

# Final Project Report to the NYS IPM Program, Agricultural IPM 2003-2004

## 1. Title:

Development of Bt Collard as a Trap Crop for Cabbage

## 2. Project Leader:

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## 4. Type of Grant:

Pest-resistant crops; Biological control and pest biology

## 5. Project location:

Wherever cabbage is grown. The general principle being tested (Bt-trap crops) is relevant nationally and internationally.

## 6. Abstract:

This work seeks to protect crops from insect pests by combining approaches from biological control and biotechnology. More specifically, it aims to protect cabbage from diamondback moths (DBM) through use of a collard trap crop expressing insect-resistance genes from *Bacillus thuringiensis* (Bt). Previous reports indicated that DBM laid more eggs on collard plants than on cabbage; however, the larvae that hatch from these eggs survive to damage crucifer crops. Bt-transgenic collard plants would attract DBM egg-laying and would also kill the hatched larvae, providing more effective insect control in a mixed field of cabbage and collard. We have introduced two different Bt genes (*cry1A* and *cry1C*) into the collard varieties "Champion" (non-glossy leaves) and "McCormack's Green Glaze" (glossy leaves). Lines that are highly toxic to DBM larvae have been identified. Seeds have been recovered from self-pollination of these plants. We have also obtained progeny from crosses between *cry1A* and *cry1C* plants in order to pyramid the two Bt genes. Seeds recovered from pollination of cytoplasmic male sterile (CMS) cabbage with pollen from *cry1C* plants are a first step toward production of CMS Bt-collard, which would eliminate problems of transgenic pollen flow. Initial tests of DBM egg-laying on the Bt plants and control plants indicate that the glossy collards are more attractive to the moths than non-glossy collards. The seeds already in hand and others currently being produced provide the material needed for greenhouse and field tests of the effectiveness of Bt trap crops. In addition we have produced Bt Indian mustard (*Brassica juncea* "Green Wave") as an additional possible Bt-trap crop.

## 7. Background and justification

Cabbage is a major New York State and U.S. vegetable crop with serious insect pest problems. Insecticides are the primary method of control. Managing insect pests via trap crops is an attractive biological control concept but it is often not very effective in actual implementation (Hokkanen 1991). Published reports suggest that collard has potential as a trap crop for cabbage (Mitchell et al. 2000); however, collards do not kill the larvae of insects attracted to them. Collards could be a more effective trap crop if they killed Lepidopteran insects by virtue of expression of a suitable Bt-transgene. The Earle lab has produced many types of Bt crucifers

(broccoli, cauliflower, cabbage, Chinese cabbage, rapeseed) and so was in a good position to create Bt-collard as well. The cooperators are well qualified to conduct greenhouse and field tests comparing a Bt-collard trap crop with other insect management systems for cabbage and then to take the project to an implementation stage, if appropriate.

The system proposed avoids several concerns often raised about current Bt-transgenic crops:

- 1) Public acceptance: the cabbage crop would not be transgenic.
- 2) Gene flow to other plants: male-sterile Bt-collards that produce no pollen would largely eliminate this problem. Moreover, collards are biennial and would not flower during the growing season.
- 3) Development of resistant insects: the cabbage crop would serve as a large refuge on which some susceptible insects could survive. Use of collard plants simultaneously expressing two different Bt genes would slow development of resistance on the collards plants, as demonstrated in recent studies in Shelton's program (Zhao et al. unpublished data).

This project provides an excellent test of the concept that there is no inherent conflict between GMO and IPM approaches, i.e., that transgenic plants can be part of an effective IPM system. If results are positive, several further outcomes are likely. One is deployment of Bt-collard by growers of cabbage (or other crucifer crops) with reduction of insecticide use. Research on transgenic trap crops suitable for other horticultural crops will also be stimulated. Such additional transgenic crops might incorporate either Bt genes or other types of insect control genes, as they become available. Furthermore, success in this project might help individuals or groups currently hostile to GMOs reevaluate their positions on the basis of a more environmentally friendly and less risky application of GMO technology.

## 8. Objectives:

The overall aim is to determine whether transgenic approaches can be effectively combined with other biological control methods via production of a useful transgenic trap crop. Specific objectives are as follows:

- 1) Produce collard lines with high expression of a *cry1C* and/or a *Cry1Ac* gene from *Bacillus thuringiensis*. These genes both encode proteins that kill Lepidopteran insects.
- 2) Compare oviposition, larval mortality, and insect damage on cabbage plants grown alone or together with non-transformed or Bt-transgenic collard plants.
- 3) Produce cytoplasmic male-sterile Bt-transgenic collard lines.
- 4) Conduct field trials in Ithaca, Geneva, and Charleston, SC to determine the ratios, arrangements, and timings of cabbage and Bt-collard plantings that give best control of Lepidopteran pests.
- 5) Conduct field trials comparing insect control using Bt-collard as a trap crop with other insect control methods used for cabbage.
- 6) Evaluate the efficacy of this approach and, if appropriate, develop plans for larger scale testing and implementation of the transgenic trap crop strategy.

Note: These objectives will clearly require more than one year of work, but the full list of objectives is presented to indicate the scope of the whole project.

## 9. Procedures:

The procedures are listed for objectives 1), 2) and 3), which are the ones addressed in the work to date.

- 1) Two collard (*Brassica oleracea* var. *acephala*) lines were used: Champion (non-glossy leaves) and McCormack's Green Glaze (glossy leaves). *Agrobacterium tumefaciens*-mediated transformation of seedling explants was used to introduce a *cry1C* or a *cry1Ac* Bt gene into these lines. Putative transformants were identified via their resistance to hygromycin or kanamycin, associated with the *cry1C* or *cry1Ac* gene, respectively. Integration of the genes was confirmed by polymerase chain reaction assays, using primers specific for each Bt gene. Bt protein production was measured by ELISA assays. Resistance to second instar diamondback moth larvae was assayed by scoring leaf damage and larval mortality on detached leaves after 5 days. Standard susceptible DBM larvae and larvae resistant to Cry1A or Cry1C Bt proteins were used. The details of the procedures used are presented in Cao et al. (2002).
- 2) Initial ovipositional tests were done by Shelton's group using a two-choice method comparing each plant species to cabbage, using three replicates of each choice test. A single leaf of each plant type was placed into a 50 ml flask filled with water, and the lip of the flask was sealed with Parafilm. A flask of one plant type was placed into a 1 m<sup>3</sup> chamber along with a flask of a cabbage leaf. Care was taken to use only leaves of a similar size. Newly emerged diamondback moth adults (3 female and 3 male) were introduced into each chamber. Moths were allowed to mate and lay eggs for 24 hours after which the eggs were counted. Each leaf was placed with its flask into smaller chamber for 7 days at which time the number of larvae was counted.
- 3) *Cry1C* Champion plants with high expression of Cry1C protein were vernalized for 10 weeks at 4° C to induce flowering. Vernalized non-transgenic cytoplasmic male sterile (CMS) cabbage plants were shipped to Ithaca from South Carolina by collaborator Farnham. Pollen from the Champion plants was used to pollinate the CMS cabbage plants. *Cry1C* and *cry1A* collard plants were also self-pollinated (via bud pollination) or crossed to create progeny carrying both Bt genes.

## 10. Results and discussion:

### PRODUCTION OF COLLARD PLANTS CARRYING BT GENES

Twenty-eight hygromycin-resistant collard plants were obtained from two transformation experiments using the *cry1C* + hygromycin construct (16 Champion and 12 McCormack's Green Glaze [MGG]). Ten kanamycin-resistant plants were obtained from two transformation experiments using the *cry1Ac* + kanamycin construct (6 Champion and 4 MGG).

### ANALYSIS OF PLANTS OBTAINED IN THE TRANSFORMATION EXPERIMENTS

#### Presence of Bt genes in the antibiotic-resistant plants

Polymerase chain reaction (PCR) assays of 11 hygromycin Champion plants and 5 MGG plants indicated that they contained the *cry1C* Bt gene. Southern blot analyses of these plants further confirmed the presence and integration of the *cry1C* gene into the collard genome. Similarly, PCR assays of the 10 kanamycin-resistant plants showed that they contained the *cry1Ac* Bt gene.

#### Production of Bt proteins in the plants

Sixteen Champion and 3 MGG hygromycin-resistant plants were assayed to determine their levels of Cry1C protein. Seven Champion plants had a high level of Cry1C protein (**over 1000 ng/mg total soluble proteins [TSP]**), five had a moderate level (about 600 ng/mg TSP), and the other four had a low level. Two of the 3 MGG plants produced a high level of Cry1C protein and the other had a moderate level. ELISA assay of the plants carrying the *cry1Ac* gene showed that only four Champion plants produced Cry1A protein, one at a moderate level and three at a low level.

## **Insect Resistance of the plants carrying Bt genes**

Thirteen Champion plants and 3 MGG plants expressing the *cry1C* gene were used in bioassays examining control of DBM larvae. The transgenic plants with high expression of Cry1C protein suffered no leaf damage from the larvae. Plants with moderate or low Cry1C protein levels showed slightly higher leaf damage, ranging from 0 to <5% and 1-25%, respectively.

Nevertheless, all of the Bt- transgenic plants caused 100% mortality of susceptible and Cry1A-resistant DBM larvae, regardless of the level of Bt protein. As expected, the *cry1C*-transgenic plants did not control DBM larvae with high resistance to Cry1C protein.

Similar patterns were seen in bioassay of *cry1Ac* Champion plants with susceptible, Cry1C-resistant or Cry1A-resistant DBM larvae. These plants caused 100% mortality of susceptible and Cry1C-resistant DBM larvae although moderate or low Cry1Ac expressers had 0–3% or 0 - 20% defoliation, respectively. As expected, the *cry1Ac* Champion plants did not control Cry1A-resistant DBM larvae.

## **RECOVERY OF PROGENY**

Substantial progress has been made on recovery of seeds from the Bt-collard materials. This required vernalization of the plants, followed by bud pollination to overcome self-incompatibility. Self-pollinations already done should provide us with about 500 seeds from *cry1C* MGG plants producing a high level of Cry1C protein and about 200 seeds from *cry1Ac* Champion plant producing a moderate level of the Cry1Ac protein. Reciprocal crosses were made between *cry1Ac* Champion and *cry1C* MGG in order to obtain progeny with pyramided Bt genes that provide superior insect control (Zhou et al. 2003). About 800 seeds from this cross have already been harvested and at least 500 more are expected. Additional *cry1C* Champion and MGG plants have been vernalized and grown to flowering. More seeds will be produced from self- or cross-pollinations.

**In addition, two CMS cabbage plants were pollinated with pollen from Champion plants producing high levels of Cry1C protein. More than 7000 CMS hybrid seeds have been harvested from these crosses.**

## **OVIPOSITIONAL TESTS**

**Initial tests of oviposition using a two-choice method indicated that DBM preferred the glossy collards to non-glossy collard or cabbage. The non-Bt collard plants allowed substantial survival of larvae hatched from the eggs laid while Bt collards allowed no larval survival. Indian mustard (*Brassica juncea*) was even more preferred for DBM oviposition but allowed very high larval survival.**

<u>Plant material</u>	<u>Ovipositional ratio<sup>a</sup></u>	<u>Larval survivorship (%)<sup>b</sup></u>
Indian mustard	11.1a	79a
Glossy collard	7.9c	31c
Glossy collard + <i>cry1C</i> gene	7.6c	0d
Non-glossy collard	1.3d	67b
Non-glossy collard + <i>cry1C</i> gene	1.2d	0d
Cabbage/cabbage (check)	1.1d	65b

<sup>a</sup> The number of eggs laid on the leaf of the plant, compared to the number of eggs laid on the cabbage leaf in the same chamber for each replicate.

<sup>b</sup> Percent of larvae that survived from the eggs laid on each leaf after 7 days.

## PRODUCTION OF INDIAN MUSTARD PLANTS EXPRESSING THE *CRY1C* GENE

Because the ovipositional ratio for Indian mustard was even higher than that of glossy collard, we decided to introduce the *cry1C* gene into this plant. Transformation of seedling explants of cv. "Green Wave" produced 15 *cry1C* plants from 724 explants. The transformation frequency was 2%. Nine of these plants were assayed for control of susceptible DBM larvae. Six plants caused complete larval mortality with little or no defoliation while three failed to control DBM larvae and suffered 70-90% defoliation. All 15 transformed lines are now in soil, with a total of 23 plants including clones. Seeds will be recovered from these plants for further work, including possible development of CMS lines.

## DISCUSSION AND FURTHER WORK PLANNED

We have now developed many of the plant materials required for this project, including Bt-collard plants from two different varieties, one with non-glossy leaves and one with glossy leaves. Two different Bt genes are represented, at different levels of expression. We have multiple lines that completely control DBM larvae. Seed progeny of selfed plants, crosses between *cry1A* and *cry1C* plants, and CMS Bt collard-cabbage hybrids are either already in hand or will soon be available. The materials in hand will allow field tests examining insect damage in plots with different arrangements of the cabbage and Bt-collards. Initial tests have suggested that glossy collards are more attractive for DBM oviposition than the non-glossy type and are therefore more promising as Bt-trap crops. We will therefore focus on the glossy collards for further work. Attempts to produce MGG plants with good expression of Cry1Ac protein are in progress. We have also expanded the materials to include Bt-Indian mustard, which may also be of interest as a Bt-trap crop because of its high ovipositional ratio. Introduction of a Bt gene will make the plant not only highly attractive for oviposition but also toxic to hatched larvae. This is what one would desire in a trap crop. Pollen flow and seed set during the growing cycle would be more of an issue with the Indian mustard than with collard, but these concerns could be dealt with by introduction of CMS. Bt Indian mustard and Bt collard will differ in other aspects of their agronomy, life cycle, and other characteristics, so having two different possible Bt trap crops for Lepidopteran pests of crucifers will be beneficial. Successful implementation of the Bt-trap crop strategy will provide a new way to protect crucifer crops against insect damage.

## 11. References:

- Cao J, Zhao JZ, Tang JD, Shelton AM, Earle ED. 2002. Broccoli plants with pyramided *cry1C* and *cry1Ac* Bt genes control diamondback moths resistant to Cry1A and Cry1C proteins. *Theor. Appl. Genet.* 105:258-264
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- Mitchell ER, Hu G, Johanowicz D. 2000. Management of diamondback moth (Lepidoptera: Plutellidae) in cabbage using collard as a trap crop. *HortScience* 35:875-879
- Zhao J, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. *Nature Biotechnology* 21:1493-1497