FINAL REPORT

Project Title: An Alternative Management Strategy for Codling Moth: Autocidal Biological Control

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Objectives:
The primary objective of this project is to genetically engineer codling moth to contain a single point alteration of the genome to render the insect conditionally sterile under normal environmental conditions. Optimal system will have a selectable marker gene (EGFP) and a conditional lethal mutation which would allow for mass production under controlled environmental conditions, but would be lethal to offspring under normal environmental conditions [truncated *Notch* (N^{60G11})]. The truncated *Notch* (N^{60G11}) is a dominant mutation which is triggered at temperatures below 20°C and prevents embryogenesis. In order to obtain the primary objective, it was necessary to develop a reliable transformation technology for codling moth which included the development of a microinjection technique to successfully deliver DNA into the early embryo. It was also necessary to determine the optimal transposable element and vector construct to give stable transformation and integration into genomic DNA. Once the initial transgenic insects, expressing only the marker gene were obtained, it was necessary to determine the integration site in the moth genome and the exact sequence of surrounding the insertion site.

After successful transformation was determined and the system was optimized, a DNA vector with the conditionally lethal N^{60g11} mutation was used to create a strain of codling moth which could be reared above the critical temperature in the laboratory and perform temperature experiments with transformed codling moth, homozygous and heterozygous for the N^{60g11} mutation.

Significant Findings:
Injected DNA containing green fluorescent protein (EGFP) resulted in transient expression of the fluorescent gene following injection in codling moth embryos. Both *piggyBac* and *hermes* transposons were used to insert the DNA into the chromosomes.
The *piggyBac* transposable element was the most successful in inserting the marker gene with reliable excision of the plasmid.
The *hermes* containing plasmid resulted in the generation of a number of eye mutants, but low expression of the EGFP.
Successful transformations of Oriental Fruit Moth and Lesser Apple worm were obtained using the *piggyBac*/EGFP vector.
Evidence using polymerase chain reaction (PCR, amplified DNA) indicates that the green fluorescent gene was present in codling moths raised from eggs that were injected with an EGFP construct and in the offspring of those moths, indicating inheritance of the injected gene.
Evidence using blotting analyses (unamplified DNA) indicates that the green fluorescent gene was present in Generation 5 of five codling moth lines which originated from DNA-injected individuals.
Two lines of EGFP expression codling moth resulted in positive PCR for 25 generations and positive DNA blots up to generation 13.
Sequencing of the DNA surrounding the insertion site of EGFP lines using inverse PCR is now under way.
Comparisons of oviposition, egg hatch, and survivorship of the EGFP lines with wild type show not

deleterious effects of the EGFP insertion into the genome.

A draft of the confined field release (caged tree studies) has been filed with USDA-APHIS Biotechnology permitting office. A final draft will be filed in January 2002.

Successful transformation of codling moth with the EGFP/N^{60g11} was obtained this summer. There are 4 lines obtained from different plasmid constructs. Expression of the EGFP is very high in two lines and are PCR positive. Lines are currently up to generations 4 and 6.

The EGFP/N^{60g11} lines have been split into two equal parts. One half will be used to continue the lines and the other half is in the temperature sensitivity experiments.

RESULTS:

Expression of the injected green fluorescent protein gene has been noted for codling moth embryos using both the *hermes* and *piggyBac* element with the fluorescent gene driven by a moth gene promoter (Tables 1 and 2). This transient gene expression lasts until the larva hatches. Helper DNA does not affect transient expression but becomes important in the insertion and inheritance of the marker DNA. The percent expression using these two vectors was similar, but the hatch rate was considerably higher when using the vector containing *hermes* (Table 1). Thus, both *piggyBac* and *hermes* DNA containing a silkworm gene promoter for GFP function well in codling moth eggs.

The *piggyBac* transposable element was used to transform both Oriental Fruit Moth and Lesser Appleworm (demonstrating that this technology, Autocidal Biological Control, coule also be developed for these pest and perhaps other Tortricid pests.

Based on polymerase chain reaction analyses (amplified DNA) performed during the past year, the GFP gene was present in moths raised from eggs injected with GFP (Generation 0) and in subsequent generations (Table 4). GFP-positive moths in Generations 11 and 12 indicate that the GFP gene was inherited in codling moth. Furthermore, blotting analyses, in which the DNA is NOT amplified revealed the presence of the GFP gene in Generation 5 and 8 pooled DNA samples of several moths (Table 4). The DNA originally injected was *piggyBac*/silkworm promoter/GFP. As evident from the table, the results may be variable between the two types of DNA analyses and between generations of a single line. The two outstanding lines (Lines 9 and 7) are being analyzed more closely with DNA sequencing analysis to determine where the inserted DNA landed in the chromosome. Three other lines were positive for marker DNA in Generation 5, but not always positive in preceding generations.

The transformation of codling moth with the EGFP/N^{60g11} is the first successful transformation of a Lepidopteran with a conditional lethal mutation. This puts the codling moth program well ahead of the pink bollworm program. Demonstration of the temperature sensitivity of this transgene to render these lines conditionally sterile under normal environmental conditions is still needed. If these tests are successful, an application for confined field releases will be made. Both lines of transgenic codling moth will be used in the development of an Environmental Impact Statement, the first required by USDA-APHIS for any transgenic organism. There is interest in both USDA-ARS and USDA-APHIS to complete this aspect of the program, and to also complete the first environmental impact study (EIS) for a transgenic organism. The final draft of the confined field release application will be sent to USDA-APHIS in January 2002, and posted on the Federal Register at that time. The caged field trials will be conducted over the 2002-2003 seasons (two field seasons). We will be filing for a Biotechnology Risk Assessment grant (USDA-CSREES-NRI) to complete the information for the EIS.

Conclusions:

The successful transformation of codling moth with both marker and conditionally sterile constructs

has fulfilled the primary objective of this project. Research will continue to demonstrate the temperature sensitivity of the conditional sterile construct. The development of a system to transform other Lepidopteran pests may lead to the generation of more candidates for Autodical Biological Control, a 21st Century approach to pest eradication.

Table 1. Codling moth injection data with the DNA vector containing *hermes*, a silkworm gene promoter, and green fluorescent protein gene*.

	# injected	# green embryos (% of injected)	# hatched (% of injected)	# hatched that were green (% of hatched)
without helper	377	234 (62.1)	179 (47.5)	147 (82.1)
with helper*	260	155 (59.6)	82 (31.5)	56 (68.3)
Total	637	389 (61.1)	261 (41.0)	203 (77.8)

*DNA from S. Thibault, Exelixis Pharmaceuticals, South San Francisco, CA.

Table 2. Codling moth injection data with the DNA vector containing *piggyBac*, a silkworm gene promoter, and enhanced green fluorescent protein gene*. Table contains both 1998 and 1999 data.

	# injected	# green embryos (% of injected)	# hatched (% of injected)	# hatched that were green (% of hatched)
without helper	804	464 (57.7)	191 (23.8)	138 (72.3)
with helper**	1373	857 (62.4)	396 (28.8)	299 (75.5)
Total	2177	1321 (60.7)	587 (27.0)	437 (74.4)

*DNA from S. Thibault, UC Riverside).
**Helper plasmids from S. Thibault, UC Riverside, and Al Handler, Gainesville, FL.

Table 3. Injection data for oriental fruit moth and lesser appleworm compared to codling moth using the DNA vector containing *piggyBac*, the silkworm gene promoter, and enhanced green fluorescent protein gene.

Moth	# injected	# green embryos (% of injected)	# hatched (% of injected)	# hatched that were green (% of hatched)
OFM	96	56 (58.3)	17 (17.7)	17 (100)
LAW	28	17 (60.7)	9 (32.1)	9 (100)
CM	1522	962 (63.2)	449 (29.5)	346 (77.1)

OFM = Oriental fruit moth, LAW = Lesser appleworm, CM = codling moth

Table 4. Summary of results from polymerase chain reaction (PCR) analyses (amplified DNA) and blotting analyses (unamplified DNA) on adult codling moths. DNA originally injected contained *piggyBac*, a silkworm gene promoter, and EGFP. The analyses probed for the presence of a portion of the EGFP gene that was part of the vector injected into Generation 0 embryos.

Analysis	Generation	# Sublines Positive for GFP / #Screened (Positive Sublines)
PCR	5	0/3
PCR	6	4/4 (39,43,77,82)
PCR	7	1/5 (66)
PCR	8	1/7 (39)
PCR	9	2/8 (77)
PCR	10	6/6 (82, 25, 66, 77)
PCR	11	1/3 (66, 82)
PCR	12	0/2
PCR	13	1/2 (39)
PCR	14	1/1 (43)
Southern	5	9/20*
Southern	8	16/12**

[1]Pooled DNA samples from each line for Southern blots.
Line 7 è Sublines 66,74,77,82
Line 9 è Sublines 25,28,30,39,43
*(Sublines 25,30,24,36,50,46,74,28,42)

Figure 1. Expression of enhanced green fluorescent protein in the eyes and body of adult codling moth transformed with the EGFP/N^{60g11} plasmid construct (left) compared to wild-type (right). Note the bright green fluorescence in the transformed lines as compared to the dull green autofluorescence of the wild type.

EGFP/N^{60g11} Wild-type

Project total cost: $132,957