Expression of Cry 1Ac in Biotech Cotton

The International Cotton Advisory Committee (ICAC) estimates that 51% of world cotton production in 2007/08 was from biotech varieties planted on 44% of the world area. This 44% of the total area refers to the legally grown biotech cotton in most countries, but unofficial reports from a number of countries suggest that biotech cotton is grown illegally on a much larger area. Based on the official numbers, ICAC also estimates that 48% of the cotton traded internationally in 2007/08 will be biotech cotton produced in nine countries. The commercially approved biotech cottons are BXN™, Roundup Ready®, Roundup Ready® Flex, LibertyLink®, Bollgard®, Bollgard® II, WideStrike™, Guokang, Roundup Ready® + Bollgard®, Roundup Ready® Flex + Bollgard® II, Event 1, WideStrike™ + Roundup Ready®, WideStrike™ + Roundup Ready® Flex and LibertyLink® + Bollgard® II.

Each type of biotech cotton is approved in a limited number of countries. However, all biotech cottons, except Guokang and Event 1, are approved in the United States. In general, insect-resistant biotech cotton is approved in more countries than herbicide-resistant biotech cotton. Herbicide- resistant biotech varieties are grown on the greater part of the biotech cotton area in the United States. Biotech varieties were grown on 93% of the total cotton area in the United States in 2007/08 and it is estimated that, of that 93%, only 1-2% was planted to straight insect-resistant varieties. All the rest was planted to herbicide-resistant varieties, either alone or in combination with the insect- resistant gene. It is estimated that in 2007/08 approximately 1.5 million hectares in the United States were planted to Bollgard® II, which was almost double the area planted to it in 2006/07. Monsanto estimates that Bollgard® II will account for almost two million hectares in 2008/09, and 80% of that will be Bollgard® II stacked with Roundup Ready® Flex. The area planted to Bollgard varieties is decreasing, but the Cry 1Ac gene will still be around in the form of Bollgard® II.

The Bt toxin is expressed in all plant parts and throughout the life of the plant. However, reports show that the quantity of toxin declines in the older leaves and other plant parts, particularly close to the maturity stage. Many researchers have proved the effect of genotype on toxin expression, but the quantity of the toxin produced remains extremely high, above the quantity needed to kill the target pests effectively. After the harvest, cotton stalks are either mulched into the soil or used as fuel. The cotton seed also retains a limited expression of the protein (0.007% dry weight of the seed), but results have shown that such a low expression level provides an adequate margin of safety for any expected human exposure. Moreover, according to Monsanto, processing removes or deactivates all proteins from commodities intended for human consumption. Thus cottonseed oil and linters pose no residue concerns connected with human consumption of edible commodities produced from Bollgard® II cotton. These are the issues that will be discussed in the present article.

Mode of Action and Toxicology

Bollgard® II contains two genes, Cry 1Ac and Cry 2Ab, and their controlling sequences/promoters. The genes and their controlling sequences produce endotoxins in biotech varieties by expression in the cells of the cotton plant. The endotoxins are very specific in their mode of action. Toxins are effective against Lepidoptera and, to some extent, Cry 2Ab is effective against Dipetra (e.g. flies and mosquitoes).

Biotech cotton resistant to lepidopteron pests will have completed 12 years of successful commercial production in the United States and Australia when it is planted in 2008/09. The mode of action of Cry proteins is highly specific to species of insects due to a number of factors that must be present for the endotoxin to be effective. The endotoxin will not have its full impact in the absence of these factors, which include: high pH in the midgut, which enables the protein to be dissolved in or absorbed by tissues; existence in the midgut of the proper binding receptors, without which the endotoxin will not be dissolved in the tissues; and, in addition, specific midgut proteases must be present to convert the Cry 1Ac protoxin into the active core toxin. These conditions do exist in the target lepidopteron insects and that is why the Cry proteins are effective against these target pests. Receptors bind the toxin, opening ion-specific pores and disrupting digestive processes. Fortunately, the human gastrointestinal tract has an acidic environment resulting in a very low pH (around 1.2), rendering the Cry 1Ac and Cry 2Ab proteins effectively insoluble and, therefore, ineffective against humans or any

other living organism that does not meet the abovementioned conditions.

Australian Approach to Avoiding Resistance to Bt Toxins

Australia adopted Bollgard® (locally called Ingard) biotech cotton in 1996/97. Acting on the lessons learnt from the high resistance to insecticides developed during the 1970s and 80s, the Australian government implemented more stringent measures to prevent the development of resistance to Bt genes. The use of a refuge crop was mandatory, as it was in all other countries, but Australia also put a limit on the area to be planted to Ingard on each farm. The restriction was in force until the approval of Bollgard® II, which replaced Ingard in most of the biotech area. This was done as an added protection against the development of resistance to the single gene in Ingard varieties. With the replacement of Ingard with Bollgard® II varieties, resistance models showed that the chances of developing resistance to both genes were very slim and, consequently, in 2004/05 the area restriction was lifted. For the last three seasons since 2005/06, Bollgard® II has been sown on no less than 75% of the total planted area, the rest being under herbicide-resistant varieties.

The fact that insect-resistant biotech cotton reduces insecticide use is no surprise because that is the reason that Bollgard® II and other insect-resistant biotech cottons are grown. The percentage by which insecticide sprays may be reduced will depend on the pressure from the target pests. *H. armigera* and *H. punctigera* have been the main pests in Australia, so pesticide savings have been as high as 75% on cotton.

It is incorrectly assumed, however, that insect-resistant biotech cotton is immune to bollworms. Insertion of Cry 1Ac and Cry 2Ab does not make cotton bollworm-proof. Larvae of the target bollworms can still survive on biotech cotton, but in much lower numbers. The target bollworms have a tendency to develop resistance to the Bt genes, particularly if the resistance management strategy recommended by Monsanto is not followed. Nevertheless, studies in the United States and Australia have shown that, after more than ten years of planting biotech cotton, no change has occurred in the frequency of resistant alleles nor has any trace of resistant bollworm populations been found in the field. Resistance has not been detected in China (Mainland) either. The production system there is more complex than in Australia and the United States, but the work done in China (Mainland) on laboratory-reared bollworm populations has shown that resistance is a possibility. Studies (Barber, 2008) have shown that genes conferring resistance to Cry 1Ac are very rare. Alleles conferring resistance to Cry 2Ab are more common than expected, but also rare.

Bollworm Survival on Bollgard® II Cotton

There is no doubt that the target bollworms do survive on Bollgard® II cotton. The surviving population on Bollgard® II is not resistant to either of the Bt genes. It is thought, rather, that they survive on pollen grains that do not carry the Bt genes. This is in addition to the late season decline in efficacy attributed to lower levels of Cry 1Ac toxin occurring late in the season. There might also be other reasons, such as population shifts from non-biotech fields or alternate host crops, but whatever the reason may be, the fact is that even reduced levels of a bollworm population continue to produce losses in yield. Such losses can be checked only with additional spraying, and that is why the table below shows 3% insecticide sprays on Bollgard® II cotton (Doyle *et al.*, 2005). Any spraying against target insects on biotech cotton will have economic consequences when savings on insecticides fail to compensate for the technology fee.

Genotypic, Environmental and Agronomic Effects on Cry 1Ac Expression

One of the objectives of having Cry 2Ab in Bollgard® II is to enhance late season control of Heliothines, in addition to serving as a resistance management tool. The literature shows that both objectives have been achieved so far, but toxin expression varies and a number of factors have been proven to influence the amount of toxin expressed by plant tissues. Rochester (2006) assessed the impact of nitrogen levels, plant population density, light intensity, water management, herbicide application, soil fertility, plant growth regulator application and cultivar choice on Cry 1Ac expression in field and glasshouse experiments. He performed enzyme-linked immunosorbent assays (ELISA) on green leaves collected from plants with different treatments.

Porcentage of Insecticides / Miticides Sprayed on Conventional and Bollgard® II Cotton in Australia - 2004/05

Cotton	Insect Pests						
	***Helicoverpa* spp.**	**Mirids**	**Aphids**	**Green veg. Bug**	**Mites**	**Thrips**	**Others**
Conventional (%) (Total no. of sprays = 11.4)	93.0	0.9	4.2	0.9	0.2	1.2	0.4
Bollgard® II (%) (Total no. of sprays = 3.0)	3.0	55.0	21.0	12.0	4.0	3.0	2.0

Mid Flowering Concentration of Cry 1Ac in Cotton Grown at Various Nitrogen Levels

Nitrogen Applied (Kg/ha)	Nitrogen Status	Cry 1Ac (mg/kg)		
		Node 8	Node 13	Node 18
0	Deficient	0.48	0.53	0.44
100	Adequate	0.71	0.43	0.42
200	Excessive	0.76	0.51	0.46

Concentration of Cry 1Ac in Cotton Grown at Various Nitrogen Levels in Low and High Fertile Soils

Nitrogen Applied (Kg/ha)	Cry 1Ac (mg/kg)	
	Low N	High N
0	2.23	2.39
100	2.87	2.52
200	2.66	2.61

Cry 1Ac Protein in Cotton Leaves at Various Stages of Crop Development

Nitrogen Applied (Kg/ha)	Cry 1Ac (mg/kg)		
	Flowering	Mid Boll-fill	20% Open Bolls
0	2.39	4.87	2.96
70	2.35	5.05	2.76
140	2.63	4.07	3.45
210	2.37	4.71	3.58
280	2.72	4.82	3.73

Rochester (2006) observed that nitrogen application under low, as well as high, nitrogen status increased Cry 1Ac concentration in leaves. Higher plant population increased leaf concentration of the Cry 1Ac protein at lower node positions. Some data on the effects of nitrogen on Cry 1Ac expression are shown in the tables above.

Cry 1Ac protein concentration was determined in six varieties/genotypes two weeks prior to application of chemical defoliants. Varieties/genotypes proved to be a major source of variation in leaf Cry 1Ac expression. The quantity of Cry 1Ac ranged from 2.03 mg/kg in one variety to 5.02 mg/kg in another. In 15 trials conducted over a five-year period, Cry 1Ac protein concentrations ranged from 0.27 to 6.01 mg/kg. There was even considerable variation among individual plants within a single cultivar. Cry 1Ac protein expression was highly heritable ($h^2 = 0.94$), as parent plants produced progeny with a similar level of Cry 1Ac protein expression. Cry 1Ac protein expression was higher in older (lower) leaves. Treatment effects were also often more evident in older than in younger leaves. Short episodes of water logging, shading, herbicide application, or plant growth regulator application did not significantly affect leaf Cry 1Ac protein expression, but severely wilted plants did exhibited reduced Cry 1Ac expression. Cry 1Ac protein expression was reduced under conditions that affected cotton growth and development or plant survival, such as drought or sodic/saline soil that severely impaired crop nutrition. Cry 1Ac protein synthesis may be limited or the protein metabolized in plants subjected to environmental or edaphic stresses.

Greenplate *et al.* (2001) tested 35 candidate commercial varieties over time at 11 locations for levels of Cry 1Ac. They used quantitative bioassay methods to estimate the presence of Cry 1Ac protein in terminals, squares and young bolls. Samples were collected starting at two weeks after the pinhead square stage and continued at two-week intervals for the next 10 weeks. The results showed that field site, sampling time and variety were responsible for significant variation in Cry 1Ac concentrations in terminals and squares. For terminals, sampling time was the largest source of variation, followed by site and variety. For squares, both sampling time and site were responsible for most of the variation in the Cry protein. Bolls exhibited significantly lower levels of Cry 1Ac protein compared to terminal tissues, but only slightly lower than squares. For fruiting structures, field site was a much larger contributor to Cry 1Ac variability than varietal background. The authors observed, based on the reported data, that differences in field efficacy for less sensitive species like fall armyworm *Spodoptera frugiperda* and cotton bollworm *Helicoverpa zea* are likely functions of differences in terminal/foliar levels of Cry 1Ac protein as influenced by plant age. Greenplate *et al.* (2001) also observed that in spite of statistically measurable differences in Cry 1Ac levels among varieties, the data are insufficient to suggest that measurable differences among varieties will be found in field efficacy against the target insects.

Cry Protein Expression Levels in Various Tissues for Selected Transgenic Crops

Crop	Protein	Leaf (µg/g)	Root (µg/g)	Seed (µg/g)	Whole Plant (µg/g)
Bt 11 Corn	Cry 1b	3.3	2.2-37.0 (extractable)	1.4	
Mon 810 corn	Cry 1Ab	10.34		0.19-0.39	4.65
Corn	Cry 1F	56.6-148.9		71.2-114.8 total protein	250
Mon 863 corn	Cry 3Bb1	30-93	3.2-6.6	49-86	13-54
Cotton	Cry 1Ac	2.04		1.62	
Potato	Cry 3A	28.27	0.39 (tuber)		3.3

The work done in India (Kranthi *et al.*, 2005) also showed that expression of Cry 1Ac protein is dependant on genotype (hybrids in India) and plant parts. Kranthi and his team (2005) observed that toxin expression in the boll-rind, squares and in the ovary of the flowers was clearly inadequate to confer full protection to the fruiting parts throughout the life of the plant. They correlated increasing levels of *H. armigera* survival with the reduction of toxin levels below 1.8 µg/g in plant parts. Kranthi *et al.* (2005) tested eight Bt hybrids and their isogenic non-Bt lines in replicated trials during 2003/04. Cry 1Ac concentration was estimated using the commercially available 'Bt-Quant' ELISA kit produced by Innovative Biosciences in India. The results showed that Cry 1Ac was high, 4.42-6.61 µg/g, in the upper canopy leaves early in the season at 30 days after sowing. A gradual decline in expression was observed over time in all Bt hybrids. The decline started rather early in some hybrids compared to others, but ultimately, Cry 1Ac declined to below 0.47 µg/g in all hybrids, although it never declined to undetectable levels. There were significant differences in expression levels between plant age intervals.

Cry 1Ac expression was found to be highest in leaves, followed by squares, bolls and flowers. Kranthi *et al.* (2005) pointed out that *Helicoverpa* species are at least ten times more tolerant to the Cry 1Ac protein as compared to the tobacco budworm, *Heliothis virescens*, which is a major pest on cotton in the United States. Bt cotton varieties in the United States cause as high as 99–100% mortality in susceptible *H. virescens*. This is partly due to that fact that aside from the fruiting parts, *H. virescens* also feeds on leaves. In contrast, *H. armigera*, which is the major target pest of Bt cotton in Australia, India, China (Mainland) and Pakistan, is primarily a bollworm and prefers feeding on fruiting parts, seldom on foliage. Thus, a higher level of expression in leaves is more advantageous to Bt cotton in the United States, where *H. virescens* is the major pest, compared to those countries where *H. armigera* is the major pest on cotton. Therefore, biotechnology efforts in these countries should focus on developing biotech cotton varieties with tissue-specific promoters to enhance the expression of toxin in fruiting parts.

The paper by Kranthi *et al.* (2005) created some controversy in India and the Genetic Engineering Approval Committee (GEAC), the supreme biotech agency for approval of biotech crops in India, discussed the issue at a number of meetings. Ultimately, the GEAC admitted the possibility of variation in Cry 1Ac expression in specific tissues as a function of the genetic background of the host and, consequently, has accepted the possibility that under certain conditions control of target pests by biotech cotton might be insufficient. However, some NGOs and agencies that are not sympathetic to the technology have used the paper to serve their own purposes and reject the technology.

It is generally accepted that the simplest and most effective impact of insect-resistant biotech genes takes place when the Bt proteins are directly consumed by the target species. In the case of lepidoptera on cotton, direct consumption will result exclusively from feeding on bolls/fruiting forms and leaves. The toxin concentration in the roots, stems or branches is of no consequence in cotton. The amount of Cry protein expression in the plant tissue is the result of two factors, the event and the promoter. The event is the actual act of inserting the gene coding for Bt production into the genetic material of the plant. The insertion location determines where in the plant tissue the Bt protein is expressed and in what concentrations. For its part, the promoter tells the gene when and where to produce the Bt protein. Several promoters are used, and this also affects the quantity of Bt expression. The data in the table above reflects tissue expression levels submitted to the US Environmental Protection Agency (EPA) for some Bt crops (Clark *et al.*, 2005).

The data below show that in cotton Cry 1Ac expression in the leaves is minimal as compared to other crops and other plant parts. Some earlier studies had suggested that Bt proteins are at their highest expression at the seedling stage, but with the significant effects that the newly identified factors have on

Cry 1Ac Expression Changes (µg/g fresh weight) at Different Stages of Crop Development (Average of eight Bt hybrids)

Days After Sowing	Upper Leaves	Mid-canopy leaves	Bottom leaves
30	5.51	-	-
58	3.31	3.48	5.49
70	2.17	3.18	3.60
85	1.96	3.06	3.07
95	0.95	1.55	2.19
110	0.30	0.54	1.10
124	0.13	0.13	0.39
138	0.23	0.23	0.17
148	0.05	0.05	0.10

expression of the protein, this conclusion may have changed. However, no one can doubt that the maximum quantity of Bt protein/hectare or unit area occurs at the stage when the plant biomass in the field is greatest. As far as cotton is concerned, the same biomass may even have variable quantities of the Cry proteins. It is important to determine the quantity of the protein not only to monitor the protein efficacy, but also to determine what effects, if any, the protein may have on non-target species. Here again, the observations made by Kranthi *et al.* (2005) on the main target species in the area and their feeding behavior are also valid. Additionally, one must take into account the fact that cotton stalks are mulched into the soil along with their content of Bt protein.

Sims and Ream (1997) calculated that a mature transgenic cotton crop would add approximately 1,174 g/ha or 1.6 µg/g of Bt protein to the soil. Some other data from the US Environmental Protection Agency (2001) suggest that only 3.56 g/ha of Cry 1Ac would be added to the soil. There is no hard evidence available to support one or the other estimate, but the three factors that would determine the quantity of the protein added to the soil are: concentration in the plant parts, plant population and plant size. Sims and Ream (1997) used a concentration of 34 µg/g in leaf tissues and the US EPA may have used whole plants and different stages of the crop, but both used a plant stand of 60,000 per hectare. Furthermore, to determine the level of protein incorporation into the soil from Bt cotton it is also necessary to take into account the depth in the soil at which toxin concentrations are measured.

Petals are wrapped around the stigma and style in a flower bud, but, as soon as the flower is ready for fertilization, the petals open outward exposing both stigma and anthers for cross-pollination. Depending upon growing conditions, most of the time that the stigmas are exposed takes place when self-pollination has already occurred. Once the cotton flower is fertilized, the petals surrounding the ovary close in once again, change color and start their detachment process from the flower base. The fertilized flower has become a tiny boll and as it starts growing bigger, it begins to push the petals off the flower base in a vertical direction. Petals start drying on the boll tip where they remain for varying lengths of time. The boll tips where the petals remained for the longest time are said to carry lower amounts of Cry 1Ac than those where petals were shed rather quickly. This observation is consistent with other conclusions to the effect that leaves low in chlorophyll content have lower amounts of Cry 1Ac. On the other hand, it is also claimed that the corn ear worm, *Helicoverpa zea* prefers to enter the boll from the boll tip. Thus breeders should be selecting varieties that shed petals comparatively quickly.

A study carried out at the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences in Beijing on biotech varieties with a Cry 1Ac/Cry1Ab fused gene found that the toxin content in Bt cotton varieties changed significantly over time, depending on plant part, growth stage and variety. The toxin was expressed at higher levels early in the season, declined in mid-season and rebounded late in the season. These findings have not been confirmed in the rest of the literature, but if this is the case, it could help to avoid late-stage spraying against target pests. On the other hand, this variation might help the target population to develop resistance to the toxin.

In conclusion, it may safely be said that genotypes are the most important factor in determining Cry 1Ac expression. However, a number of other factors related to growing conditions also affect toxin expression, so the need persists to measure toxin expression and even make some changes in production practices if the maximum benefits are to be derived from use of the technology.

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