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BOLLGARD II®: A STEP CHANGE FOR INSECT CONTROL IN COTTON

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Abstract

Bollgard® cotton, which contains the Cry1Ac gene for in plant production of the *Bacillus thuringiensis* endotoxin, was introduced to the cotton market in 1996. This transformation resulted in very high insecticidal expression to the tobacco budworm (*Heliothis virescens)*, pink bollworm (*Pectinophora gossypiella*) and European corn borer (*Ostrinia nubilalis* Hubner) and also provided good activity to cotton bollworm (*Helicoverpa zea)* in cotton. To augment the performance of Bollgard, a second gene Cry2Ab, was ballistically inserted into plant material containing the Cry1Ac gene. Prodigy from this insertion event was designated MON 15985 and contained the D&PL 50 Bollgard® background. Trade named Bollgard II™, it contains genes for production of both the Cry1Ac and Cry 2Ab insecticidal active proteins. This two-gene product, which received EPA approval December 23, 2002, provides substantially improved activity against cotton bollworm, beet armyworm (*Spodoptera exigua*), soybean looper (*Pseudoplusia includens*), fall armyworm (*Spodoptera frugiperda*) and several other lepidopteran species in cotton. Along with greater spectrum of activity, the two proteins expressed in Bollgard II are sufficiently dissimilar to greatly deter resistance development in field populations. Laboratory data also demonstrate the propensity of cross-resistance of Cry1Ac and 2Ab to be minimal based on studies using *Helicoverpa zea* (Jackson, 2002), *Heliothis virescens* (Gould, 1995) and pink bollworm (*Pectinophora gossypiella)* (Head, 2001). By comparison, the Cry2Ab protein present in Bollgard II cotton is expressed at a high dose for tobacco budworm and, in combination with Cry1Ac, presents two "high dose" proteins for control of the tobacco budworm in Bollgard II cotton. Overall, Bollgard® II provides increased efficacy to (*Helicoverpa zea)*, has an expanded spectrum of activity including armyworm and soybean loopers (*Pseudoplusia includens*), is a better resistance management tool, offers better yield potential and can ease and simplify insect control for growers.

Sequence Homology of Cry1A versus Cry2A Proteins

The amino acid sequence identity is generally very high within the individual Cry classes of proteins (over 80% in most cases). The percent identity between these two groups (Cry1A and Cry2A), however, is less than 20%. Of the identified classes of Cry proteins, these two classes are among the most divergent (Crickmore *et al*., 1998).

Analyses of resistance to B.t. Cry proteins indicate that when cross-resistance occurs, it occurs most often when the proteins are similar in structure. Work with diamondback moth has related these patterns to domain II of the Cry proteins (Tabashnik et al., 1996). With other insect species, different domains, particularly domain III, appear to be implicated in toxicity and specificity [e.g., the beet armyworm, *Spodoptera exigua* (Bosch et al., 1994); the gypsy moth, *Lymantria dispar* (Lee et al., 1995)]. This indicates that structurally related Cry proteins act through similar insecticidal mechanisms, and proteins that are very different in structure do not. In the case of the Cry1A and the Cry2A proteins, the low sequence homology suggests that these two classes of Cry proteins operate through different insecticidal mechanisms, and it is unlikely that a single resistance mechanism would confer resistance to both proteins.

Structural Comparison of Cry1Ac and Cry2Ab Proteins

The lack of substantial sequence similarity between the Cry1Ac and Cry2Ab proteins suggests that there is a difference in their tertiary structure. This conclusion is supported by direct measures of Cry2Aa structure, which suggest that Cry2A proteins are very different from Cry1A proteins (Morse *et al*., 2001). Another way to compare the tertiary structure of two proteins is to assay for cross reactivity of polyclonal antibodies raised to each protein. If the overall structure of the two proteins differs, then the epitope binding sites for antibody recognition should differ. Data show that the anti-Cry2Ab antibodies do not cross-react with the Cry1Ac protein, nor do the anti-Cry1Ac antibodies cross-react with the Cry2Ab2 protein (Kolwyck *et al*., 2000), suggesting a difference in the tertiary structure between the two proteins. When sampled by ELISA for Cry2Ab, both non-transgenic and Cry1Ac-containing Bollgard cotton samples were below the limit of detection (2.65 µg/g fresh leaf) for the Cry2Ab ELISA. This demonstrates that the anti-Cry2Ab2 antibodies do not cross react with the Cry1Ac protein at a level that is detectable by ELISA. Similarly, when a Cry2Ab2 standard was analyzed using a Cry1Ac ELISA, no Cry1Ac was detected (< 0.4 ng/ml). In addition, there was no significant difference between the level of Cry1Ac protein detected in single positive controls and double positive test events, indicating that cross-reactivity of the anti-Cry1Ac antibody with Cry2Ab2 protein is not detectable in the Cry1Ac ELISA. These data are further supporting evidence that the two proteins act differently in their specific pesticidal mode of action.

Mechanism of Action and Binding Characteristics

The generally recognized insecticidal mechanism of *B.t.* Cry proteins has several distinct steps after ingestion by the insect: solubilization, proteolytic stability, binding to the midgut epithelium, formation of ion channels in the midgut cells, and finally lysis of these cells (English and Slatin, 1992). Each of these steps is governed by the amino acid sequence-dependent structure of the protein and the insect-specific midgut environment. The contribution of each step in the mode of action to the overall toxicity varies considerably (Slaney *et al*., 1992), although toxin binding has received the most attention. The Cry1 toxins are highly amenable to binding analysis, *i.e.*, they are relatively soluble, produce a stable active toxin, and bind with high affinity (Hoffman *et al*., 1988). Yet, not all toxins rely as strongly on this binding step to achieve toxicity.

In a comparative analysis of Cry1Ac and Cry2Aa, English *et al*. (1994) demonstrated that Cry2Aa did not bind to a specific, high affinity receptor capable of specific binding of Cry1Ac. Binding of Cry2Aa was, in fact, non-saturable; regardless of the amount of toxin added to the assay, it was not possible to bind all of the sites available to the toxin. Among these non-saturable binding sites, there are likely interactions which promote toxicity, because Cry2Aa was a highly effective toxin against the cotton bollworm. These effective binding sites may have been masked by either a large number of non-specific sites on the midgut tissue or Cry2Aa recognized other Cry2Aa molecules, creating higher order structures and thereby subverting the utility of the assay. In a more careful analysis of Cry2Ab on the brush border of the same insect, no specific or saturable binding could be detected. In these experiments, interactions between like Cry2Ab molecules prevented discernment of specific receptors. However, saturable and hence specific Cry1Ac binding was still evident, thereby validating the assay.

These data suggest that Cry2Aa and Cry2Ab do not depend on a large number of high affinity interactions with brush border proteins for potency as do Cry1 proteins. Instead, this class of proteins relies on interactions with other protein molecules to create unique and potent ion channels. Theoretically, if the affinity of interactions with a large number of brush border proteins diminishes, the specific activity of a toxin may be maintained by increasing the potency of the ion channels formed by the toxin.

English *et al*. (1994) demonstrated that, unlike Cry1Ac, Cry 2Aa formed voltage-dependent currents in thin "Montal" membranes. In Black Lipid Membranes (BLMs), both Cry2Aa and Cry2Ab form voltage-independent currents. Ion channels, in general, rely on the voltage, either the imposed voltage or the electrochemical gradient, to drive current. In this sense, all channels depend on voltage (Hille, 1992). The term "voltage-dependent", however, means the channel can selectively sense specific voltages to turn on or off. Thus, Cry2 toxins (more certainly Cry2Aa) may be able to "tune-in" to the membrane potential of the midgut cells and perhaps take advantage of this property to drive the destructive current. One can reasonably argue that whenever a hole is created in the membrane, colloid osmotic lysis will kill the cell independent of voltage dependence. It is not possible at this time to say conclusively whether voltage dependence enhances the activity of Cry2a toxins over Cry 1Ac.

Cry2Aa formed ion channels with reduced ion selectivity (English *et al*., 1994) and, in preliminary experimentation on Cry2Ab, the Cry2Ab channels were not highly selective. The reduced ion selectivity observed for Cry2Aa and suggested for Cry2Ab channels indicate that potency is enhanced by disrupting cellular homeostasis of anions and cations instead of being limited to the cation selective Cry1Ac; thus, the difference in membrane interaction between Cry1Ac and Cry2 proteins is significant.

Cry2Aa formed conductances greater than 800 pS in Black Lipid Membranes, but Cry2Ab produced conductances greater than 8000 pS. These conductances are most likely the result of very large ion channels compared with the relatively small 600 pS conductances of Cry1Ac. The propensity of Cry2Ab to form large aggregates not seen with Cry1Ac may be responsible for, or at least contribute to, the formation of these large conductance states. The size of the ion channels are likely responsible for the potency of the Cry2 proteins, which would be consistent with the findings for Cry3B2 proteins (English *et al*., 1998).

Collectively, these data are consistent with the conclusion that Cry2Ab, and Cry2 toxins, in general, produce highly potent ion channels to compensate for binding either to themselves or to a large collection of non-specific binding sites. Alternatively, Cry1Ac compensates for relatively poor ion channel properties with specific binding. In conclusion, biochemical analyses demonstrate that the Cry1Ac and Cry2A proteins differ significantly with respect to solubility, presence of a protoxin, saturable binding kinetics and pore formation. These properties suggest different general mechanisms of action for the Cry1 and Cry2 classes of pesticidal proteins and therefore support the conclusion that these proteins are complementary for insect resistance management.

Lab Efficacy Studies

Studies to evaluate the LC50 values of these two genes (Table 1) show very high expression of the Cry1Ac protein to tobacco budworm (*Heliothis virescens*), European corn borer (*Ostrinia nubilalis* Hubner) and good expression to cotton bollworm (*Helicoverpa zea*) with limited activity to fall armyworm (*Spodoptera frugiperda*) and beet armyworm (*Spodoptera exigua*).

LC50 values for the Cry2Ab protein indicate a substantial increase in activity against beet armyworm and fall armyworm, but appear to be less active against tobacco budworm, cotton bollworm and European corn borer than Cry1Ac.

In 1998 and 1999, field trials were conducted to assess the efficacy of Bollgard II cotton against the tobacco budworm. Although the Cry2Ab has slightly higher LC50 values, its in plant protein production of is over 10 fold greater than that of Cry1Ac (Table 2). This produced an overall mean lepidopteran activity level 3.5-fold greater for Bollgard II than for Bollgard cotton alone. Anatomical studies to observe expression in differential plant parts indicate 3 to 5-fold increase in activity across leaves and squares respectively (Table 3), indicating improved expression throughout the plant. Additional lab studies demonstrate a significant increase of activity of the 2-toxin isoline over the single toxin Bollgard® isoline (Cry1Ac-only), with Cry2Ab contributing the larger portion of activity in the 2-toxin line (Table 4) (Greenplate, 2002). This relative increase in mean lepidopteran activity levels was seen at every sampling time (from two weeks post-pinhead square to eight weeks post-pinhead square) and at all field sites. Although the unit activity of Cry2Ab against TBW is less than for that of Cry1Ac, the overall insecticidal activity of Cry2Ab is higher in the plant since this protein is present at greater levels within the plant.

Activity of Cry2Ab against Cry1Ac Resistant Tobacco Budworm (TBW) Colonies

A series of studies on two tobacco budworm colonies selected for resistance to Cry1Ac indicates that these insects have much greater susceptibility to the Cry2Ab protein than to Cry1Ac (Gould, 1995). One of these colonies, YHD2, had approximately 20,000-fold resistance to the Cry1Ac protein *in vitro*, but minimal cross-resistance to other Cry proteins. In tests with neonate larvae, this colony had close to 100% survival after seven days on cotton leaves expressing Cry1Ac alone. No insects survived, however, on cotton tissue expressing Cry2Ab or both the Cry1Ac and Cry2Ab proteins. A second resistant colony, KCBhybrid, had lower resistance to the Cry1Ac protein *in vitro* but the resistance was relatively broad-based, with similar levels for Cry1A and Cry2A proteins. These insects had lower survival rates than those from YDH2 on Cry1Ac, although about 60% did survive. When placed on plant tissue expressing both the Cry1Ac and Cry2Ab proteins, few or no insects survived, and any survivors had not developed beyond first instar. Even third instar tobacco budworm from these two different resistant colonies were unable to gain weight on plant lines expressing both Cry1Ac and Cry2Ab. This indicates that Cry2Ab expressed at the levels attained in the Bollgard II cotton lines will be capable of controlling pest insects with a range of resistance mechanisms and thus will provide value in the resistance management program.

Activity of Cry2Ab against Cry1Ac Resistant Cotton Bollworm (CBW) Colonies

A Cry1Ac-resistant colony of the cotton bollworm was tested for its ability to survive on Bollgard II cotton, Bollgard cotton, and conventional cotton. The bollworm strain was collected in the field and then selected in the laboratory over thirteen generations for tolerance to Cry1Ac (Jackson, 2002). Neonate larvae from the field strain and the resistant laboratory strain were placed on fruiting structures of 100-day-old cotton plants. Larval survival and fruit damage ratings were taken seven days after infestation.

For the field strain, 40% of the larvae survived on conventional cotton compared to 4% on Bollgard cotton and 0% on the two lines of Bollgard II cotton. For the lab-selected strain, 47% survived on conventional cotton compared to 19% on Bollgard cotton, thus demonstrating some resistance to the Cry1Ac toxin in Bollgard cotton. When tested against the Bollgard II cotton lines, less than 5% of the larvae survived.

No differences were seen among cotton genotypes when comparing damage to the fruit surface, which was expected because the bollworm larvae usually initiate feeding on the surface of cotton fruit without actually penetrating the fruit. However, the results were significantly different when comparing fruit penetration. For the field strain, 28% of the fruit were penetrated for conventional cotton compared to 7% for Bollgard cotton. Bollgard II cotton showed no fruit penetration damage. For the Cry1Ac-selected laboratory strain, 33% of the fruit were penetrated on conventional compared to 12% for Bollgard cotton. Bollgard II cotton showed a ten-fold reduction in the percentage of fruit penetrated, with values of 1.8% and 1.7% for the two Bollgard II cotton lines, respectively.

These data clearly demonstrate that Bollgard II cotton provides better control of both the field and laboratory-selected bollworm strains than does Bollgard cotton alone. These data predict that the Cry2Ab protein in Bollgard II cotton would be effective in the management of bollworm populations that develop resistance to the Cry1Ac toxin.

Activity of Cry2Ab against Cry1Ac Resistant Pink Bollworm (PBW) Colonies

Bollgard II cotton was evaluated for its ability to control pink bollworm strains that were previously selected in the laboratory for resistance to the Cry1Ac protein. Survivorship and damage to bolls were measured (Head, 2001). In duplicate experiments, 22.2% and 18.5% of the resistant pink bollworm were able to survive on Bollgard cotton. When tested against the Bollgard II cotton line 15985, none of the pink bollworm larvae survived. These results demonstrate that the high levels of resistance to Cry1Ac in the laboratory-selected strain do not confer cross-resistance to Bollgard II cotton, which produces

both the Cry1Ac and Cry2Ab proteins. Therefore, these studies demonstrate that plants expressing high levels of Cry2Ab can effectively control pests which develop resistance to Cry1Ac; thus, the two-gene product provides a valuable resistant management tool for control of pink bollworm.

Overall, these experiments demonstrate that the combination of the Cry1Ac and Cry2Ab proteins in Bollgard II cotton can be an effective resistance management tool for control of the primary lepidopteran pests of cotton. In the event of resistance development to a single protein, the presence of the second protein in Bollgard II cotton can provide control of the resistant strains of insect pests.

Field Data

Field efficacy data trends positively correlate to data sets observed in the lab. Numerous field efficacy trials in cotton over the past 4 seasons underscore the phase insecticidal activity increase provided by Bollgard II. Data from Bradley (Table 5) in a small plot efficacy trial demonstrated substantial decrease in damaged fruit ove the core sample dates from late July through August of 2002. Unprotected conventional plots (DPL 50) allowed 95% fruit damage while Bollgard (DPL 50B) allowed 29% damage and Bollgard II (DPL 50 BGII) only allowed 3% fruit damage from a wild population of CBW larvae. A similar trial conducted by Tunipseed (Table 6) in 2002, also with beneficial arthropods removed, highlighted the seasonal protection of Bollgard II through larval counts. In this trial, unprotected conventional cotton (DPL 50) produced 13.4 CBW larvae / 3 drop sample while Bollgard (DP 50B) produced 2.5 and Bollgard II (DPL 50BGII) allowed only 0.6 larvae.

Studies directed toward Spodoptera species also show very good activity from Bollgard II against BAW and FAW. In a pair of trials conducted in 2000 (Table 7) in Hale Center, TX and Leland, MS against Beet armyworm, Bollgard II virtually eliminated larvae found in unprotected plots. Averaged across these two trials, conventional cotton produces an average of 7.8 larvae / 3 row feet while Bollgard allowed 5.4 larvae and Bollgard II only 0.1 larvae / 3 row foot sample.

In a cages study conducted by Coots (Table 8) (Coots, 2003) where large number of FAW pupae were released on a weekly basis, Bollgard II allowed only 6% 1st and 2nd position fruit loss compared to 44% in Bollgard and 62% in conventional cotton. This data indicates that Bollgard II will have very good field activity against this pest species.

A study conducted by Smith, 2000 (Table 9) illustrates Bollgard II's excellent activity against soybean looper in cotton. In this test Bollgard II did not produce any larvae while conventional cotton recorded 28 and Bollgard cotton 23 larvae per 3-drop sample.

Refuge Plan for Bollgard II

Product stewardship and appropriate IRM plans are essential for the deployment of this two-gene product. Mathematical modeling by Roush (1994, 1998) predicts that toxin mixtures could effectively delay resistance relative to the single toxins if the mortality of susceptible insects relative to each of the individual toxins is high and resistance is somewhat recessive. The one deployment strategy that was shown to be clearly inferior was the creation of a spatial mosaic of the two toxins by using both as single toxin products in different cotton fields. For transgenic crops, the most effective strategy was to pyramid the toxins within a single product.

Similarly, Caprio (1998) used models to contrast resistance management strategies employing multiple toxins that included alternating toxins, sequential use of the toxins, and combining toxins. In combination with the use of refuges, all three strategies delayed the development of resistance, but rotations and combining toxins were more effective than replacing the toxins over time. For example, with a 5% refuge, rotations delayed resistance four times longer and combining toxins delayed resistance eight times longer than the sequential use of the same toxins. While resistance management issues with Bollgard II are highly favorable compared to a single gene product, for the initial stages of Bollgard II introduction, the IRM plan will be the same as for Bollgard.

Summary

Bollgard II is anticipated to benefit the cotton industry by providing increased efficacy on cotton bollworm, by exhibiting an expanded spectrum of activity against caterpillar pests including fall armyworm, beet armyworm and soybean looper, by providing a better resistance management tool through the two gene approach, by enabling growers to implement better integrated pest management strategies, by offering better yield potential through better insect control and by simplifying cotton insect control for growers.

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Table 1. LC50 Values against Specified Lepidopteran Species.

Lepidopteran Specie	Cry1Ac Toxin	Cry 2Ab Toxin
Heliothis virescens	0.02	0.44
Helicoverpa zea	2.11	16.75
Pectinophora gossypiella	0.01	0.04
Spodoptera exigua	>100	43.81
Spodoptera frugiperda	>100	76.31

Table 2. Cry1Ac Expression (ELISA Activity – (ug/g dry weight))

	Cry 1Ac	Cry 2Ab
Bollgard	4.8	---
Bollgard II	4.8	95

Table 3. Cry1Ac Equivalent Protein Activity Levels in Plant (ug/g dry weight)

	Leaves	Squares
Bollgard	21.71	10.0
Bollgard II	66.10	57.15

Table 4. Relative Efficacy of 1985 Isoline from Greenhouse Leaf Tissue Cry 1Ac Equivalent Protein Activity - Levels in Plant (ug/g dry weight)

Protein	Greenhouse Leaf Tissue
Cry 1Ac	13.4
Cry2Ab	40.1
Cry1Ac + Cry 2Ab	46.9

Table 5. Damaged Fruit Evaluations – Seasonal Average. J.R. Bradley – Rocky Mount, NC 2002

Line	% Bollworm Feeding Damage
Conventional	94
Bollgard	29
Bollgard II	3

Table 6. Bollworm Larval Counts Turnipseed / Hagerty – Blackville, SC - 2002

Line	Larvae / 3 row feet
Conventional	13.4
Bollgard	2.5
Bollgard II	0.6

Table 7. Beet Armyworm Larval Counts. Hale Center, TX and Leland, MS - 2000

Line	Larvae / 3 row feet
Conventional	7.8
Bollgard	5.4
Bollgard II	0.1

Table 8. Bolls Lost to FAW. Coots – Loxley, AL - 2002

Line	% Bolls Lost to FAW*
Conventional	62
Bollgard	44
Bollgard II	6

*1st and 2nd position

Table 9. Soybean Looper Counts. Smith – Fairhope, AL 2000

Line	Larvae / 3 drop samples
Conventional	28
Bollgard	23
Bollgard II	0