other organisms (Biron 2003). GFP and similar fluorescent proteins are established as a marker for transgenesis because they enable non-destructive, visual identification of transgenic versus non-transgenic organisms. GFP's fluorescence is due to a chromophore autocatalyticaly created within the protein. The chromophore results from post-translational cyclization, dehydration, and oxidation of three residues, Ser65-Tyr66-Gly67 (Cody *et al.* 1993).

Some fluorescent protein applications involve the intentional release into the environment of organisms genetically modified to express GFP, DsRed or related proteins indicating widespread acceptance that organisms modified to produce these proteins pose little risk to the environment. Examples of these uses include transformation markers for the development of genetically modified agricultural plants and animals (Brandizzi *et al*. 2002), as a marker for detection of

harmful bacteria on food products, the detection of bacteria in sewage, as marker of human and animal cells, proteins and genes for studies of disease, for genetic testing, drug development, and for basic research of animal, plant, microbes and fungi (Chalfie, 1995, Chalfie *et al.* 1994, Chiu *et al.* 1996, Christensen *et al.* 1996, Cruz *et al.* 1996, Eberl *et al.* 1997, Epel *et al.* 1996, Flotte *et al.* 1998, Garamszegi *et al.* 1997, Grebenok *et al.* 1997, Grossman *et al.* 2001, Harding *et al.* 1997, Ikawa *et al.* 1995, Kaether *et al.* 1997, Kalejta *et al.* 1997, Leff and Leff 1996, Lo *et al.* 1998, Misteli and Spector 1997, Niswender *et al.* 1995, Pesnyakevich and Lyon 1998, Pines 1995, Ramiro *et al.* 1998, Skillman *et al.* 1998, Stewart *et al.* 2000, Zhang *et al.* 1996).

Use of Fluorescent proteins as reporter genes in agricultural crops is common (Eady *et al.* 2005, Aboul-Soud *et al* 2004, Zhang *et al* 2001, Arazi *et al* 2001, Tombolini *et al.* 1999).
APHIS reports 58 approved permit requests for field testing organisms with GFP in the U. S since 1997 (http://www.isb.vt.edu) and no adverse incidents or escapes have been reported. GFP has been field tested for use in the environment as a marker to monitor the movement of pollen of transgenic plants (Harper 1999) or as a transformant selection marker to replace previously used antibiotic resistance transformant markers. This change from antibiotic resistance as a marker to a fluorescent protein marker is an improvement in the safety of transgenic plants. Fluorescent protein markers should not confer any selective advantage that could increase the invasiveness of a crop or a non-target species that acquired the gene unlike antibiotic resistance genes as selectable markers. In addition, it is highly unlikely that horizontal transmission of these genes would occur as a result of predation etc. Vertebrates (*e. g.* fish and turtles) prey and have preyed upon Cnidarians that produce GFP and other fluorescent proteins for millions of years. But GFP (or any other) fluorescence is not known from Salmon and sea turtles that prey upon jellyfish. This strongly suggests that GFP genes would not be transferred from GFP-positive prey to such predators.

GFP is neither a novel nor uncommon protein as it and other fluorescent proteins have likely been present in Cnidarians in the ocean environment for at least 100 million years. It is not a novel transgene in the environment either, as there has been at least one free environmental release of recombinant GFP. Such an important release into the environment was with transgenic or transformed bacteria used for biotechnological applications. In a field demonstration in October 1998 in South Carolina, Prof. Burlage and his ORNL colleagues Martin Hunt, Steve Hicks, Mike Maston, Mike Keleher, and Keith Williams (all of the Instrumentation and Controls Division of ORNL) successfully demonstrated a GFP transgenic microbial technique to detect five out of five simulated landmine targets in a 300 M^2 field. The researchers sprayed bacteria that had been transformed with a recombinant DNA construct so these bacteria would express GFP only in the presence of TNT explosives. Land mines containing TNT were placed in the field weeks previous to the application of the bacteria onto the soil during the trial. Where TNT was present the bacteria would fluoresce in the characteristic GFP wavelengths allowing the detection of the buried munitions. Transgenic microbial mine detection is much closer to commercialization than we anticipated:

http://www.ornl.gov/info/ornlreview/v32_2_99/green.htm

That this study was done and no untoward nor unexpected effects nor ectopic expression of GFP were detected from the release of GFP transgenic microbes strongly suggests that releases of GFP-transgenic and radiation-sterilized insects would be highly unlikely to present any risk to predators or saprophytic organisms that would feed upon GFP transgenic PBW or other GFP transgenic insects. Additionally, no other significant environmental effects should be expected.

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APPENDIX VI

EGFP Pink Bollworm Rearing and Handling Procedures

To ensure that no cross contamination occurs in rearing the following procedures have been prepared by F. D. Stewart:

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Building

The doors to all rooms that involve EGFP will be secured by a double security door or temporary "soft" sealing which will allow for emergency egress.

Oviposition

1. Individual oviposition cages will be taped shut to prevent accidental spillage.
2. A large screened isolation cage with a double screened door will be installed around a section of cages in the oviposition Room that is now in use (1X).

Incubation of Eggs
1. We will use a dedicated incubator for the EGFP strain.
2. For security reasons the incubator will be locked until eggs are needed. Only the oviposition and egg treatment personnel will have access to the key.
3. The egg pads will be in secured paper bags and should only be opened immediately prior to implant.

Egg Treatment
1. Treatment of eggs will follow the same SOP that is currently in place.
2. In the sequence of sterilizations the EGFP eggs will be done last each day.
3. Individual eggs that have become detached from the egg pads will be rinsed or otherwise removed from the walls of the formaldehyde tanks after each separate sterilization. The walls of the tank should be checked for the presence of eggs immediately before the next sterilization.
4. Isolation should be arranged for the drying of the GFP egg pads on the drying racks. It is important to preclude larvae of any group from being placed/transferred into a bag which will later be used for infest. All cross contamination will be avoided.
5. Prior to placing egg pads in a bag, the bag will be checked for tears or other damage or conditions which may allow larvae to escape.
6. The bag opening will be folded shut with three folds and stapled shut to prevent any larvae from escaping from the bag.
7. Bags will then be transported to the dedicated incubator.

Implant Sequence
1. Production strain.
2. EGFP strain.

Implant Security
1. Egg pads should not be removed from the bag for infest until all loose eggs are removed/destroyed from the surface where they are placed in the form-fill machine.
2. Once a strain of insects is implanted for the day, all the empty cells should be blown out with pressurized air to remove any eggs that might have fallen into them.
3. Different strains will be isolated on their own cart each day.

Dark Room and Cutout Incubation and Pupal Stripping
The 2X Cutout Room will be partitioned in proportion to the fan coil units to accommodate larval and pupal, and incubation and harvesting of pupae. The west door will be closed off and all openings sealed off to prevent larval egress.

Dark Room Area
This will consist of the west two-fifths of the room and will have within its boundaries 4 fan coil units, two on each side. The partition will consist of dark cloth or black plastic hung from a height that will keep out light. The overhead bulbs will be removed to help provide the dark conditions.

Rearing will be at 86 degrees F.

Cutout Room Area
This area will also consist of two-fifths of the entire room and have 4 fan coil units within. Lights will be left on to simulate moonlight conditions. Pupae will be transported in containers with secured covers to prevent accidental spillage to both the oviposition room and the moth collection room.

Pupal Incubation and Harvest Area
These areas will reside in the eastern one-fifth of the room. The entrance will have a double entrance, the inner door chamber will be sealed to preclude larvae and any moths from leaving or entering the room. The doors will have custom seals to prevent moths from escaping.

Before 8 a.m. every morning, the carts that are to be removed from the room will be cleaned with a blower within the room to remove all remaining larvae from the rack; then the rack will be rolled outside and placed in the solar/steam kill room where it will remain for 8 hours at a temperature exceeding 200 degrees F.

All larvae and pupae that fall on the floor will be concentrated with electric blowers and vacuumed up and the contents will be placed in a plastic bag within a paper bag to be frozen for a minimum of 48 hours, or autoclaved, or placed in the solar/steam chamber mentioned above.

Four-day old pupae will be collected and transported to the oviposition room in closed containers. Quality control on pupae will begin with pupae collected in the EGFP cutout area, the sampling containers are sealed and then transported to the QC lab where they will be weighed and counted and then destroyed by submersion in water or autoclaving.

Moth Collection
Four day old pupae will be set up to preclude any eclosion prior to filling emergence trays and setting up emergence boxes used for containing the moths prior to collection.

The filling trays will occur after all other strains have been processed for moth collection. The room will be thoroughly cleaned of any pupae that might have spilled. No EGFP moths from the emergence room will be used for strain propagation.

Isolated moth collection lines, and a cold room will be used to emerge and collect EGFP moths. This will be done in the separate 2X moth collection room where no other insects will be held.

Quality control samples will be taken in the cold room in vials. The vials will be subjected to a minimum one-hour freezing to kill all moths before the quality control work of weighing and counting occurs. The weighing of bulk moths for irradiation will be done in the 2X cold room.

Emergence boxes will be held for nine days on the collection lines. These boxes will be spaced such that they can be easily checked for leakage and handling when the time comes to tear them down for steaming and washing. As each box is removed from a line, a fine mesh screened cap

will be placed on the end of it to preclude escapes and allow the steam to freely enter.

Moths to be irradiated will be transported to the 40^0 F irradiation pass-through room in a standard cardboard canister used for irradiation. All containers used for the EGFP strain will be color coded for instant identification. Each canister will be tacked shut to prevent escape should it be accidentally dropped.

Irradiation Security

The canisters for immediate irradiation will be placed in the pass-through box and irradiated in turn at 10 kr. Following irradiation, the insects will be passed through to the packaging room where they will be put into a dedicated shipping box which will not contain moths from any other strain. Moths will then be shipped to their destination.

APPENDIX VII

Threatened and Endangered Species Analysis

The following table is the current threatened and endangered species list for Pima County, AZ. Upon review of the nature of the species, their habitats, and other comments concerning their distribution, none of the threatened and endangered species of Pima County are expected to incur any risk or jeopardy by EFGP genetically modified PBW that have been treated with 10 kr radiation. In addition, these threatened and endangered species are not expected to frequent agricultural fields where cotton is grown.

Threatened and Endangered Species List for Pima County, AZ (From: http://www.fws.gov/arizonaes/Documents/CountyLists/Pima.pdf)

Common Name	Scientific Name	Status	Description	County	Elevation Range	Habitat	Comments
Bald eagle	*Haliaeetus leucocephalus*	Threatened	Large, adults have white head and tail. Height 28-38"; wingspan 66-96". 1-4 yrs dark with varying degrees of mottled brown plumage. Feet bare of feathers.	Apache Cochise Coconino Gila Graham La Paz Maricopa Mohave Navajo Pima Pinal Santa Cruz Yavapai Yuma	Varies	Large trees or cliffs near water (reservoirs, rivers, and streams) with abundant prey.	Some birds are nesting residents while a larger number winters along rivers and reservoirs. An estimated 200 to 300 birds winter in Arizona. Once endangered (32 FR 4001, 03-11-1967; 43 FR 6233, 02-14-78) because of reproductive failures from pesticide poisoning and loss of habitat, this species was down listed to threatened on August 11, 1995. Illegal shooting, disturbance, and loss of habitat continues to be a problem. Species has been proposed for delisting (64 FR 36454), but still receives full protection under the ESA.

Cactus ferruginous pygmy-owl	*Glaucidium brasilianum cactorum*	Endangered	Small (Approx. 7"), diurnal owl, reddish brown overall with cream-colored belly, streaked with reddish brown. Some individuals are grayish brown.	Cochise Gila Graham Greenlee Maricopa Pima Pinal Santa Cruz Yuma	<4000 ft	Mature cottonwood/willow, mesquite bosques, and Sonoran desert Scrub	Range limit in Arizona is from New River (North) to Gila Box (East) to Cabeza Prieta Mountains (West). Only a few documented sites where this species persists are known. Additional surveys are needed. Proposed critical habitat occurs in Pima and Pinal counties (67 FR7 1032; 11-27-02).
California brown pelican	*Pelecanus occidentalis californicus*	Endangered	Large dark gray-brown water bird with a pouch underneath its long bill and webbed feet. Adults have a white head and neck, brownish black breast, and silver gray upper parts.	Apache Cochise Coconino Gila Graham Greenlee La Paz Maricopa Mohave Navajo Pima Pinal Santa Cruz Yavapai Yuma	Varies	Coastal land and islands; species found around many Arizona lakes and rivers	Subspecies is found on Pacific Coast and is endangered due to pesticides. It is an uncommon transient in Arizona on many Arizona lakes and rivers. Individuals wander up from Mexico in summer and fall. No breeding records in Arizona.

Chirica-hua leopard frog	*Rana chiricahuensis*	Threat-ened	Cream colored tubercules (spots) on a dark background on the rear of the thigh, dorsolateral folds that are interrupted and deflected medially, and a call given out of water distinguish this spotted frog from other leopard frogs.	Apache Cochise Coconino Gila Graham Greenlee Navajo Pima Santa Cruz Yavapai	3300-8900 ft	Streams, rivers, backwaters, ponds, and stock tanks that are mostly free from introduced fish, crayfish, and bullfrogs	Require permanent or nearly permanent water sources. Populations north of the Gila River may be closely related, but distinct, undescribed species. A special rule allows take of frogs due to operation and maintenance of livestock tanks on State and private lands.
Desert pupfish	*Cyprinodon macularius*	Endan-gered	Small (2 inches) smoothly rounded body shape with narrow vertical bars on the sides. Breeding males blue on head and sides with yellow on tail. Females and juveniles tan to olive colored back and silvery sides.	Graham La Paz Maricopa Pima Pinal Santa Cruz Yavapai	<5,000 ft	Shallow springs, small streams, and marshes. Tolerates saline and warm water.	Critical habitat includes Quitobaquito Springs, Pima County, portions of San Felipe Creek, Carrizo Wash, and Fish Creek Wash, Imperial County, California. Two subspeices are recognized: Desert Pupfish (*C.m. macularis*) and Quitobaquito Pupfish (*C.m. eremus*).

Gila chub	*Gila intermedia*	Endangered	Deep compressed body, flat head. Dark olive-gray color above, silver sides. Endemic to Gila River Basin.	Cochise, Gila, Graham, Greenlee, Maricopa, Pima, Pinal, Santa Cruz, Yavapai	2,000 - 4,500 ft	Pools, springs, cienegas, and streams.	Found on multiple private lands, including the Nature Conservancy, the Audubon Society, and others. Also occurs on Federal and state lands and in Sonora, Mexico. Critical habitat occurs in Cochise, Gila, Graham, Greenlee, Pima, Pinal, Santa Cruz and Yavapai counties.
Gila top-minnow	*Poeciliopsis occidentalis occidentalis*	Endan-gered	Small (2 inches), guppy-like, live bearing, lacks dark spots on its fins. Breeding males are jet black with yellow fins.	Gila Graham La Paz Maricopa Pima Pinal Santa Cruz Yavapai	<4,500 ft	Small streams, springs, and cienegas vegetated shallows.	Species historically occurred in backwaters of large rivers but is currently isolated to small streams and springs.
Huachuca water umbel	*Lilaeopsis schaffneriana ssp. recurva*	Endan-gered	Herbaceous, semi-aquatic perennial in the parsley family (Umbelliferae) with slender erect, hollow, leaves that grow from the nodes of creeping rhizomes. Flower: 3 to 10 flowered umbels arise from root nodes.	Cochise Pima Santa Cruz	3500-6500 ft	Cienegas, perennial low gradient streams, wetlands.	And in adjacent Sonora, Mexico, west of the continental divide. Populations also on Fort Huachuca Military Reservation. Critical habitat in Cochise and Santa Cruz Counties (64 FR 37441, July 12, 1999)

Jaguar	*Panthera onca*	Endan-gered	Largest species of cat native to Southwest. Muscular, with relatively short, massive limbs, and a deep-chested body. Usually cinnamon-buff in color with many black spots. Weights ranges from 40-135 kg (90-300 lbs).	Cochise Santa Cruz Pima	1,600--->9,800 ft	Found in Sonoran desert scrub up through subalpine conifer forest	Also occurs in New Mexico. A Jaguar conservation team is being formed that is being led by Arizona and New Mexico state entities along with private organizations.
Kearney blue star	*Amsonia kearneyana*	Endan-gered	A herbaceous perennial in the dogbane family (Apocynaceae). Thickened woody root and many pubescent (hairy) stems that rarely branch. Flowers: white terminal inflorescence in April and May.	Pima	3600--3800 ft	West-facing drainages in the Baboquivari Mountains.	Plants grow in stable, partially shaded, coarse alluvium along a dry wash in the Baboquivari Mountains. Range is extremely limited. Protected by Arizona Native Plant Law.
Lesser long-nosed bat	*Leptonycteris curasoae yerbabuenae*	Endan-gered	Elongated muzzle, small leaf nose, and long tongue. Yellowish brown or gray above and cinnamon brown below. Tail minute and appears to be lacking. Easily disturbed.	Cochise Gila Graham Greenlee Pima Pinal Maricopa and Santa Cruz	<6000 ft	Desert scrub habitat with agave and columnar cacti present as food plants.	Day roosts in caves and abandoned tunnels. Forages at night on nectar, pollen, and fruit of paniculate agaves and columnar cacti. This species is migratory and is present in Arizona usually from April to September and south of the border the remainder of the year.

Masked bobwhite	*Colinus virginianus ridgewayi*	Endan-gered	Males brick-red breast and black head and throat. Females are generally nondescript but resemble other raves such as the Texas bobwhite.	Pima	1000--4000 ft	Desert grasslands with diversity of dense native grasses, forbs, and brush.	Species is closely associated with *Acacia angustissima*. Formerly occurred in Altar and Santa Cruz Valleys, as well as Sonora, Mexico. Presently only known from reintroduced populations on Buenos Aires.
Mexican spotted owl	*Strix occidentalis lucida*	Threat-ened	Medium sized with dark eyes and no ear tufts. Brownish and heavily spotted with white or beige.	Apache Cochise Coconino Gila Graham Greenlee Maricopa Mohave Navajo Pima Pinal Santa Cruz Yavapai	4,100--9,000 ft	Nests in canyons and dense forests with multi-layered foliage structure.	Generally nests in older forests of mixed conifer or ponderosa pine/gambel oak type, in canyons, and various use habitats for foraging. Sites with cool microclimates appear to be of importance or are preferred. Critical habitat was finalized on August 31, 2004 (69 FR 53182).
Nichol Turk's head cactus	*Echinocactus horizonthal-onius var. nicholii*	Endan-gered	Blue-green to yellowish-green, columnar, 18 inches tall, 8 inches in diameter. Spine clusters have 5 radial and 3 central spines; one downward short; 2 spines upward and red or uasally gray. Flower: pink fruit: woolly white.	Pima Pinal	2400--4100 ft	Sonoran desert scrub	Found in unshaded microsites in Sonoran desert scrub on dissected alluvial fans at the foot of limestone mountains and on inclined terraces and saddles on limestone mountainsides.

Ocelot	*Leopardus (=Felis) pardalis*	Endan-gered	Medium-sized spotted cat whose tail is about 1/2 the length of head and body. Yellowish with black streaks and stripes running from front to back. Tail is spotted and face is less heavily streaked than the back and sides.	Cochise Pima Santa Cruz	<8000 ft	Humid tropical and sub-tropical forests, savannahs, and semi-arid thorn-scrub.	May persist in partly-cleared forests, second-growth woodland, and abandoned cultivation reverted to brush. Universal component is presence of dense cover. Unconfirmed reports of individuals in the southern part of the state continue to be received.
Pima pineapple cactus	*Coryphantha scheeri var. robustispina*	Endang ered	Hemispherical stems 4-7 inches tall 3-4 inches diameter. Central spine 1 inch long, straw colored and hooked surrounded by 6-15 radial spines. Flower: yellow, salmon, or rarely white narrow floral tube.	Pima Santa Cruz	2300--5000 ft	Sonoran desert-crub or semi-desert grassland communities.	Occurs in alluvial valleys or on hillsides in rocky to sandy or silty soils. This species can be confused with juvenile barrel cactus (*Ferocactus*). However, the spines of the later are flattened, in contrast with the round cross-section of the *Coryphantha* spines. Also, the aeroles (spine clusters) of *Coryphantha* are on tubercles (bumps), while the areoles of *Ferocactus* are on ridges (ribs). 80-90% of individuals occur on State and private land.

Sonoran prong-horn	*Antilocapra americana sonoriensis*	Endan-gered	Buff on back and white below, hoofed with slightly curved black horns having a single prong. Smallest and palest of the pronghorn subspecies	Maricopa Pima Yuma	500--2,000 ft	Broad intermountain alluvial valleys with creosote-bursage and palo verde-mixed cacti associations	Typically, bajadas are used as fawning areas and sandy dune areas provide food seasonally. Historic range was probably larger than exists today. This sub-species also occurs in Mexico.
Southwest ern willow fly-catcher	*Empidonax traillii extimus*	Endan-gered	Small passerine (about 6 inches) grayish-green back and wings, whitish throat, light olive-gray breast,and pale yellowish belly. Two wingbars visible. Eye-ring faint or absent.	Apache Cochise Coconino Gila Graham Greenlee La Paz Maricopa Mohave Navajo Pima Pinal Santa Cruz Yavapai Yuma	<8500 ft	Cottonwood/ willow and tamarisk vegetation communities along rivers and streams.	Migratory riparian obligate species that occupies breeding habitat from late April to September. Distribution within its range is restricted to riparian corridors. Difficult to distinguish from other members of the *Empidonax* complex by sight alone. Training required for those conducting flycatcher surveys. Critical habitat was proposed on October 12, 2004 (50 CFR 60706, October 12, 2004) and can be viewed at http://arizonaes.fws.gov