HYBRID *BACILLUS THURINGIENSIS* δ-ENDOTOXINS PROVIDE ENHANCED SPECTRUM OF ACTIVITY AGAINST LEPIDOPTERAN PESTS OF COTTON

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Abstract

Studies were conducted to fully characterize the relative activities of Cry1Ac, Cry2Ab2, Cry1Fa, variant of Cry1Ca, and selected hybrid proteins against Lepidopteran cotton insects in diet bioassays using purified proteins. Bollworms (cotton bollworm, *Helicoverpa zea*; pink bollworm, *Pectinophora gossypiella*; and tobacco budworm, *Heliothis virescens*) and armyworms (*Spodoptera exigua* and beet armyworm, *Spodoptera frugiperda*) were used as test insects. The activity profile of four primary proteins - Cry1Ac, Cry2Ab2, Cry1Fa, and variant of Cry1Ca and those of the hybrid proteins of Cry1Ac and Cry1Fa and Cry1Ac and variant of Cry1Ca were determined in laboratory bioassays.

Based on LC_{50} values, Cry1Ac, has good to excellent activity on all bollworm species and little or no activity on armyworms. Cry1Fa on the other hand, is not toxic to *H. zea*, but has good activity on other more susceptible bollworms as well as on armyworms. In comparison, variant of Cry1Ca has no activity on *H. zea*, exhibits good activity on *S. exigua* but is not active on *S. frugiperda*.

The Cry1Ac/Cry1Fa hybrid proteins tested in these experiments extend the spectrum of activity of the two parent proteins, Cry1Ac and Cry1Fa. In contrast, the Cry1Ac/1Ca hybrid, is not toxic to *H. zea* at 100 μg/ml (highest dose tested), but was found to be toxic to both *Spodoptera* species, although the native Cry1Ac and variant of Cry1Ca were not toxic to *S. frugiperda*

Square and leaf tissue from R_1 cotton plants expressing a Cry1Ac/Cry1Fa hybrid protein were tested for activity against 3rd instar *H. zea* and *S. frugiperda*, respectively. The results obtained were very promising, and the line tested compared very favorably with the Cry1Ac/Cry2Ab2 line which was used as a positive control in this experiment.

Introduction

Many strains of *Bacillus thuringiensis* produce parasporal crystal proteins, or δ-endotoxins, which are toxic to specific lepidopteran, coleopteran an dipteran larvae. Commercial formulations of *B. thurigiensis* isolates have been used for decades for the control of agricultural pest insects. With the advent of advanced molecular tools, we have witnessed the introduction of transgenic crop plants, which contain and express genetically engineered δ-endotoxin genes (USP 6,110,464, 2000).

In recent years, protein engineering efforts have provided the hope of constructing δ-endotoxins with an extended spectrum of insecticidal activity (Lee et al., 1995; Honee et. al., 1991). It is logical to presume that this would only be possible by engineering carefully selected target regions. Methods have been described for the construction of several novel hybrid δ-endotoxins composed of aminoacid sequences from. native Cry1Ac and Cry1Fa, and from Cry1Ac and variant of Cry1Ca. These hybrid proteins, while retaining the insecticidal activity of the native proteins, showed activity against insect species insensitive to the native proteins.

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Three hybrid proteins, two Cry1Ac and Cry1Fa hybrids, and one Cry1Ac and variant of Cry1Ca hybrid, were used in these studies. Their spectrum of bioactivity was determined against key Lepidopteran pests of cotton. The parent proteins and Cry2Ab2 were used for comparison. Preliminary data on R_1 cotton plants expressing a Cry1Ac/Cry1Fa hybrid protein are also presented in this paper.

Experimental Protocol

Insects

Laboratory reared *H. virescens, H. zea, S. exigua,* and *S. frugiperda* were obtained from Ecogen, Inc., Langhorne, Pennsylvania. and *P. gossypyella* was obtained from the insect rearing facility at the Western Cotton Research Laboratory, Phoenix, Arizona. All insects used in the studies had been reared in laboratories in the absence of any insecticidal pressure for over 20 generations.

Purified Endotoxins

Recombinant strains of *Bacillus thuringiensis* were used to express the primary proteins, Cry1Ac, Cry2Ab2, Cry1Fa and variant of Cry1Ca, and three hybrid proteins, two Cry1Ac and Cry1Fa hybrids, and one Cry1Ac and variant of Cry1Ca hybrid. The proteins were then isolated and purified from sporulated lysed cultures utilizing standard procedures (Donovan *et. al*. 1992, Malvar *et. al*., 1994). The crystalline preparations of the proteins were then treated with high *pH* buffer to solubilize the proteins after which they were run on SDS PAGE gels (4-20% acrylamide) and quantified against bovine serum albumin (BSA) standard (Dankocsik *et. al*. 1990)

Diet Incorporation Studies

Dose-response studies on the susceptibility of the different insect species to various proteins were performed by diet incorporation (Stone *et al.* 1989). A series of 6 to 8 concentrations prepared by serial dilution was used in each instance. Neonates were infested onto the diet. Mortality measurements were recorded seven days after infestation. Larvae that were dead or were still at the neonate stage were considered dead in tabulating larval responses to the individual proteins. Concentration-mortality regressions were estimated assuming the probit model (SAS software 1995). Results were expressed as LC_{50}s in μg/ml diet.

Plant Tissue Studies

Squares and leaf disks from young cotton leaves were obtained from the R_1 Cry1Ac/Cry1Fa hybrid line, and from Cry1Ac, Cry1Ac/Cry2Ab2, and non-expressing control lines on three sampling dates (from 8-, 10- and 12-week-old plants). Bioassays were conducted in 32-well C-D International rearing trays (Pitman, NJ). The wells were lined with 0.2% agar, and the plant tissues was placed at the center of each well. Each treatment consisted of 32 squares or leaf disks. Third instar *H. zea* and *S. frugiperda* were infested (one larva per well) onto the squares and leaf disks, respectively. The plates were incubated at 27^0C, and mortality readings were taken six days after infestation. Averages of results from the three test dates were compiled to compare the efficacy of different lines against the test insects.

Results and Discussion

Comparisons of Toxicity Data on Primary and Hybrid Proteins Against Major Lepidopteran Pests of Cotton

The results obtained from several replicated experiments have been presented graphically in Figs. 1 and 2. Based on LC_{50} values, It is clear that Cry1Ac, has good to excellent activity on all bollworm species and little or no activity on armyworms. Cry1Fa on the other hand, is not toxic to *H. zea,* but has good activity on other more susceptible bollworm species as well as on armyworms. In comparison, variant of Cry1Ca has no activity on *H. zea*, exhibits good activity on *S. exigua* but is not active on *S. frugiperda*.

The Cry1Ac/1Fa hybrid proteins tested in these experiments have excellent to good activity on all Lepidopteran pests of cotton, thus extending the spectrum of activity of the two parents, Cry1Ac and Cry1Fa. In contrast, the Cry1Ac/1Ca hybrid is not toxic to *H. zea* at 100 μg/ml (highest dose tested), but was found to be toxic to both *Spodoptera* species, although its 'parents' Cry1Ac as well as variant of Cry1Ca were not toxic to *S. frugiperda*.

Investigations on lepidopteran pests in India have revealed that the hybrid proteins have extended spectrum of activity against Asian Lepidopterous cotton pests also. The two bollworm species (*H. armigera* and the spotted bollworm, *Earias vitella*) as well as the cotton leafworm (an armyworm, *S. litura*) are controlled by the hybrid proteins (K. S. Mohan, Personal Communication).

Plant Tissue Studies

The results obtained from these studies are summarized in Figs. 3 and 4. The efficacy of the Cry1Ac/Cry1Fa line on *H. zea* was determined to be significantly better than that of the Cry1Ac line ($p < 0.05$). Results from the *S. frugiperda* studies clearly demonstrate the excellent bioactivity of the Cry1Ac/Cry1Fa line against the armyworms.

Conclusions

Activity profile on Lepidopteran pests of cotton are given in this paper. Comparative efficacy of the various proteins on cotton pests have been discussed.

As we are well aware, Cry1Ac is very efficacious against bollworms. Cry1Fa on the other hand is very effective on the armyworms. The hybrid proteins derived from these native proteins had inherited the toxicity profile of both 'parents' and cover the full spectrum of Lepidopteran pests of cotton.

The Cry1Ac/1Ca hybrid was very toxic to the armyworms, but had no effect on *H. zea* at the doses tested. Since *H. zea* is a major pest of cotton in the US and world over *H. armigera* in the Old World), this particular hybrid protein would not be appropriate for use. It is interesting to note that the variant of Cry1Ca is only effective against *S. exigua* and not against *S. frugiperda*. That is, although both Cry1Ac as well as variant of Cry1Ca are not effective on *S. frugiperda*, the hybrid was found to be as effective as the other chimeras (Cry1Ac/1Fa) on *S. frugiperda*. This is an instance where the hybrid protein possesses properties not demonstrated by either of the 'parental' proteins.

These results demonstrate that other combinations of *Bt* proteins may be more effective in controlling all Lepidopteran target pests of cotton (Figs. 5 and 6).

References

Dankocsik, C., W. P. Donovan and C. S. Jany. 1990. Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subspecies *kurstaki* by gene fusion and determination of the crystal protein insecticidal specificity. Mol. Microbiol. 4: 2087-2094.

Donovan, William P., Mark J. Rupar, Annette C. Slaney, Thomas Malvar, M. Cynthia Gawron-Burke and Timothy B. Johnson. 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to *Coleoptera* species. Appl. Environ. Microbiol. 58: 3921-3927.

Honee, G., D. Convents, J. Van Rie, S. Jansens, M. Peferoen and B. Visser. 1991. The *C*-terminal domain of the toxic fragment of a *Bacillus thuringiensis* crystal protein determines receptor binding. Mol. Microbiol. 5: 2799-2806.

Lee, M. K., B. A. Young and D. H. Dean. 1995. Dsomain III exchanges of *Bacillus thuringiensis* cry1A toxins affect binding to different gypsy moth midgut receptors. Biochem. Biophys. Res. Comm. 216: 306-312.

Malvar, Thomas, Cynthia Gawron-Burke and James A. Baum. 1994. Overexpression of *Bacillus thuringiensis* HknA, a histidine protein kinase homology, bypasses early Spo$^-$ mutations that result in CryIIIA overproduction. J. Bacteriol. 176: 4742-4749.

SAS Institute. 1995. JMP Statistical Discovery Software. Cary, NC.

Stone, T. B. and S. R. Simms. 1991. Insect Rearing and the development of bioengineered crops. *In* T. E. Anderson & N. C. Leppla [eds.], Advances in insect rearing for research and pest management. West-view Press.

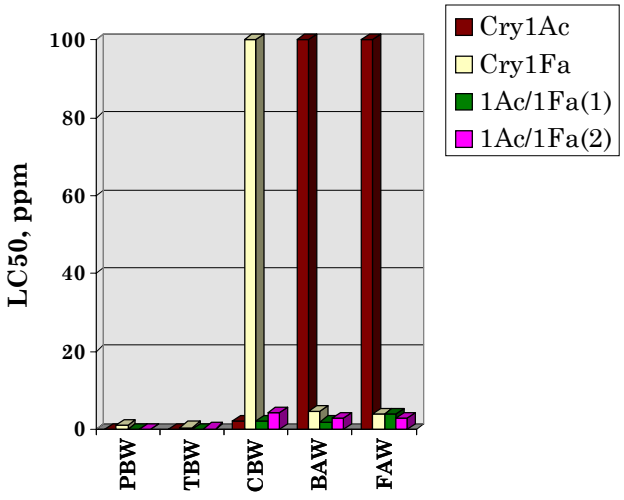

Figure 1. Toxicity data (LC_{50}) for primary and hybrid proteins (Cry1Ac/Cry1Fa) against bollworm and armyworm pests of cotton in the US.

LC_{50}: Lethal concentration in μg/ml, at which 50% of the larvae are dead or not moulted. TBW: *Heliothis virescens*, CBW: *Helicoverpa zea*, PBW: *Pectinophora gossypiella*, BAW: *Spodoptera exigua*, FAW*: Spodoptera frugiperda*. LC_{50} of 100 ppm denote that LC50 was >> 100 ppm.

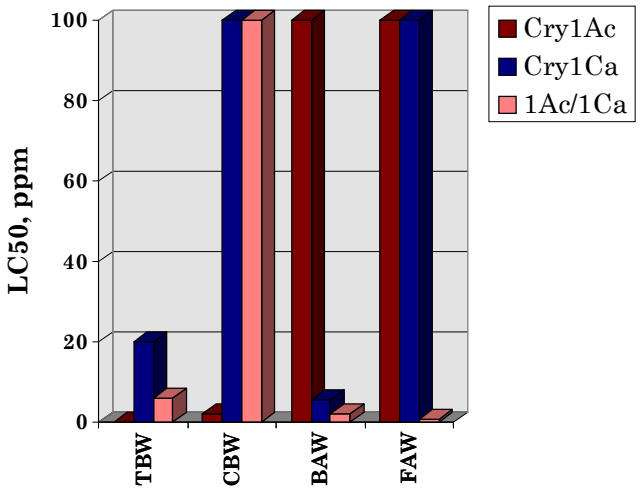

Figure 2. Toxicity data (LC_{50}) for primary and hybrid proteins (Cry1Ac/ variant of Cry1Ca) against armyworm pests of cotton in the US.

LC_{50}: Lethal concentration in μg/ml, at which 50% of the larvae are dead or not moulted. TBW: *Heliothis virescens*, CBW: *Helicoverpa zea*, PBW: *Pectinophora gossypiella*, BAW: *Spodoptera exigua*, FAW*: Spodoptera frugiperda*. LC_{50} of 100 ppm denote that LC50 was >> 100 ppm.

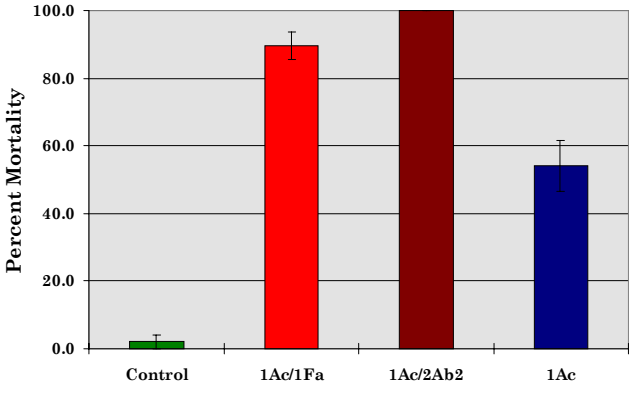

Figure 3. Mortality data for R_1 cotton plants expressing Cry1Ac/Cry1Fa hybrid protein when infested with 3rd instar cotton bollworm larvae.

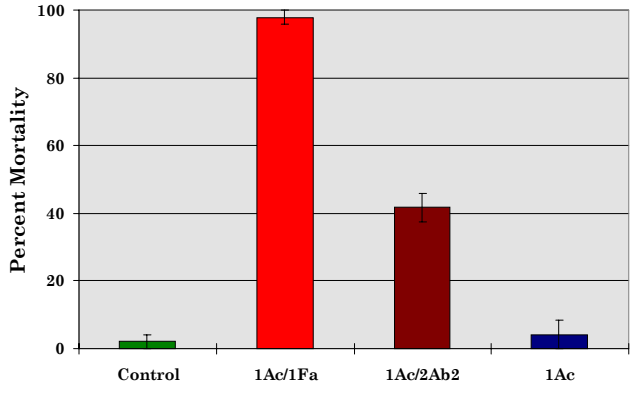

Figure 4. Mortality data for R_1 cotton plants expressing Cry1Ac/Cry1Fa hybrid protein when infested with 3rd instar fall armyworm larvae.

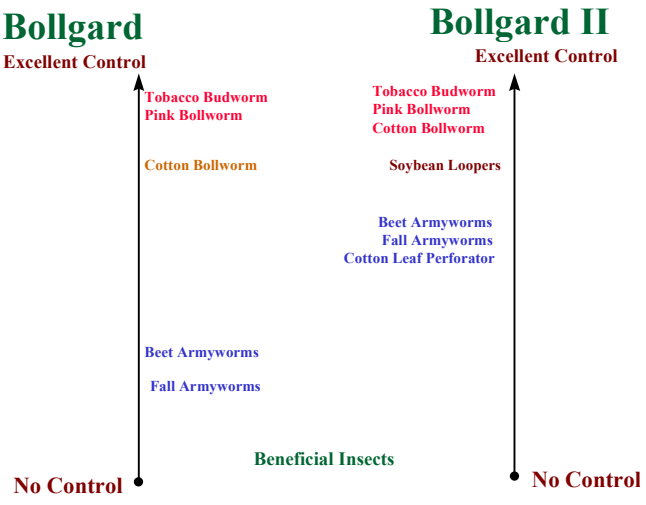

Figure 5. Toxicity profile of Bollgard® and Bollgard® II against Lepidopteran Pests of cotton.

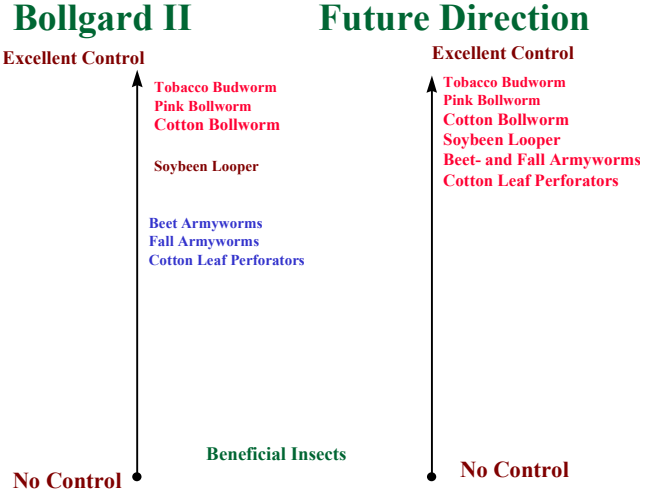

Figure 6. Possible direction for improvement in Lepidopteran activity of future Bollgard® technology.