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PRE-MARKET ENVIRONMENTAL RISK ASSESSMENT OF TRANSGENIC PLANTS: A CASE-STUDY APPROACH UTILIZING MON 15985 COTTON

The development and French translation of this case study has been supported in part by funding from the United States Agency for International Development.

August 2007

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COTTON EVENT MON 15985 CASE STUDY

1. INTRODUCTION TO THE CASE STUDY

This case study has been developed as a tool for providing risk assessors with a practical illustration of the concepts of risk assessment discussed in the previous sections. For the purposes of this training tool, Monsanto Company has generously consented to the use of some of the information provided in their regulatory submissions for insect-resistant transgenic cotton event MON 15985 (Bollgard II®). It must be noted, however, that in order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original applications. Certain information has been reduced to summaries and the data as presented in the case study are only a subset of that which was actually submitted. The case study does not constitute a complete application nor is it to be considered a complete risk assessment. To that end, the use of this information in the form of a training tool does not constitute an endorsement of the information or product nor should it be considered as a reflection of any of the original submissions.

Transgenic cotton event MON 15985 (OECD unique identifier MON-15985-7) was developed through biolistic transformation of Bollgard® cotton (OECD identifier MON-ØØ531-6) with DNA containing the *cry2Ab* gene from *Bacillus thuringiensis*. Thus, event MON 15985 cotton produces two insecticidal proteins, Cry1Ac and Cry2Ab, and provides protection against a range of Lepidopteran species including: tobacco budworm (*Heliothis virescens*), pink bollworm (*Pectinophora gossypiella*), cotton bollworm (*Helicoverpa zea*), cabbage looper (*Trichoplusia ni*), saltmarsh caterpillar (*Estigmene acrea*), cotton leaf perforator (*Bucculatrix thurbeiella*), soybean looper (*Pseudoplusia includens*), beet armyworm (*Spodoptera exigua*), fall armyworm (*Spodoptera frugiperda*), yellowstriped armyworm (*Spodoptera ornithogolli*) and European corn borer (*Ostrinia nubilalis*).

Cotton event MON 15985 was first deregulated in the United States in 2002 and since that time has been authorized for general (commercial) cultivation in Australia, India and South Africa. In addition, several other countries have authorized the use of products derived from event MON 15985 cotton (*e.g.*, refined oil, cottonseed cake and meal) in livestock feed and/or human food. These countries include Canada, European Union, Japan, Korea, Mexico, and the Philippines (Table 1).

Table 1 Summary of regulatory approvals for event MON 15985 (June 2007)

Country	Environment (Year)	Food and/or Feed (Year)	Food (Year)	Feed (Year)
Australia	2002		2002	
Canada			2003	2003
European Union		2005		
India	2006			
Japan			2002	2003
Korea			2003	2004
Mexico		2003		
Philippines			2003	2003
South Africa	2003			
United States	2002		2002	

Notes:
Australia: Commercial production limited to New South Wales and southern Queensland.
Canada: Not grown in Canada. Not subject to variety registration.
European Union: Notified as an existing product on 18 April 2005.

The following presentation of the data generated to support the environmental safety of cotton event MON 15985 primarily address considerations around environmental risk assessment of a transgenic plant and do not specifically address the additional issues pertinent to human food or livestock feed safety of derived products.

2. BACKGROUND INFORMATION

In order to be able to assess the environmental safety of a transgenic plant, one must be familiar with both the biology of the plant itself, as well as the agricultural or silvicultural practices employed in its cultivation.

2.A THE HOST ORGANISM

A brief description of the host organism, *Gossypium hirsutum* (cotton), focussing mainly on its reproductive biology, is provided below. Where possible, information pertinent to the biology, cultivation and uses of cotton in Africa has been presented.

2.A-1 Taxonomic Description

Cotton refers to four species in the genus *Gossypium* of the family Malvaceae - *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L. - that were domesticated independently as a source of textile fibre (Brubaker *et al*., 1999). Globally, the *Gossypium* genus comprises about 50 species (Brubaker *et al*., 1999), 18 of which can be found in Mexico, 14 in north-east Africa and Arabia and 17 in Australia.

Most commercially cultivated cotton is derived from two species, *G. hirsutum* (Upland cotton, 90% of world plantings) and *G. barbadense* (Pima, or Long-staple cotton, which accounts for 3% of world production). *Gossypium hirsutum* is the species planted in Burkina Faso, Tanzania and Kenya, with *G. barbadense* not contributing significantly to cotton production. Two other species, *G. arboreum* and *G. herbaceum*, are cultivated in Asia, but are not grown commercially in West and East Africa.

2.A-2 Centres of Origin and Genetic Diversity for Cotton

DNA sequence data from extant *Gossypium* species suggests that the genus arose about 10–20 million years ago (Wendel and Albert, 1992; Seelanan *et al.*, 1997). The

geographic centre of origin of the genus has not yet been identified. The genus radiated into a number of geographic centres of diversity including Africa-Arabia, Australia, and Mesoamerica (Peruvian, Ecuadorian, Bolivian region). Globally, the *Gossypium* genus comprises about 50 species (Brubaker *et al.*, 1999). The place of origin of the genus is not known, however the primary centres of diversity for the genus are west-central and southern Mexico (18 species), north-east Africa and Arabia (14 species) and Australia (17 species).

2.A-3 Ploidy of Cotton, its Progenitors and any Sexually Compatible Species

The 50 species of the genus *Gossypium* differ greatly in morphology, ecology, and physiology, and the evolution of these differences was accompanied by extensive chromosomal evolution. Today, there are 45 diploid (2n=26) and 5 allotetraploid species in the genus divided into eight recognized genomes: Genomes A, B, E, and F are found in the African-Arabian-Asian region; C, G, and K are found in Australia; and the D genome is found in the New World. The AD genome is recognized as the result of a hybridization event between the A and D genomes which resulted in the allotetraploid species.

G. hirsutum and *G. barbadense* are both allotetraploids. They have been found to contain two genomes: A and D. Current hypothesis holds that the A and D genomes must have been in proximity at some point in the Earth's history, despite their current oceanic separation (A genome is found in Africa-Arabia and D-genome in Mexico). Molecular data indicates that the hybridization event leading to the allotetraploids occurred one to two million years ago. Other members of the AD genome grouping include: *G. mustelinum*, found in a remote region of northeast Brazil; *G. darwinii*, found only on the Galapagos Islands and *G. tomentosum*, found on the Hawaiian Islands. *G. hirsutum* and *G. barbadense* have large ranges including Central and South America, the Caribbean and the Pacific islands of the Solomons and Marquesas. The putative point of origin of the AD allotetraploids is thought to be the Isthmus of Tehuantepec.

It is thought that the closest living model of the paternal D-genome donor to the AD complex is *G. raimondii*, found in Peru. Another possible D-genome donor is thought to be *G. gossupioides*. It is not known what species contributed the A-genome, as the ancestor is presumed to be extinct. It is known that *G. herbaceum* is genomically more similar to the A subgenome present in the allotetraploids than the other member of the A-genome, *G. arboretum*.

2.A-4 Cotton Breeding, Seed Production, and Agronomic Practices

In nature, *G. hirsutum* is a perennial shrub that grows to about 1.5 meters in height. Commercially, however, *G. hirsutum* is cultivated as an annual, with destruction of plants after harvesting the fruit for seed and fibre. Second season re-growth (ratooning) of cotton is practiced in some areas, but is expressly prohibited in others where it is believed to compound pest pressures. Cultivated cotton is grown either as a dryland crop that relies on rainfall or as an irrigated crop where a reliable water supply is available.

Typical cotton farming practices include soil preparation, planting, managing weeds, pests and watering during the growing season. Cotton growers may also plant other

crops during the off-season period. The timing of cotton cultivation varies depending on climate. Cotton is planted when the soil temperature reaches 14°C at a depth of 10 cm for at least three days.

Agronomically, the growth of cotton can be divided into three key developmental phases: germination and seedling establishment; leaf area and canopy development; and reproduction and dispersal. Total developmental time, from germination to maturation of the first fruit, is usually about 15–17 weeks, although this may be affected by temperature and other environmental variables. Key growth stages for cotton in the United States are shown in Table 2.

Table 2 Cotton growth stages (BIO, 2006)

Growth Stage	DD60's (GDDs)	Nodes	Days after planting
Emergence	50	0	5 – 15
4th true leaf	250	4	20 – 30
1st square (pinhead)	350 – 450	5 – 8	30 – 45
1st bloom	800 – 850	15 – 18	50 – 80
Cutout	1300 – 1500	20 – 24	80 – 120
Defoliation	1800 – 2000	21 – 28	120 – 170
Harvest	1900 – 2600	21 – 30	130 – 180

2.A-5 Consumption and uses of Cotton

Cotton is grown primarily for its seed bolls that produce the lint, or fibre, that is the raw material for numerous textile products. About two thirds of the harvested cotton crop is seed which is separated from the lint during ginning. The cotton seed is crushed to produce cottonseed oil, cottonseed cake (meal) and hulls. Cottonseed oil is used primarily as cooking oil, in shortening and salad dressing, and is used extensively in the preparation of snack foods such as crackers, cookies and chips. The meal is used as a food source in some countries; meal and hulls are an important protein concentrate for livestock and may also serve as bedding and fuel. Linters, or fuzz, which are not removed in ginning, are used in felts, upholstery, mattresses, twine, wicks, carpets, surgical cottons, and in industrial products such as rayon, film, shatterproof glass, plastics, sausage skins, lacquers, and cellulose explosives.

2.A-6 Reproductive Biology

Floral Morphology:

Gossypium spp. have complete, hermaphroditic, solitary, axial flowers that begin to form four to five weeks after planting (Watson and Dallwitz, 1992; Oosterhuis and Jernstedt, 1999; Macfarlane *et al.*, 2002). The floral buds, also known as "squares", form apically and flower approximately 25 days after they first appear. Flowering follows a distinct pattern: first flowers open low on the plant at the first position on the fruiting branch and approximately three days later a flower will open on the next higher fruiting branch at the same position. About six days after the first flower on a branch opens, the second on that branch will open. This same pattern will continue until defoliation or first frost, provided the plant continues to actively grow. Flowers open at dawn and remain open for a single day (Oosterhuis and Jernstedt, 1999). Flowers on *G. hirsutum* are creamy white upon opening but turn pink-red the following day after anthesis and pollination have occurred (Oosterhuis and Jernstedt, 1999).

Flowers are composed of a calyx, corolla, androecium and gynoecium enclosed in three photosynthetic bracts with three to six nectaries located at the base of the bracts (Watson and Dallwitz, 1992; Oosterhuis and Jernstedt, 1999; Macfarlane *et al.*, 2002). The calyx is lobulate with five sepals fused along most of their length (Oosterhuis and Jernstedt, 1999). The corolla is sympetalous, with five petals, fused at their base (Oosterhuis and Jernstedt, 1999).

The androecium, or male organ, is composed of a staminal column that surrounds the style. There is an indefinite number of unilocular stamens, ranging between 50 and 100. The stamens which occur in pairs are also fused along much of the length of their filaments (Oosterhuis and Jernstedt, 1999; Macfarlane *et al.*, 2002). The gynoecium, or female organ, is composed of a superior ovary, a single, simple and apical style, and one two to five-lobed stigma (Oosterhuis and Jernstedt, 1999). The ovary is composed of three to five syncarpous carpels, each of which constitutes one locule. Each locule contains eight to ten ovaries, but will only produce approximately eight seeds each (Oosterhuis and Jernstedt, 1999).

Pollen and Pollination:
Soon after anthesis, the anthers of cotton flowers dehisce, discharging their pollen. Cotton pollen is relatively large and heavy, and not easily dispersed by wind (Jenkins 1992). Cotton is a facultative self-pollinator and an opportunistic out-crosser when insect pollinators are present (Oosterhuis and Jernstedt, 1999). Cotton pollen remains viable for about 12 hours (Govila and Rao, 1969). Fertilization of ovules occurs about 12–30 hours after pollination.

G. hirsutum flowers are entomophilous (attractive to insects). Pollination occurs either by insect dispersal or by self-fertilisation as pollen is too heavy and sticky to be transported by wind (Llewellyn and Fitt, 1996). Hymenopterous insects are the most common order of pollinators with *Bombus* spp. (bumblebees) and *Apis* spp. (honeybees) being the most significant genera (Umbeck *et al.*, 1991). When insect vectors are present, cross-pollination can be as much as 50 to 80% within a stand.

Out Crossing:
Insect prevalence strongly influences out-crossing rates for cotton (Elfawal *et al.*, 1976; Moresco *et al.*, 1999), and varies with location and time (Moffett *et al.*, 1975; Elfawal *et al.*, 1976; Moffett *et al.*, 1976). Insect visitation rates, however, may overestimate cross-pollination rates because many potential pollinators preferentially target nectaries rather than the pollen (Moffett *et al.*, 1975; Rao *et al.*, 1996). Many field-based assessments estimate out-crossing at 10% or less (Meredith and Bridge, 1973; Gridley, 1974; Theron and van Staden, 1975; Elfawal *et al.*, 1976; Umbeck *et al.*, 1991; Llewellyn and Fitt, 1996). Higher estimates (16.5% to 25%) have been reported in a few cases (Smith, 1976; Moresco *et al.*, 1999).

The frequency of cross-pollination decreases with distance from the pollen source. Umbeck *et al*. (1991) used a selectable marker to examine cross-pollination from a 30 × 136 meter source of transgenic cotton. Cross-pollination decreased from five to less than one percent from one to seven meters, respectively, away from the source plot. A low

level of cross-pollination (less than one percent) was sporadically detected to the furthest sampling distance of 25 meters. Additionally, in a study with various field designs, Llewellyn and Fitt (1996) also found low levels of cross-pollination in cotton, decreasing to below 0.3% at 16 meters from the source. Berkey *et al*. (2002) reported that cross-pollination between fields separated by a 4 meter road decreased from 1.89% in the row nearest the source to zero percent at approximately 23.2 meters into the tested field.

These low outcrossing rates were further confirmed by out-crossing studies conducted at confined field trials of MON 15985 in Burkina Faso (Table 3).

Table 3 Out-crossing frequencies of MON 15985 in Burkina Faso

Distance (m)	Border Unsprayed[1]	Border Sprayed[2]
2	5.50%	8.30%
5	1.90%	4.20%
10	0.80%	5%
15	0.40%	0%

1. The border of the field was unsprayed with insecticide. N=4140
2. The border of the field was sprayed with insecticide. N=120

Fruit Development:

The growth and development of cotton fruit, known as 'bolls', begins immediately following fertilization although the most rapid period of growth occurs after about 7–18 days (Oosterhuis and Jernstedt, 1999). During development, the bolls are spherical to ovoid and pale green. Maximal boll size is achieved about 25 days after fertilization, with full maturity achieved approximately 20 days later. Mature bolls are thick and leathery, and dry rapidly to become brittle and brown. Such fruit often split open, revealing the seeds and associated fibres.

Seed Morphology:

Cotton is grown primarily for its fibres, which are produced by epidermal cells of the seed coat. Prior to ginning and delinting, the seed coat bears two types of fibres: long lint fibres valued by the textile industry and short, fuzzy fibres, known as linters used in various products including foods. After ginning, the cotton seed is still covered in linters and is known as 'fuzzy seed'. Cotton seeds are ovoid in shape, slightly pointed, about 10 mm long x 4 mm wide, and dark brown in colour (called 'black seed'). Each boll produces about 20 to 25 seeds.

Seed Dispersal:

As cotton does not generally reproduce vegetatively (Serdy *et al.*, 1995), spread within the environment occurs by seed dispersal. Dispersal of cotton seeds is a physical process. Observations of dispersed seeds and the occurrence of volunteer plants in northern Australian cotton trials indicate that delinted black seed has the lowest risk of unintentional spread within the environment (OGTR, 2002). When dispersal of black seed occurs, it is associated with spillage at sowing in cotton production areas.

Fuzzy seed is commonly used as livestock feed and therefore has a high potential for dispersal to non-cotton production habitats. Unprocessed 'seed cotton' that retains all of the fibres attached to the seedcoat, also has a high potential for dispersal within the environment. Data from Monsanto (OGTR, 2002) suggest that volunteers from dispersed

seed cotton were relatively common in irrigation channels and drains, and along roadsides. Roadside volunteers most likely established following seed cotton spillage during transport of cotton modules from the paddock to the gin.

Post-dispersal, seeds that do not germinate are likely to be removed by seed predators or rot, rather than become incorporated into a persistent soil seed bank.

Occurrence of Intraspecific or Interspecific Hybrids:
Insect-mediated cross-pollination between *G. hirsutum* plants is the most likely means by which cotton genes may be dispersed in the environment. Gene transfer between adjacent *G. hirsutum* individuals does occur, albeit at relatively low frequencies. Llewellyn and Fitt (1996) estimated that cross-pollination between cotton plants in adjacent rows accounted for only 1 to 2% of seeds. Fertile progeny are also produced when *G. hirsutum* is cross-pollinated with *G. barbadense* (Brubaker *et al.*, 1999), thereby potentially providing another ready means by which *G. hirsutum* genes may be spread in the environment. However, in West and East Africa, cultivation of *G. barbadense* is relatively rare.

Gene flow from cultivated *G. hirsutum* to feral *G. hirsutum* populations is possible and viable seeds would be generated if this occurred. Ensuring geographic distance between feral cotton populations and cultivated cotton plantations would reduce this possibility. If cotton volunteers establish in areas adjacent to existing feral populations, such as may occur along certain transportation routes, the potential for spread of the transgenes to these feral populations could increase. The potential for cultivated *Gossypium hirsutum* to hybridize with feral cotton populations in Burkina Faso is unlikely since such feral populations have not been reported.

2.A-7 Distribution of Related Species, including any Evidence of Weediness
Cotton has been grown for centuries throughout the world without any reports that it is a serious weed pest. Modern cotton cultivars do not possess any of the attributes commonly associated with problematic weeds, such as seed dormancy, persistence in soil seed banks, germination under adverse environmental conditions, rapid vegetative growth, a short life cycle, very high seed output, high seed dispersal and long-distance dispersal of seeds (Keeler, 1985; Keeler, 1989).

G. hirsutum and *G. barbadense* may occur as escapees from agriculture and/or as small populations of naturalised exotic species (Lazarides *et al.*, 1997; Sindel, 1997). Where such populations have established, however, they are not considered a threat to agricultural productivity or native biodiversity.

In the Africa area there are 13 known species of indigenous *Gossypium* (Table 4). There has not been a comprehensive survey of *Gossypium* species in East Africa published to date, although Vollesen (1987) published an account of African species in the Kew herbarium collection. From that work it was noted that three species have been found in East Africa in the past: *G. longicalyx*, *G. benadirense*, and *G. somalense*. All the known East African species are diploid. *Gossypium longicalyx* is cytogenically distinct, the only

member of the F-genome (Percival *et al.*, 1999). *G. benadirense* and *G. somalense* are members of the E-genome.

Hybridization between members of distinct genomes within *Gossypium* species requires human intervention and produces functionally infertile offspring (Percival *et al.*, 1999). Each of two potential barriers must be overcome before gene flow can occur successfully. Pre-zygotic barriers include geographic separation, differences in floral phenology, different pollen vectors and different mating systems such as stigmatic or stylar incompatibility systems. Post-zygotic barriers include genetic incompatibility at meiosis, selective abortion, lack of hybrid fitness and sterile or unfit backcross progeny (Brown *et al.*, 1997). Even if there are common pollinators present, the flowers are open simultaneously and receptivity is coordinated, and stands of plants are in close proximity, chromosomal incompatibility between diploid species and allotetraploid species precludes the possibility of natural interspecific hybridization between the wild African species and cultivated cotton.

Table 4 Known distribution of *Gossypium* species in Africa (Vollesen, 1987; Percival *et al.*, 1999)

Genome	Species	Known Range
A	G. *herbaceum*	Mozambique, Zimbabwe, Botswana, Angola, Namibia, Swaziland, Transvaal province and Natal (South Africa)
B	G. *anomalum*	Angola, Namibia (poss. Niger, Chad, Sudan)
B	G. *triphyllum*	Angola, Botswana, Namibia
B	G. *capitatis-viridis*	An island endemic, Cap Verde Islands
F	G. *longicalyx*	Sudan, Uganda, Tanzania
E	G. *benadirense*	Ethiopia, Somalia, Kenya
E	G. *bricchettii*	Somalia
E	G. *vollesenii*	Somalia
E	G. *stocksii*	Somalia, Oman
E	G. *somalense*	Niger, Chad, Sudan, Ethiopia, Somalia, Uganda, Kenya (sparse distribution)
E	G. *areysianum*	South Yemen
E	G. *incanum*	South Yemen
?	G. *trifurcatum*	Somalia

Other members of the tribe Gossypieae present in Africa include the genera *Cienfuegosia*, *Thespesia* and *Gossypiodes*. *Cienfuegosia* and *Thespesia* are limited to tropical Africa. *Gossypiodes* (*G. kirkii*), are found in East Africa and Madagascar (Wendell and Cronn, 2003). *Ceinfuegosia* and *Thespesia* are diploid species (2n=20 and 2n=26, respectively) and are not likely to be chromosomally compatible with cultivated cotton. *Gossypiodes* is considered the closest sister genus to *Gossypium*, having diverged from the *Gossypium* clade in the Pleistocene era, but is also a diploid species (Wendell and Cronn, 2003).

Gene transfer to unrelated plant species is highly improbable because of pre- and post-zygotic genetic incompatibility barriers that are well documented for distantly related plant groups. No evidence for horizontal gene transfer from cotton to any other organism or microorganism has been identified.

2.A-8 Common Pests and Diseases of Cotton

Of the 30 pests of cultivated *G. hirsutum*, the most important are the caterpillars of *Helicoverpa armigera* and *Helicoverpa punctigera*, and the spider mite *Tetranychus urticae* (Shaw, 2000; Pyke and Brown, 2000).

The cotton bollworm (*H. armigera*) is a noctuid moth that occurs throughout the Australasia-Pacific region, in Africa and in Western Europe. It has a wide host range and its caterpillars attack many field and horticultural crops. Over the past thirty years it has been largely controlled by synthetic pesticides, leading to widespread evolution of resistance to many of these chemicals. For example, typically 80–90% of the insects are now resistant to synthetic pyrethroids. In cotton, the adult moth lays its eggs on young terminal branches, and the eggs hatch into larvae (caterpillars) within two to three days. The caterpillars attack young leaves and flower buds (squares) and can burrow into the developing fruit, consuming developing seeds and fibres. The caterpillar stage lasts for 15–20 days and *H. armigera* cotton bollworm may go through four to five generations during the cotton-growing season. The last generation goes into a period of suspended development or 'diapause' over winter, burrowing into the soil around the base of the plants. The over-wintering pupae emerge from the soil in the following spring. Mechanical cultivation of the soil at the end of the cotton-growing season disturbs the exit tunnels made by the larvae when they burrow into the soil. This strategy, known as "pupae busting", can kill over 90% of the pupae in the soil. This is an effective mechanism for reducing the number of moths that emerge in the spring and for delaying development of insects with resistance to insecticides used on cotton.

In Burkina Faso, *H. armigera* breeds in two types of asynchronous agrosystems (Nibouche, 1994). During the rainy season, from mid-June to October, the pest colonizes rain fed crops (mainly cotton and maize) and weeds. Throughout the dry season, from October to mid-April, *H. armigera* attacks irrigated crops. During the 2-month period between mid-April and mid-June, irrigated crops are harvested and no population of *H. armigera* is noticed. Biological studies have shown that diapause occurs in Burkina Faso, but at very low rates (Nibouche, 1994). Seasonal migrations could occur between rain fed crops and irrigated crops within Burkina Faso or, on a greater scale, between the Sudanese climatic area (Burkina Faso) and the Guinean climatic area (Ivory Coast). Such migrations following the seasonal movements of the inter-tropical convergence zone have been documented in Heteroptera of the genus *Dysdercus* (Duviard, 1981) and in *Agrius convolvuli* L. (Lepidoptera, Sphingidae) (Bowden, 1973).

The cotton whitefly (*Bemisia tabaci*) is a serious pest of fibre, horticultural and ornamental crops worldwide. It can cause extensive damage through direct feeding, honeydew production and as a viral vector.

Diseases in cotton may affect the quality of the fibre and seed, as well as the yield and cost of production of the cotton crop (Bell, 1999). The main diseases affecting cotton include: seedling diseases; fungal wilt diseases (Fusarium wilt or Verticillium wilt); and leaf spots.

Verticillium wilt and Fusarium wilt are fungal diseases caused by *Verticillium dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum*, respectively. The fungi infect the plant root tips, enter the xylem vessels and proliferate throughout the xylem vessels of the plant. This plugs the vessels and plants develop the wilt symptoms. Verticillium wilt is widespread in most cotton growing areas, and has a wide host range, including many common weeds. In Tanzania, there has been a dramatic increase in the incidence of Fusarium wilt since 1969, with an average increase of 8%, Verticillium wilt was also present (ICAC, 2003).

Seedling diseases can be caused by several fungi, commonly *Pythium* and *Rhizoctonia*. The diseases can cause seed rot and damping-off, and are most likely to occur when cool, wet weather occurs soon after planting.

Leaf spots can be caused by fungi (Alternaria leaf spot, caused by *Alternaria macrosporia* or *A. alternata*) or bacteria (bacterial blight caused by *Xanthomonas campestris*). Alternaria leaf spot is present in Tanzania and Uganda (ICAC, 2003). In addition, false mildew has been reported in Tanzania and Uganda (ICAC, 2003).

Nematodes, particularly *Meliodogyne* spp. and *Pratylenchus* spp. are also present in cotton in East Africa (ICAC, 2003). In Tanzania, *M. incognita*, *Pratylenchus* spp., *Rotylenchulus* spp., *Xiphinema* spp., A*phelenchus* spp., and *Tylenchus* spp. have been identified (ICAC, 2003). Uganda is known to have *M. incognita*, *M. acronea*, *Pratylenchus* spp., and *Rotylenchulus* spp.

2.A-9 Potential Interactions with other Organisms

In its growing environment, cotton has ongoing interaction with numerous species. More than 1,326 species of insects have been reported in commercial cotton fields worldwide but only a small proportion of these are pests (Matthews and Tunstall, 1994).

2.B THE DONOR ORGANISM(S)

Information about the natural history of the donor organism for any expressed gene products is required, particularly if the donor or other members of its genus normally exhibit characteristics of pathogenicity or environmental toxicity, or have other traits (*e.g.*, source of significant allergens) that affect human health.

2.B-1 Identification of Donor Organisms

Bacillus thuringiensis:

Event MON 15985 contains modified versions of the *cry1Ac* and *cry2Ab* genes, both derived from strains of *B. thuringiensis*. The native *cry1Ac* and *cry2Ab* genes were recreated synthetically to optimize for expression in plants.

Escherichia coli:

Event MON 15985 also expresses the *nptII* and *uidA* genes, both derived from *E. coli*. The *nptII* gene was derived from the Tn5 transposable element and encodes the enzyme neomycin phosphotransferase II. The *nptII* gene was used as a selectable marker during the creation of event MON 531, the parent line used during the plant transformation to create event MON 15985. The *uidA* (synonym *gus*) gene, isolated from *E. coli* strain

K12, encodes the enzyme ß-D-glucuronidase (GUS), and was included as a scorable marker allowing colorimetric identification of MON 15985 transformants following histochemical staining.

2.B-2 Safety of the Donor Organisms

Bacillus thuringiensis:

Bacillus thuringiensis is a crystalliferous spore-forming gram-positive bacterium that has been used commercially over the last 40 years to control insect pests. These microbes are found naturally in soil worldwide. Strains of *B. thuringiensis* control insect pests by the production of crystalline insecticidal proteins known as delta-endotoxins, each of which have a specific range of activity against target insects (Table 5). To be active against the target insect, the protein must be ingested. In the insect gut, the protein binds to specific receptors on the insect mid-gut, inserts into the membrane and forms ion- specific pores. These events disrupt the digestive processes and cause the death of the insect. There are no receptors for the protein delta-endotoxins of *B. thuringiensis* subspecies on the surface of mammalian intestinal cells; therefore, humans are not susceptible to these proteins. This has been confirmed in numerous safety studies carried out in laboratory animals, which are traditionally experimental surrogates for humans. The results of some of these studies have been published in scientific reviews (Ignoffo, 1973; Shadduck *et al*., 1983; Siegel and Shadduck, 1989). Results of unpublished safety studies generated by registrants of *B. thuringiensis* commercial preparations have also been summarized in EPA Registration Standard for *Bt* Formulations (EPA, 1988).

These scientific considerations demonstrate the history of safe use of *B. thuringiensis* preparations. Based on the available scientific data, EPA and other regulatory scientists worldwide have determined that use of registered *B. thuringiensis* products pose no significant risks to human health or non-target organisms.

Table 5 Bt δ-endotoxins and their activity against specific insect species

		Major target insects	
Cry protein	**Origin (Bt subspecies)**	**Order[2]**	**Common names**
Cry1Aa	*kurstaki*	L	silk worm, tobacco horn worm, European corn borer
Cry1Ab	*berlineri*	L,D	tobacco horn worm, cabbage worm, mosquito
Cry1Ac	*kurstaki*	L	tobacco budworm, cabbage lopper, cotton bollworm
Cry1Ad1	*aizawai*	L	several Lepidoptera
Cry1Ae1	*alesti*	L	tobacco budworm
Cry1Ba1	*thuringiensis*	L	cabbage worm
Cry1Bc2	*morrisoni*	L,D	several Lepidoptera
Cry1Ca3	*entomocidus*	L	cotton leaf worm, mosquito
Cry1Cb1	*galleriae*	L	beet army worm
Cry1Da1	*aizawai*	L	beet army worm, tobacco horn worm
Cry1E	*kenyae*	L	cotton leaf worm
Cry1Eb1	*aizawai*	L	several Lepidoptera
Cry1Fa	*aizawai*	L	European corn borer, beet army worm
Cry2Aa	*kurstaki*	L,D	gypsy moth, mosquito
Cry2Ab	*kurstaki*	L	gypsy moth, cabbage lopper, tobacco horn worm
Cry2Ac	*shanghai*	L	tobacco horn worm, gypsy moth
Cry3Aa	*san diego*	C	Colorado potato beetle
Cry3Aa3	*tenebrionis*	C	Colorado potato beetle
Cry3Ba	*tolworthi*	C	Colorado potato beetle
Cry7Aa	N/A[3]	C	spotted cucumber beetle
Cry4Aa	*israelensis*	D	mosquito (*Aedes* and *Culex*)
Cry4Ba	*israelensis*	D	mosquito (*Aedes*)
Cry9Aa	*galleriae*	L	greater wax moth
Cry9Ba	*galleriae*	L	greater wax moth

1. Adapted from Krattiger (1997).
2. L: Lepidoptera; C: Coleoptera; D: Diptera
3. N/A: Not available.

Escherichia coli:

Escherichia coli strains have been used for the last 60 years in the study of bacterial physiology and genetics. Historically, wild-type strain K12 was used in early studies on conjugation and recombination (Swartz, 1996). The use and study of strain K12 continued to predominate due to its use in the study of recombination and the generation and mapping by conjugation of a large number of mutants in metabolic pathways that aided both the studies of bacterial genetics and physiology. In a study of *E. coli* strains including representatives of the K12 strain, polymerase chain reaction (PCR) amplification demonstrated the absence of defined virulence genes that are present in known pathogenic isolates of this genus (Kuhnert *et al*., 1997). The authors concluded that the K12 strains commonly used in the laboratory are devoid of virulent factors and should be considered nonpathogenic. Similarly, in a more direct study of the pathogenic potential of K12 strains conducted using both a BALB/c mouse and chick gut model, it was concluded that the K12 strains do not possess recognized pathogenic mechanisms and should be considered nonpathogenic (Chart *et al*., 2000). Based on these studies and the fact that *E. coli* K12 has been used extensively in research and in many laboratories for decades without causing any harm, *E. coli* K12 is generally recognized as safe.

2.C THE RECEIVING ENVIRONMENT

Information about the receiving environment is critically important to assessing the possible impacts of environmental introduction (cultivation) of the transgenic plant. It

provides a baseline against which to assess environmental impacts. For this case study, the information on the receiving environment has been provided within the context of cotton cultivation in West Africa.

2.C-1 Occurrence of Sexually Compatible Species, including Feral Populations

Except for cultivated cotton varieties, outcrossing to relatives will not occur because no sexually compatible cotton varieties exist in the wild in West Africa, nor are there wild relatives that can readily interbreed with cotton in the areas of West Africa where these crops are grown. *Gossypium hirsutum* has been grown in West Africa since the 19th century. West Africa is not the centre of origin for this particular species although a near relative *Gossypium herbaceum* var. *africana* does occur. This species is a diploid while *G. hirsutum* is an allotetraploid type and no outcrossing can occur (sterile seeds would be generated if such a cross could take place in nature). Crosses in nature are considered possible only among tetraploids.

The potential for cultivated cotton species (*Gossypium hirsutum*) to hybridize with feral *G. hirsutum* cotton is unlikely since feral *G. hirsutum* cotton has not been reported in Burkina Faso.

2.C-2 Agronomic Practices

West African cotton is produced with relatively low levels of inputs, is hand picked and of high quality. Damage from lepidopteran larvae can reduce cotton harvests up to 90% (Sere, 2007). In Burkina Faso, *H. armigera* breeds in two types of asynchronous agrosystems (Nibouche, 1994). During the rainy season, from mid-June to October, the larvae colonize rain fed crops (mainly cotton and maize) and weeds. Throughout the dry season, from October to mid-April, *H. armigera* attacks irrigated crops. During the 2-month period between mid-April and mid-June, a very low rate of diapause occurs in Burkina Faso. Seasonal migrations could occur between rain fed crops and irrigated crops within Burkina Faso or, on a greater scale, between the Sudanese climatic area (Burkina Faso) and the Guinean climatic area (Ivory Coast). Such migrations have been documented in other insect species, but not in *H. armigera* (Bowden, 1973).

When available, farmers in Burkina Faso commonly apply insecticide six times a season to control feeding and sucking insects. The cost of this treatment program is approximately U.S. $69 per hectare (Sere, 2007).

Some one to two million households in West Africa cultivate cotton, with almost all cotton produced on relatively small family farms (3 to 10 hectares). Cotton production is usually part of a diverse production system involving the production of cereals, vegetables and other activities that are designed to satisfy farmers' consumption and income needs. These farms depend largely on household labour and farmers opportunistically switch types of production over time to manage risk and adapt to changing constraints (*e.g.*, climate, soil quality, *etc.*), opportunities (new urban markets, processing and marketing possibilities, *etc.*), and unexpected impacts. These family farms produce almost all of the region's staple food crops, oilseeds and cash crops, although they are also important consumers of diverse imported fruit, vegetables and processed foods (Hussein *et al*., 2005).

In Benin, Burkina Faso, Chad and Mali, cotton production is typically upland cotton, *G. hirsutum*, that is rain fed. The average yield of about one ton of seed cotton and 430 kg of lint per hectare is only 45% of the U.S. average. Recent data indicate that yields have stagnated or are decreasing in the region and a number of initiatives are aimed at reversing this trend (Bingen and Busch, 2006). These are focused on improving soil fertility and management; encouraging rotations with leguminous crops; developing appropriate water and fertilizer use and management; and developing sustainable integrated pest management systems.

3. PRODUCT CHARACTERIZATION

3.A MOLECULAR-GENETIC CHARACTERIZATION

A detailed description of the molecular characteristics of the modified plant is required in order to demonstrate that the developer has critically analyzed the plant and its products, including all novel genes and novel proteins. Characterization of a transgenic plant at the molecular level is used to provide information about: the composition and integrity of the inserted DNA; the number of copies of the inserted DNA; the number of sites of insertion; and the level of expression of the novel protein(s) over time and in different tissues. Knowledge of the introduced, or modified, genes, their regulation, and the site of integration within the host genome in the case of transgenic plants, may provide information on possible direct and indirect consequences of the genetic modification.

For example, the potential for adverse effects resulting from insertional inactivation, or activation, can be assessed by characterizing the adjacent host DNA and avoiding those products containing transgenes in close proximity to genes known to affect the production of potentially toxic or allergenic compounds. On the other hand, the molecular characterization of transgenic plants often receives a disproportionate amount of attention from regulators relative to the information it imparts in terms of food, feed or environmental safety. In part, the reason for this may be that the data generated from molecular analyses are normally less open to interpretation than data submitted to answer questions about, for example, the impact of a transgenic plant on biodiversity.

While information on the integrity and copy number of the inserted DNA are generally required by regulatory authorities, there is no evidence to suggest that transgenic plants containing multiple copies of the inserted DNA are any less "safe" than comparable plants containing only a single copy. One example of an approved event containing a high transgene copy number concerns a line of canola (*Brassica napus*; event 23-198, 23-18), which was developed by introducing a thioesterase encoding gene from the California bay tree (*Umbellularia californica*) in order to increase levels of lauric acid (12:0) and, to a lesser extent, myristic acid (14:0). The original transformation event 23 was estimated to have 15 copies of the genes, at five independent genetic loci, as shown by Southern blot (Southern, 1975) and segregation analyses.

It is important to emphasize that, while necessary, the molecular characterization of the introduced (or modified) DNA is not a sufficient means of predicting possible unanticipated consequences nor is it a replacement for direct measurements of gene

expression or changes in the levels of nutrients and antinutrients, endogenous toxicants, or potential allergens.

Because of their method of production, transgenic plants are more amenable to extensive molecular genetic characterization than are comparable plants produced using other breeding methods. In this regard, it is important to distinguish between "need to know" and "nice to know" within the context of the safety assessment. This issue is of particular relevance where the regulation of novel foods and plants with novel traits has included products derived using breeding methods for which the provision of detailed molecular information is not feasible. In these latter examples, it is difficult to argue that the safety assessment has suffered as a consequence of incomplete DNA sequence information. In short, when following a product-based approach to regulation and risk assessment, there should be a comparable standard of evidence for safety for products that are regulated alike because they present equivalent risks.

Event MON 15985 was developed by biolistic transformation of cotton meristems with purified DNA containing the *cry2Ab* and *uidA* (GUS) expression cassettes. The parental variety used in the transformation, DP50B, was derived from a conventional cross between DP50 and the transgenic Bollgard® cotton event MON 531. Because event MON 15985 was the product of two independent transformations, the following sections provide separate descriptions of the transformation method and the potentially introduced DNA for both event MON 531 (section 3.A-2) and MON 15985 (section 3.A-3).

3.A-1 Common Methods of Plant Transformation

The two principal methods for introducing new genetic material into plant cells are *Agrobacterium*-mediated transformation and microparticle bombardment. Of relevance to event MON 15985, the parental event MON 531 was a product of *Agrobacterium*-mediated transformation, while MON 15985 was the product of microparticle bombardment transformation of MON 531. Neither of these methods gives rise to specific safety concerns but each method does generally result in DNA integration patterns with different characteristics. A brief general discussion of each of these transformation methods follows, below.

Agrobacterium-mediated Transformation:

Agrobacterium tumefaciens is a soil-borne phytopathogen that uses genetic engineering processes to subvert the host plant cell's metabolic machinery. It does so to divert some of the host's organic carbon and nitrogen supplies to produce nutrients (opines), which can be specifically catabolized by the invading bacteria (Tempe and Schell, 1977). Parasitized cells are also induced to proliferate and the resulting crown gall tumour disease is a direct result of the incorporation of a region of transfer DNA, T-DNA, from a large (150-250 kb) circular Ti (tumour inducing) plasmid, carried by *A. tumefaciens*, into the host plant genome.

An understanding of this natural transformation process, together with the realization that any foreign DNA placed between the T-DNA border sequences can be transferred to plant cells, led to the construction of the first vector and bacterial strain systems for plant transformation (for a review see: Hooykaas and Shilperoort, 1992). Since the first record

on a transgenic tobacco plant expressing foreign genes (Fraley *et al*., 1983), great progress in understanding *Agrobacterium*-mediated gene transfer at the molecular level has been achieved. *A. tumefaciens* naturally infects only dicotyledonous plants and methods for *Agrobacterium*-mediated gene transfer into monocotyledonous plants have only recently been developed for rice (Hiei *et al*., 1994; Cheng *et al*., 1998), banana (May *et al*., 1995), maize (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997) and sugarcane (Enríquez-Obregón, 1997, 1998; Arencibia *et al*., 1998). A thorough analysis of the strategies for practical application of this methodology has been published (Birch, 1997).

Agrobacterium-mediated transformation of plant tissue generally results in a low transgene copy number, minimal rearrangements, and higher transformation efficiency than direct DNA delivery techniques such as microparticle bombardment (Powlowski and Somers, 1996; Gelvin, 1998).

Until 1995, it was generally assumed that the sequences between the left and right borders of the T-DNA were the only transgenic elements transferred to the recipient host. Ramanathan and Veluthambi (1995), Wenck *et al*. (1997) and Kononov *et al*. (1997) all demonstrated that plasmid backbone sequences beyond the borders of the T-DNA could also be integrated along with the genes of interest. Experiments by Kononov *et al*. (1997) demonstrated that plasmid backbone sequences could be integrated into the host genome coupled with either the right or left border sequences, or as an independent unit unlinked from the T-DNA. Matzke and Matzke (1998) state that backbone sequences that join T-DNA and host DNA appear to be especially deleterious for gene expression, an observation supported by the authors' finding that backbone fragments separated from T-DNA have been found associated with stably expressed transgenes.

Plants transformed independently with the same plasmid will commonly have different levels of expression, a phenomenon that is not always correlated with copy number (Gelvin, 1998). Instead, differential expression of transgenes has been attributed by some to "positional effects" whereby the position of the T-DNA integration site in the host genome affects the level of transgene expression. However, other research has indicated that factors in addition to, or other than, the position of the site of integration contribute to the level of transgene expression (Gelvin, 1998). This is particularly true of the variable arrangements that transgene sequences may take in the host genome.

T-DNA can integrate into the host genome in patterns other than as a single copy at a single site. Multiple copies in direct or inverted repeats and other complex patterns may also occur. The presence of multimeric T-DNA inserts, especially inverted repeat structures, is strongly linked to the phenomenon of transgene silencing (Gelvin, 1998).

Variable expression of transgenes or gene silencing is a ubiquitous phenomenon in transgenic plants whether produced by direct DNA uptake or *Agrobacterium*-mediated transformation. Gene silencing can result from interactions between multiple copies of transgenes and related endogenous genes and is associated with homology-based mechanisms that act at either the transcriptional or post-transcriptional level (Matzke and Matzke, 1998). Silencing that results from the impairment of transcription initiation is often associated with cytosine methylation and/or chromatin condensation (Fagard and

Vaucheret, 2000) while post-transcriptional silencing (co-suppression) involves enhanced RNA turnover in the cytoplasm (Matzke and Matzke, 1998).

A third category of silencing has also been proposed for the consequences of positional effects where flanking plant DNA and/or unfavourable chromosomal location exert a silencing effect on the transgene (Matzke and Matzke, 1998). According to Matzke and Matzke (1998), this type of silencing reflects the epigenetic state of host sequences flanking the insertion site or the tolerance of particular chromosome regions to insertion of foreign DNA.

Microparticle Bombardment:

Microprojectile bombardment (also known as microparticle bombardment, biolistic or particle acceleration transformation) is a technique used to directly deliver DNA to the host genome and has proven to be useful for the transformation of plant tissues recalcitrant to *Agrobacterium* infection. In short, plasmid or linearized DNA with the gene(s) of interest is fixed to tungsten or gold particles (microcarriers) which are delivered to host cells at high speed so as to penetrate the nucleus of the plant cells. In the nucleus the DNA may separate from the microcarrier and become integrated into the host genome. Microprojectile bombardment can be used to transform tissue of most plant species, however it is relatively inefficient compared to *Agrobacterium* in producing stably transformed plant cells.

Microprojectile bombardment of plant tissue results in transgene integration patterns that generally exhibit: the full-length introduced transgene; transgene rearrangements that differ in size from the full length insert; occasional concatenation of introduced plasmids carrying the transgene, and variation in copy number among the full-length and partial transgenic elements (Powlowski and Somers, 1996). Transgene copy numbers can vary from 1 through 20. Multiple copies usually co-segregate as a transgenic locus, indicating that the sequences are either integrated into tightly linked loci or into a single locus, rather than randomly integrated throughout all chromosomes (Powlowski and Somers, 1996).

Molecular characterization of transgenic plants produced through microparticle bombardment has provided evidence of extensive rearrangements of transgenic sequences (Powlowski and Somers, 1996). These rearrangements may be observed in Southern blot analyses as hybridizing fragments of a different size than the full-length DNA insert. Larger fragments are indicative of concatenation (head to head or head to tail). Concatemers of the DNA insert may be deduced by digesting genomic DNA with a restriction enzyme that cuts at a single site within the transgenic element; multiple copies of the DNA insert will then be resolved by Southern blot analysis. Concatemers may be formed by homologous recombination of the transformed DNA or by blunt end ligation of cohesive ends produced by limited exonuclease activity (Folger *et al*., 1982; Rohan *et al*, 1990, in Powlowski and Somers, 1996). Smaller than full-length fragments are evidence of deletions and truncations.

Larger than full length fragments of transgenic DNA may also be caused by interspersion of inserted DNA with host DNA. Powlowski and Somers (1998) reported that each of 13

transgenic oat lines transformed using microparticle bombardment had intact copies of the transgene, as well as multiple, rearranged, and/or truncated transgene fragments. Insertion sites varied from 2 to 12, and all fragments of the transgenic DNA co-segregated. The authors determined that the transgenic DNA was interspersed with host DNA. This phenomenon has also been reported for rice (Cooley *et al*., 1995).

3.A-2 Event MON 531 Transformation Method

Cotton event MON 531 was produced by *Agrobacterium*-mediated transformation of the cotton (*Gossypium hirsutum*) line L. cv Coker C312 with plasmid PV-GHBKO4 (Figure 1). Plasmid PV-GHBK04 contained the following elements: the 0.4 kb oriV fragment from the RK2 plasmid fused to the 3.4 kb segment of pBR322 allowing maintenance in *Escherichia coli* and in *Agrobacterium tumefaciens*. This was fused to the 360 bp DNA fragment from pTiT37 plasmid, which contained the nopaline T-DNA right border.

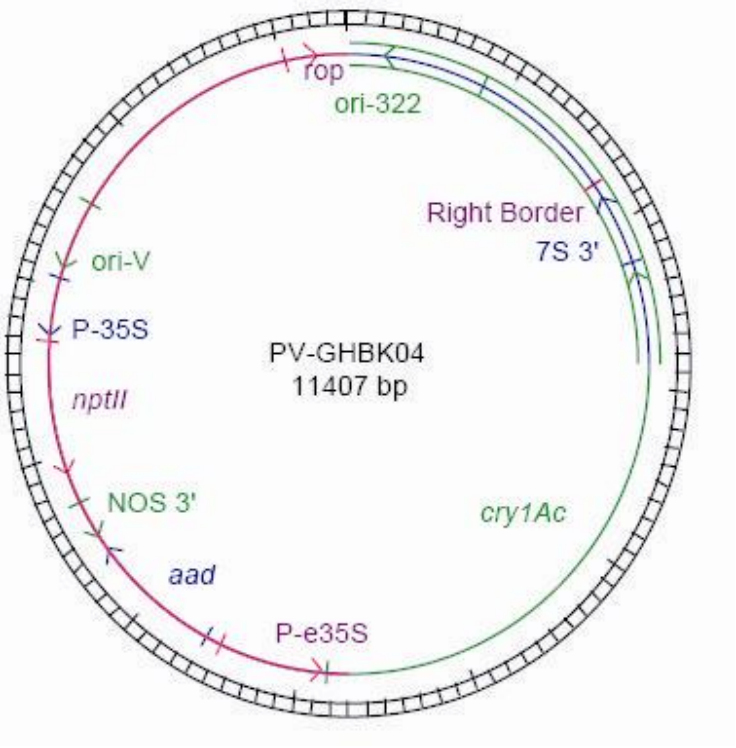

Figure 1 Plasmid map of PV-GHBK04

The remaining portion consisted of two genes engineered for plant expression, the *cry1Ac* and the NPTII encoding *nptII* (*neo*) gene. The *cry1Ac* gene was modified for optimal expression in plants and contained part of the 5' end of the *cry1Ab* gene with a portion of the *cry1Ac* gene. Expression of the modified *cry1Ac* gene was regulated by cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer region and the nontranslated region of the soybean alpha subunit of the beta-conglycin gene which provided the mRNA polyadenylation signals (7S 3' terminator sequence).

The transformation plasmid also contained the *aad* gene isolated from *E. coli* bacterial transposon Tn7, which encodes the enzyme aminoglycoside adenyltransferase (AAD)

that confers resistance to the antibiotics spectinomycin and streptomycin. The *aad* gene was under the control of its own bacterial promoter and terminator and was included in the construct as a marker to allow for selection of bacteria containing PV-GHBK04 prior to transformation of the plant cells. The *aad* gene has no plant regulatory sequences and was not expressed in plant tissues.

The *nptII* gene was located downstream of the *aad* gene and its expression was regulated using the CaMV 35S promoter and the non-translated region of the 3' region of the nopaline synthase gene (*nos*) from the pTiT37 plasmid of *A. tumefaciens* strain T37.

A summary of the genetic elements contained in plasmid vector PV-GHBK04 is presented in Table 6.

Table 6 Summary of genetic elements contained in plasmid PV-GHBK04

Genetic Element	Size (kb)	Function
right border (RB)	0.09	A DNA fragment from the pTiT37 plasmid containing the 24 bp border nopaline-type T-DNA right border used to initiate the T-DNA transfer (RB) from *Agrobacterium tumefaciens* to the plant genome (Depicker *et al*., 1982, and Bevan *et al*., 1983).
P-E35S	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1987).
cry1A(c)	3.5	The gene which confers insect resistance. The modified gene encodes an amino acid sequence that is 99.4% identical to the *cry1A(c)* gene as described by Adang *et al*. (1985).
7S 3'	0.43	A 3' non-translated region of the soybean alpha subunit of the beta-conglycinin gene that provides the mRNA polyadenylation signals (Schuler *et al*., 1982).
aad	0.79	The gene for the enzyme 3"(9)-O-aminoglycoside adenylyltransferase that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al*., 1985).
P-35S	0.32	The 35S promoter region of the cauliflower mosaic virus (CaMV) (Gardner *et al.*, 1981; Sanders *et al.*, 1987).
nptII	0.79	The gene isolated from Tn5 (Beck *et al*., 1982) which encodes for neomycin phosphotransferase type II. Expression of this gene in plant cells confers resistance to kanamycin and serves as a selectable marker for transformation (Fraley *et al*., 1983).
NOS 3'	0.26	A 3' non-translated region of the nopaline synthase gene which functions to terminate transcription and direct polyadenylation of the *nptII* mRNA (Depicker *et al.*, 1982; Bevan *et al.*, 1983).
oriV	0.62	Origin of replication for ABI *Agrobacterium* derived from the broad-host range plasmid RK2 (Stalker *et al*., 1981).
ori322/rop	1.8	A segment of pBR322 which provides the origin of replication for maintenance of the PV-GHBK04 plasmid in *E. coli*, the replication of primer (*rop*) region and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells (Bolivar *et al.*, 1977; Sutcliffe, 1978).

*Sizes given are the actual size of the genetic elements and do not include DNA border sequences, necessary for cloning purposes, unless otherwise indicated.

3.A-3 Event MON 15985 Transformation Method

Event MON 15985 was developed by biolistic transformation of cotton meristems with purified DNA containing the *cry2Ab* and *uidA* (GUS) expression cassettes. The parental

variety used in the transformation, DP50B, was derived from a conventional cross between DP50 and the transgenic Bollgard® cotton line 531.

The DNA used for transformation was an approximately 6 kb fragment containing the expression cassettes for *cry2Ab* and *uidA*, and was derived from plasmid PV-GHBK11 (Figure 2) following restriction endonuclease digestion with *Kpn*I and high pressure liquid chromatography (HPLC) purification. The purified DNA fragment did not contain any other plasmid-derived sequences, such as the bacterial origin of replication site or antibiotic resistance marker genes (Table 7). The purified *ca*. 6 kb linear fragment was precipitated onto gold particles using calcium chloride and spermidine, and introduced into cotton variety DP50B, a Delta and Pine Land Company commercial variety derived from event MON 531 (Bollgard®) containing the *cry1Ac* gene and the *nptII* marker gene, essentially as described by John (1997).

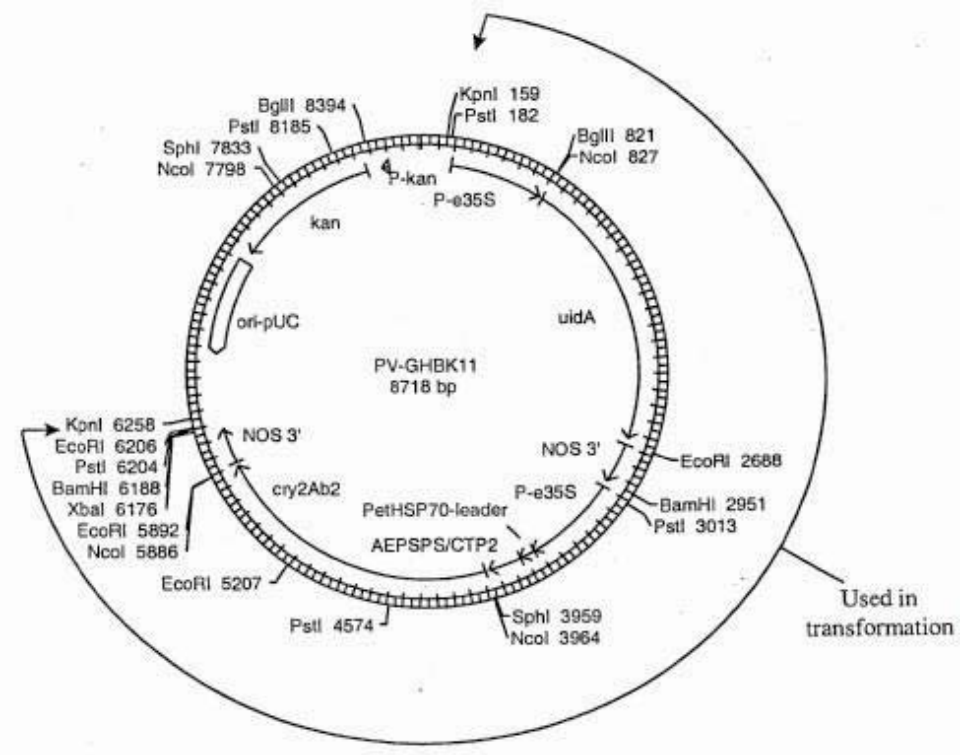

Figure 2 Plasmid map of PV-GHBK11
The region used for transformation, between the two *Kpn*I sites and containing the *cry2Ab* and *uidA* gene cassettes, is illustrated. The remaining portion is the plasmid backbone.

The *cry2Ab* construct consisted of a synthetic copy of the *cry2Ab* gene originally isolated from *B. thuringiensis* subsp. *kurstaki* (Btk) under the regulatory control of the doubly enhanced cauliflower mosaic virus 35S promoter (CaMV 35S; P-e35S) and a polyadenylation signal isolated from the 3'-terminal untranslated region of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*. Transcriptional activation was modulated by inclusion of the 5'-terminal untranslated leader sequence from the petunia heat shock 70 protein (PetHSP70). Targeting of the expressed protein to chloroplasts was

accomplished by fusing the sequence encoding the chloroplast transit peptide, isolated from the *Arabidopsis thaliana* 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene, to the 5'-terminus of the *cry2Ab* gene sequence.

The *cry2Ab* gene (1907 nucleotides) was completely re-synthesized to incorporate plant-preferred codons, and the expressed protein was 88% identical (97% similarity with conservative substitutions) to the native Cry2Ab protein expressed in *B. thuringiensis* (Figure 3). An additional amino acid (position 2, Figure 3) was introduced to create a restriction enzyme cleavage site for cloning purposes. The Cry2Ab2 protein present in event MON 15985 cotton plants is predicted to contain an additional three amino acids due to processing of the chloroplast transit peptide (underlined positions 77-79, Figure 3).

The *uidA* (synonym *gus*) gene (1808 nucleotides), isolated from *E. coli* strain K12, encodes the enzyme ß-D-glucuronidase (GUS; Figure 4), and was included as a scorable marker allowing colorimetric identification of transformed plant tissue following histochemical staining. β-D-glucuronidase is an exohydrolase that catalyzes the hydrolysis of a range of β-glucuronides into their corresponding acids and aglycones (Oshima *et al.,* 1987). Hydrolysis of the artificial substrate –pnitrophenyl-β-D-glucuronide releases a blue dye that functions as a visible marker in plant transformation processes (Jefferson *et al*., 1987). The biochemistry and catalytic activity of this protein has been thoroughly studied (Wang and Touster, 1972). The GUS protein is ubiquitous in nature, occurring in vertebrates, including humans (Jefferson *et al*., 1986), bacteria, cattle and invertebrate species (Gilissen *et al*., 1998). GUS-like activity has also been detected in various tissues in over 50 plant species, including human food sources such as potato, apple, almond, rye, rhubarb and sugar beet (Schulz and Weissenbock, 1987; Hodal *et al*., 1992; Wozniak and Owens, 1994). Expression of the *uidA* gene was regulated using the same CaMV 35S (P-e35S) promoter and NOS 3' sequences as used for the *cry2Ab* gene construct.

Table 7 Summary of genetic elements on 6 kb transforming DNA from PV-GHBK11

Genetic element	Size (kb)	Function
P-e35S (CaMV 35S)	0.6	The cauliflower mosaic virus (CAMV) 35S promoter with the duplicated enhancer region; used to drive expression of the *cry2Ab* and *uidA* genes.
PetHSP70-leader	0.1	An intron from the petunia heat shock protein *hsp70* gene; provides for an increased level of transcription.
AEPSPS/CTP2	0.23	An N-terminal chloroplast transit peptide from *Arabidopsis thaliana* EPSPS-coding gene.
cry2Ab	1.9	A synthetic *cry* gene based on a sequence from *Bacillus thuringiensis*.
NOS 3'	0.26	3' nontranslated region of the nopaline synthase which terminates transcription and directs polyadenylation.
uidA	1.8	β-D-glucuronidase (GUS) protein-encoding gene from the *E. coli* plasmid, pUC19.

```
  1  MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51  LKKSGMTLIG SELRPLKVMS SVSTACMLAM DNSVLNSGRT TICDAYNVAA
101  HDPFSFQHKS LDTVQKEWTE WKKNNHSLYL DPIVGTVASF LLKKVGSLVG
151  KRILSELRNL IFPSGSTNLM QDILRETEKF LNQRLNTDTL ARVNAELTGL
201  QANVEEFNRQ VDNFLNPNRN AVPLSITSSV NTMQQLFLNR LPQFQMQGYQ
251  LLLLPLFAQA ANLHLSFIRD VILNADEWGI SAATLRTYRD YLKNYTRDYS
301  NYCINTYQSA FKGLNTRLHD MLEFRTYMFL NVFEYVSIWS LFKYQSLLVS
351  SGANLYASGS GPQQTQSFTS QDWPFLYSLF QVNSNYVLNG FSGARLSNTF
401  PNIVGLPGST TTHALLAARV NYSGGISSGD IGASPFNQNF NCSTFLPPLL
451  TPFVRSWLDS GSDREGVATV TNWQTESFET TLGLRSGAFT ARGNSNYFPD
501  YFIRNISGVP LVVRNEDLRR PLHYNEIRNI ASPSGTPGGA RAYMVSVHNR
551  KNNIHAVHEN GSMIHLAPND YTGFTISPIH ATQVNNQTRT FISEKFGNQG
601  DSLRFEQNNT TARYTLRGNG NSYNLYLRVS SIGNSTIRVT INGRVYTATN
651  VNTTTNNDGV NDNGARFSDI NIGNVVASSN SDVPLDINVT LNSGTQFDLM
701  NIMLVPTNIS PLY
```

Figure 3 Deduced Cry2Ab2 protein sequence as produced in cotton event MON 15985
The sequence deduced from the DNA used to transform cotton. The chloroplast transit peptide is shown in italics (residues 1-79). The Cry2Ab2 protein corresponds to residues 80-713. The underlined amino acids (residues 77-79) correspond to the predicted portion of the chloroplast transit peptide remaining after processing. The amino acid at position 81 (D, aspartic acid) corresponds to the residue introduced for cloning purposes.

```
  1  MVRPVETPTR  EIKKLDGLWA  FSLDRENCGI  DQRWWESALQ  ESRAIAVPGS
 51  FNDQFADADI  RNYAGNVWYQ  REVFIPKGWA  GQRIVLRFDA  VTHYGKVWVN
101  NQEVMEHQGG  YTPFEADVTP  YVIAGKSVRI  TVCVNNELNW  QTIPPGMVIT
151  DENGKKKQSY  FHDFFNYAGI  HRSVMLYTTP  NTWVDDITVV  THVAQDCNHA
201  SVDWQVVANG  DVSVELRDAD  QQVVATGQGT  SGTLQVVNPH  LWQPGEGYLY
251  ELCVTAKSQT  ECDIYPLRVG  IRSVAVKGEQ  FLINHKPFYF  TGFGRHEDAD
301  LRGKGFDNVL  MVHDHALMDW  IGANSYRTSH  YPYAEEMLDW  ADEHGIVVID
351  ETAAVGFNLS  LGIGFEAGNK  PKELYSEEAV  NGETQQAHLQ  AIKELIARDK
401  NHPSVVMWSI  ANEPDTRPQA  AREYFAPLAE  ATRKLDPTRP  ITCVNVMFCD
451  AHTDTISDLF  DVLCLNRYYG  WYVQSGDLET  AEKVLEKELL  AWQEKLHQPI
501  IITEYGVDTL  AGLHSMYTDM  WSEEYQCAWL  DMYHRVFDRV  SAVVGEQVWN
551  FADFATSQGI  LRVGGNKKGI  FTRDRKPKSA  AFLLQKRWTG  MNFGEKPQQG
601  GKQ
```

Figure 4 Deduced amino acid sequence of the GUS protein expressed in MON 15985

3.A-4 Characterization of the Introduced DNA

For the purposes of illustration in this case study, only the characterization of the inserted DNA derived from the linear 6 kb *Kpn*I transforming DNA (*i.e.*, from plasmid PV-

GHBK11) used to produce event MON 15985 is described in detail. As previously noted, the host plant already contained a transgenic insert approved for commercial use in cotton event MON 531. Previous molecular analysis of event MON 531 had demonstrated that two copies of the T-DNA insert were integrated in a head-to-tail arrangement. One T-DNA insert contained a full-length *cry1Ac* gene and the NPTII encoding gene, and the second insert contained an inactive 3' portion of the *cry1Ac* gene. The two inserts were linked and segregated as a single locus. Similar analyses demonstrated that plasmid backbone sequences from PV-GHBK04 were not transferred into the event MON 531 genome. The *aad* gene was present but was not expressed since it was under the control of a bacterial promoter.

Number of Sites of Insertion. The number of sites at which the transforming DNA (the *cry2Ab* and *uidA* gene cassettes) was inserted into the genome of event MON 15985 was determined by Southern blot analyses of genomic DNA digested with *Sca*I restriction endonuclease. This enzyme does not cleave within the inserted DNA, therefore the number of fragments hybridizing to ^{32}P-labelled PV-GHBK11 DNA was indicative of the number of sites of insertion. Each site of insertion would be predicted to yield a single hybridizing fragment on Southern blots.

Figure 5 shows a Southern blot of genomic DNA isolated from DP50 (non-transgenic cotton, DP50B (transgenic event MON531) and MON15985 plants, digested with *Sca*I restriction endonuclease. As a positive control, a mix of DP50 genomic DNA and PV-GHBK11 plasmid DNA was digested with *Xba*I and included in the gel. The Southern blot (Figure 5) was probed with the radiolabelled PV-GHBK11 fragment illustrated.

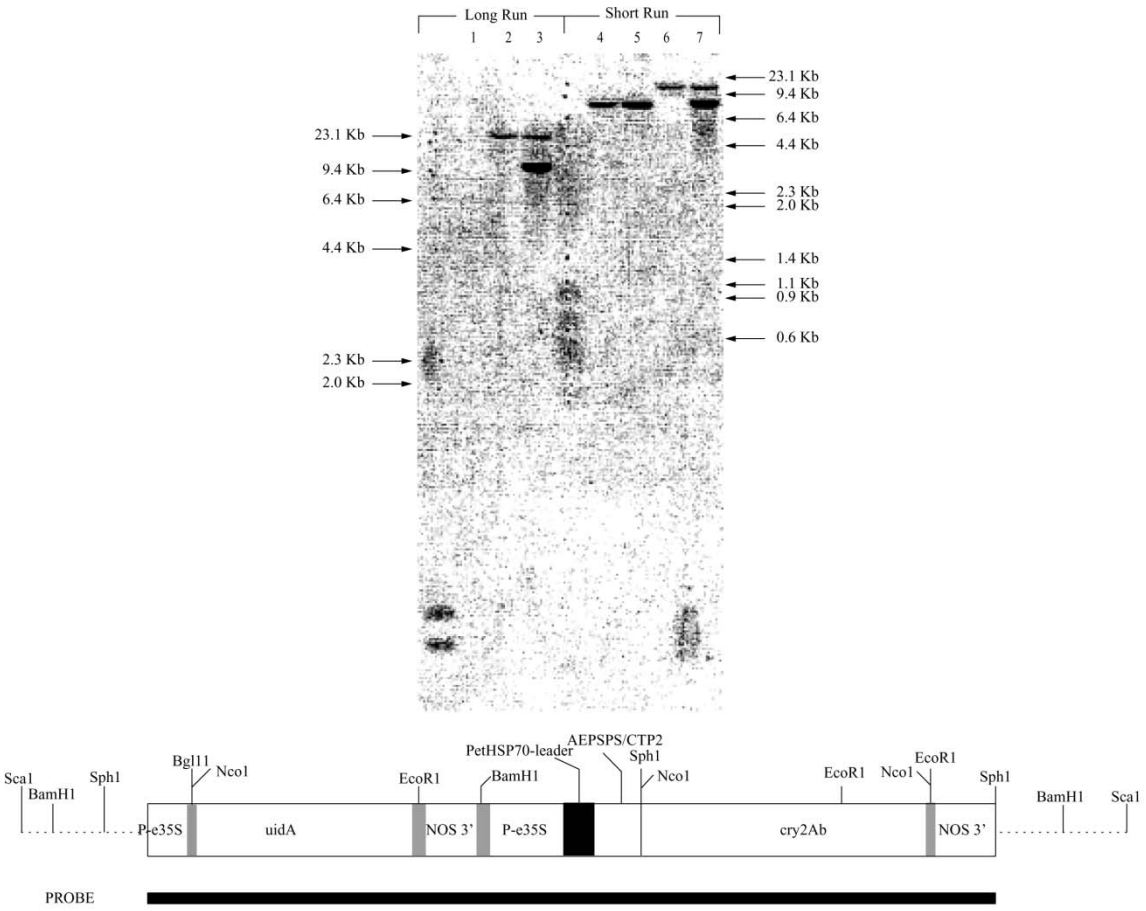

Figure 5 Southern blot analysis of MON 15985: Insertion site determination
Ten µg of DP50, DP50B and MON15985 genomic DNA isolated from leaf tissue were digested with *Sca*I. Samples were separated by alkaline gel electrophoresis, blotted onto nylon membranes and probed with the ^{32}P-labelled PV-GHBK11 DNA fragment illustrated, washed and subjected to autoradiography. Molecular size markers are indicated. Lane designation: 1) DP50, Long run; 2) DP50B, Long run; 3) MON15985, Long run; 4) DP50 spiked with 5.15 pg *Xba*I-digested plasmid PV-GHBK11 DNA, Short run; 5) DP50 spiked with 10.3 pg *Xba*I-digested plasmid PV-GHBK11, Short run; 6) DP50B, short run; 7) MON15985, Short run.

The PV-GHBK11 – DP50 DNA mix digested with ScaI and XbaI (Figure 5, lanes 4 and 5) produced a single hybridizing band at approximately 8.7 kb, the size of the plasmid (Figure 1). The probe did not hybridize with the control DP50 DNA (lane 1), but did hybridize with the ScaI-digested DP50B DNA (lanes 2 and 6) producing two bands of approximately 22 kb and 15 kb (faint). Since these bands were present in both MON 15985 and DP50B, but not in DP50 (lane 1), they were considered to be associated with the cry1Ac insert in DP50B. MON 15985 (lanes 3 and 7) produced one unique hybridization band not present in either DP50 (lane 1) or DP50B (lanes 2 and 6) at approximately 9.3 kb. This result suggested that MON 15985 contained one integrated DNA insert.

Analysis for copy number. The number of copies of the cry2Ab gene could be detected using a restriction enzyme that cuts only once within the DNA used for transformation. This would be expected to produce two hybridising bands for every copy of the insert in the genome. Genomic DNA isolated from leaf samples of MON 15985, DP50 and

DP50B, and PV-GHBK11 DNA mixed with DP50 DNA was digested with SphI, which cuts the inserted DNA (PV-GHBK11L) only once, within the cry2Ab gene (Figure 1). The Southern blot produced with this DNA was probed with the radiolabeled PV-GHBK11 fragment illustrated (Figure 6).

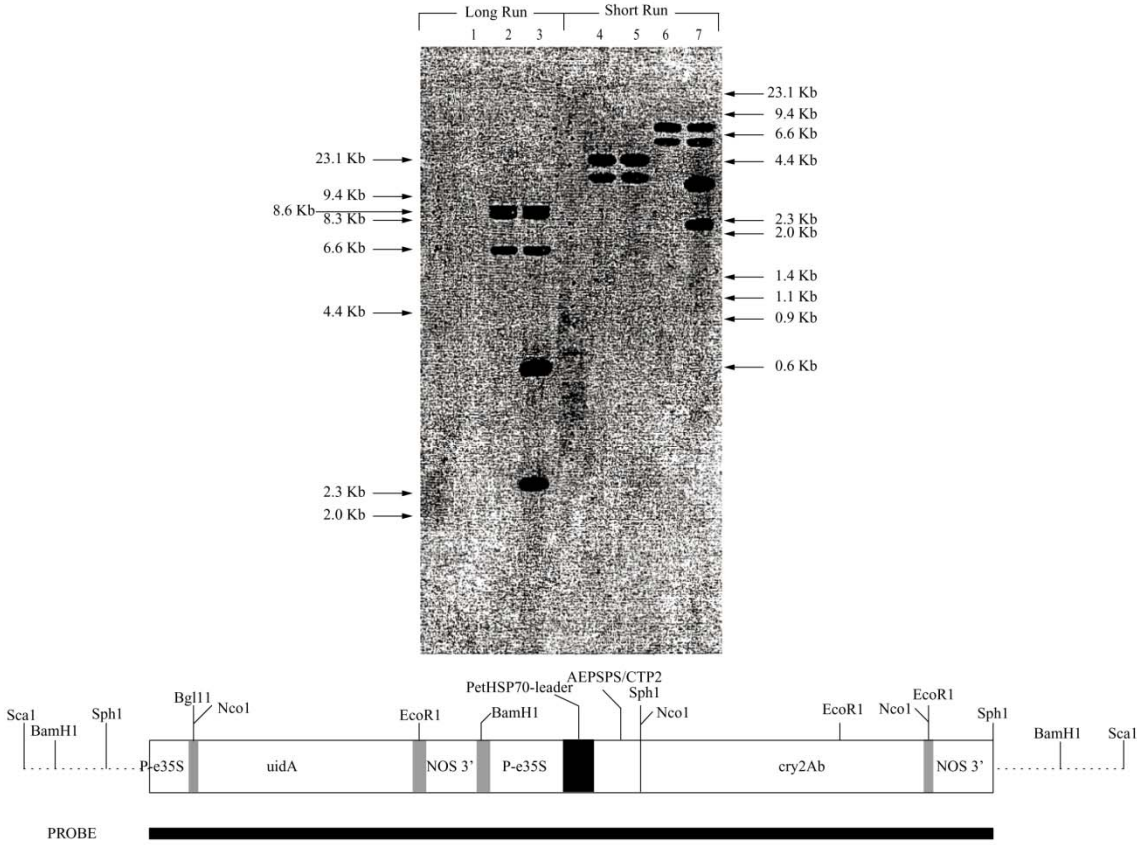

Figure 6 Southern blot analysis of MON 15985: Copy number analysis
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *SphI*. The blot was probed with the ^{32}P-labeled PV-GHBK11 fragment shown. Lane designation: 1) DP50, Long run; 2) DP50B, Long run; 3) MON 15985, Long run; 4) DP50 spiked with 5.15 pg of PV-GHBK11, Short run; 5) DP50 spiked with 10.3 pg of PV-GHBK11, Short run; 6) DP50B, Short run; 7) MON 15985, Short run.

→ symbol denotes size of DNA in kb, obtained with MW markers.

The probe did not hybridize with the non-transgenic control, DP50 (Figure 6, lane 1). Plasmid PV-GHBK11 mixed with DP50 DNA (land 4 and 5) produced hybridized bands at approximately 3.9, 4.8 and 8.7 Kb (faint). The faint ~8.7 Kb band corresponds to undigested plasmid DNA. DP50B (lanes 2 and 6) produced three hybridising bands at approximately 6.4, 8.3 and 8.6 kb. These bands were present in both MON 15985 and the DP50B control which suggested that they were associated with the cry1Ac insert. Two unique bands were apparent in MON 15985 (lanes 3 and 7) at approximately 2.3 kb and 3.5 kb. As the enzyme SphI cuts only once within the inserted DNA, these two bands suggested that MON 15985 contained one copy of integrated DNA. No additional smaller inserts were detected.

Analysis of integrity of cry2Ab coding region. Digestion with a restriction enzyme that cut at each end of the cry2Ab gene was used to determine whether the entire cry2Ab gene was inserted into MON 15985. Genomic DNA isolated from leaf samples of MON 15985, DP50 and DP50B, and PV-GHBK11 DNA mixed with DP50 DNA were digested with NcoI, to release the cry2Ab coding region. The Southern blot produced with this DNA was probed with the full-length, radiolabeled cry2Ab coding region (1.9 kb; Figure 7).

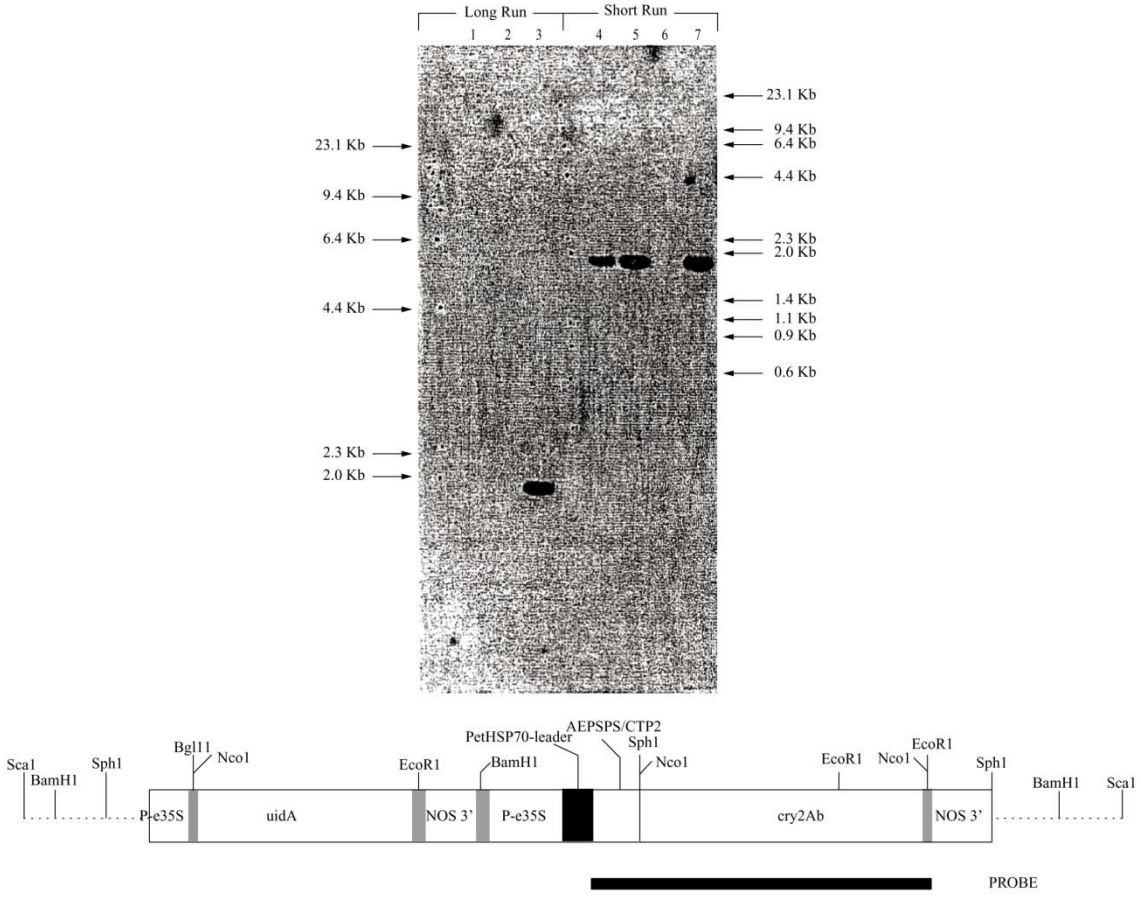

Figure 7 Southern blot analysis of MON 15985: Integrity of *cry2Ab* coding region
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Nco*I. The blot was probed with the ^{32}P-labeled *cry2Ab* coding region shown on the plasmid diagram. Lane designation: 1) DP50, Long run; 2) DP50B, Long run; 3) MON 15985, Long run; 4) DP50 spiked with 5.15 pg of PV-GHBK11, Short run; 5) DP50 spiked with 10.3 pg of PV-GHBK11, Short run; 6) DP50B, Short run; 7) MON 15985, Short run.

→ symbol denotes size of DNA in kb, obtained with MW markers.

The DP50 non-transgenic control (Figure 7, lane 1) and the DP50B control (lanes 2 and 6) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 DNA (lanes 4 and 5) produced a band of approximately 1.9 kb, which corresponded to the entire *cry2Ab* coding region (Figure 1). A single hybridization band of 1.9 kb was also produced by MON 15985 (Figure 7, lanes 3 and 7), corresponding to an intact

cry2Ab coding region. This result indicated that MON 15985 contained an intact cry2Ab gene.

In addition, the petition included analyses for the integrity of the *cry2Ab* expression cassette with the following probes: a full length *cry2Ab* cassette; an enhanced CaMV promoter; a NOS probe. These data have not been included, but all confirm the intactness of the *cry2Ab* expression cassette and the absence of additional inserts of any of the gene elements.

Analysis of the integrity of the *uidA* coding region. Digestion with restriction enzymes that cut the *uidA* gene at each end were use to determine the integrity of this inserted gene. Genomic DNA isolated from leaf samples of MON 15985, DP50 and DP50B, and PV-GHBK11 DNA mixed with DP50 DNA were digested with *Eco*RI and *Bgl*II, to release the entire *uidA* coding region. The Southern blot prepared from this DNA was probed with the full-length, radiolabeled *uidA* coding region (1.87 kb; Figure 8).

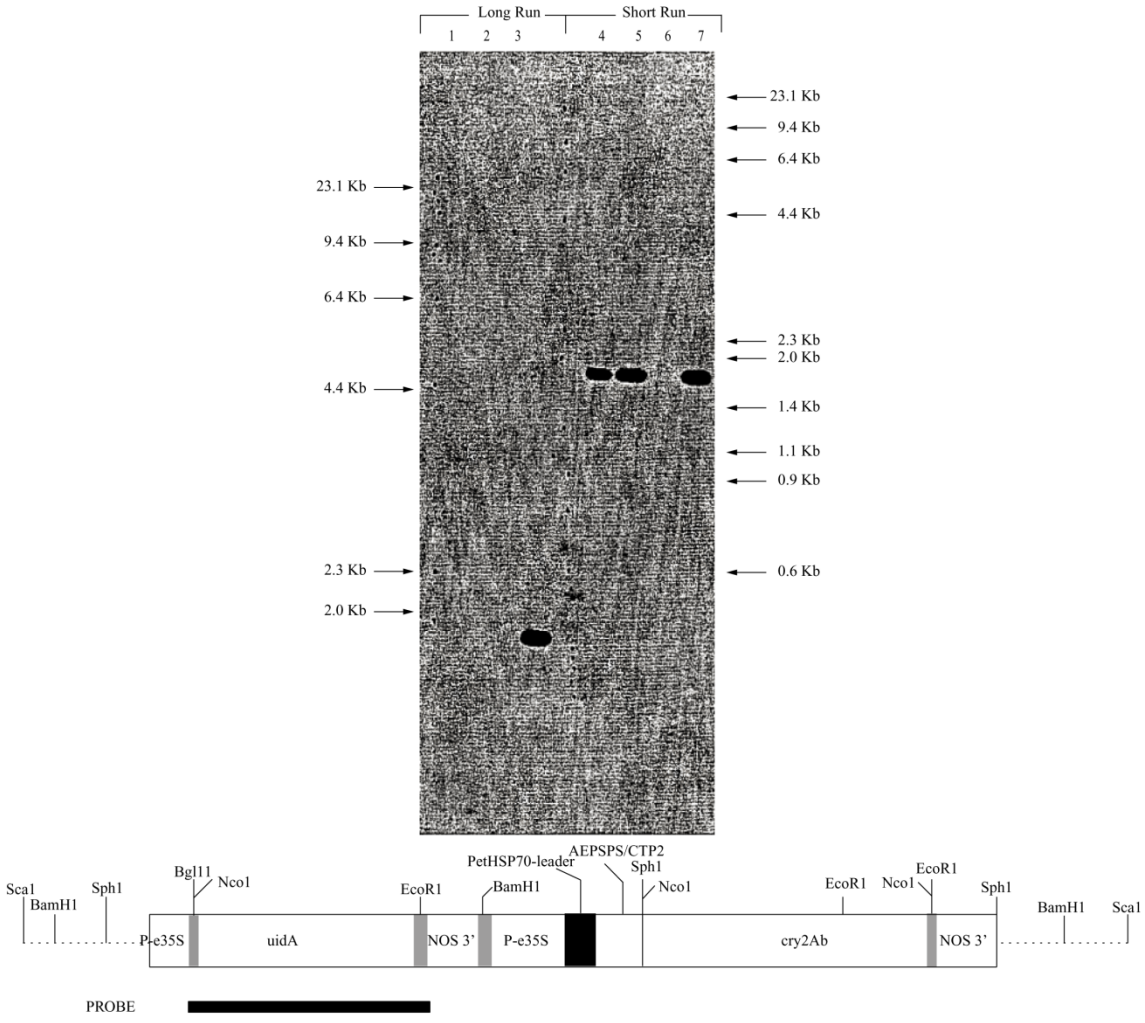

Figure 8 Southern blot analysis of MON 15985: Integrity of the *uidA* coding region

Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Eco*RI and *Bgl*II. The blot was probed with the ^{32}P-labeled *uidA* coding region shown. Lane designation: 1) DP50, Long run; 2) MON DP50B, Long run; 3) 15985, Long run; 4) DP50 spiked with 5.15 pg of PV-GHBK11, Short run; 5) DP50 spiked with 10.3 pg of PV-GHBK11, Short run; 6) DP50B, Short run; 7) MON 15985, Short run.

→ symbol denotes size of DNA in kb, obtained with MW markers.

The controls, DP50 (Figure 8, lane 1) and DP50B (lanes 2 and 6) showed no detectable hybridization bands. The PV-GHBK11 – DP50 DNA mix (lanes 4 and 5) produced a hybridization band of approximately 1.9 kb, which corresponded to the entire *uidA* coding region. MON 15985 DNA (lanes 3 and 7) also produced a single band of approximately 1.9 kb. This result indicated that MON 15985 contained an intact *uidA* coding region.

Analysis of integrity of *uidA* expression cassette. Digestion with two restriction enzymes that cut at each end of the *uid*A expression cassette was used to ensure that the full cassette was present in MON 15985. Genomic DNA isolated from leaf samples of MON 15985, DP50 and DP50B was digested with *Bam*HI and *Sph*I to release the entire *uidA* expression cassette, containing the *uidA* coding sequence, the enhanced CaMV 35S promoter and the NOS 3' polyadenylation sequence (Figure 9). PV-GHBK11 DNA was digested with *Pst*I and added to DP50 DNA for the short run sample. The Southern blot (Figure 9) was probed with the radiolabeled *uidA* coding region shown.

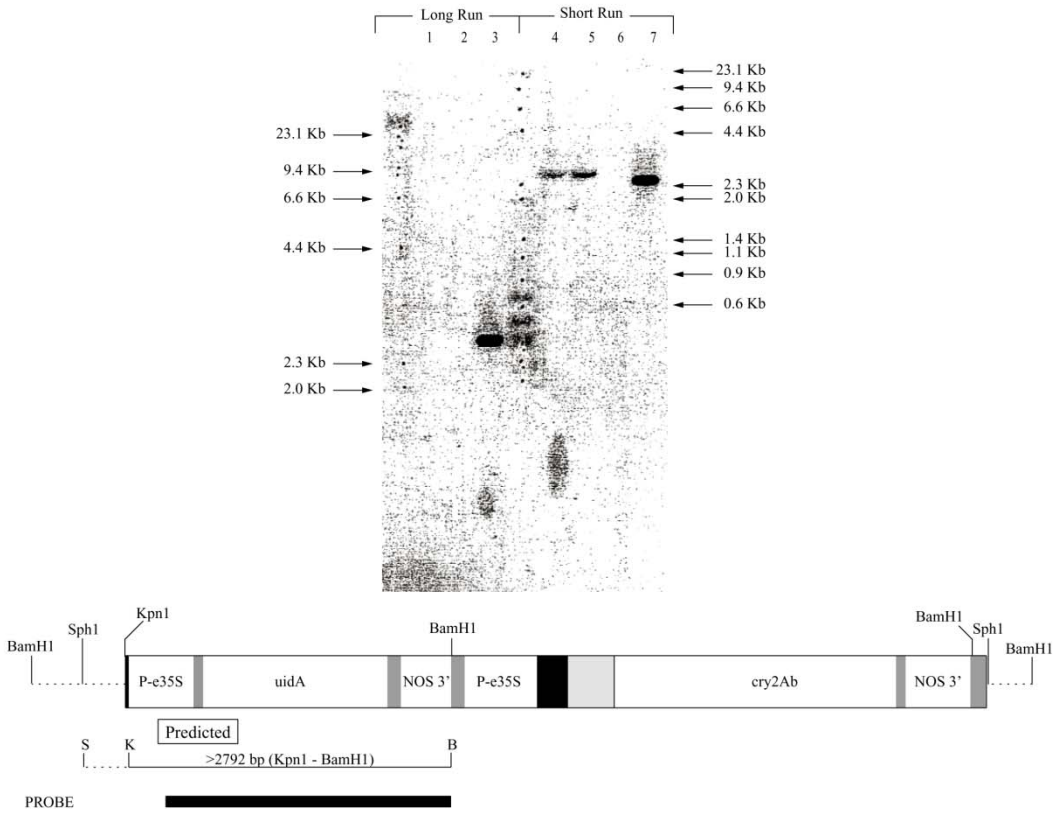

Figure 9 Southern blot analysis of MON 15985: Integrity of the *uidA* expression cassette – uidA probe

Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI and *Sph*I. PV-GHBK11 DNA was digested with *Pst*1 and added to the DP50 DNA samples prior to precipitation. The blot was probed with ^{32}P-labeled *uidA* coding region. Lane designation: 1) DP50, Long run; 2) DP50B, Long run; 3) MON 15985, Long run; 4) DP50 spiked with 5.15 pg of PV-GHBK11, Short run; 5) DP50 spiked with 10.3 pg of PV-GHBK11, Short run; 6) DP50B, Short run; 7) MON 15985, Short run.

→ symbol denotes size of DNA in kb, obtained with MW markers.

The DP50 non-transgenic DNA (Figure 9, lane1) and the DP50B DNA (lanes 2 and 6) did not show any hybridization bands. Plasmid PV-GHBK11 mixed with DP50 DNA (lanes 4 and 5) produced a 2.8 kb band which corresponded to the entire *uidA* expression cassette (illustrated as the 'Predicted' fragment). MON 15985 (lanes 3 and 7) produced a band of approximately 2.5 kb. This suggested that a portion of the *uidA* expression cassette was missing, which was confirmed by the results of PCR analysis of the 5' plant-insert junction that showed approximately 260 bp of the 5' promoter sequence and 24 bp of the polylinker were missing. Odell *et al*. (1985) showed that a deletion of this nature should not affect accurate transcription initiation. No additional bands were detected with the *uidA* coding region probe.

Analysis for the presence of plasmid backbone. To confirm that the insert did not contain plasmid DNA from PV-GHBK 11 outside of the *Kpn*I excised region used for transformation (the plasmid backbone, Figure 2), a Southern blot was hybridised with this region of the plasmid as a probe. Genomic DNA isolated from leaf samples of MON 15985, DP50 and DP50B, and PV-GHBK11 DNA mixed with DP50 DNA were digested with *Kpn*I, to release the entire DNA insert. The Southern blot was probed with the radiolabeled PV-GHBK11 backbone sequence, *i.e*., the vector DNA not used for transformation.

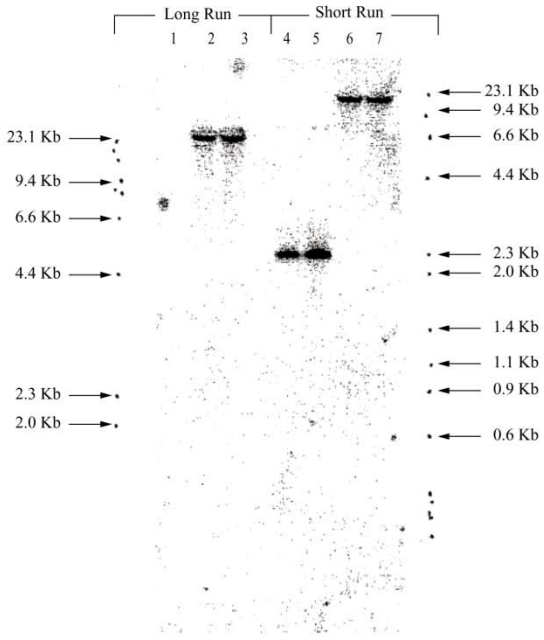

Figure 10 Southern blot analysis of MON 15985: Analysis for backbone sequences

Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Kpn*I. The blot was probed with the ^{32}P-labeled PV-GHBK11 backbone sequence (not shown). Lane designation: 1) DP50, Long run; 2) DP50B, Long run; 3) MON 15985, Long run; 4) DP50 spiked with 5.15 pg of PV-GHBK11, Short run; 5) DP50 spiked with 10.3 pg of PV-GHBK11, Short run; 6) DP50B, Short run; 7) MON 15985, Short run.

→ symbol denotes size of DNA in kb, obtained with MW markers.

The DP50 DNA (Figure 10, lane1) showed no detectable hybridization bands. Plasmid PV-GHBK11 mixed with DP50 (lanes 4 and 5) produced one band of approximately 2.6 kb, representing the entire backbone sequence in the PV-GHBK11 DNA. DP50B DNA (lanes 2 and 6) produced a single band of approximately 22 kb that is also present in MON 15985 and is considered to be background associated with the *cry1Ac* event. MON 15985 DNA (lanes 3 and 7) contained the approximately 22 kb band, but showed no additional hybridization. This result suggested MON 15985 does not contain PV-GHBK11 backbone sequences resulting from the transformation.

Analysis of plant DNA sequences flanking the insert. Identifying the junction sequences between the insert and the host genomic DNA is useful for event identification. Genome Walker technology was used to determine the genomic DNA sequence flanking both ends of the inserted DNA and PCR primers were used to amplify the junction regions (Figure 11). As controls, DNA from DP50 (non-transgenic), DP50B (MON531) and an alternate *cry2Ab* event (MON15813) were used.

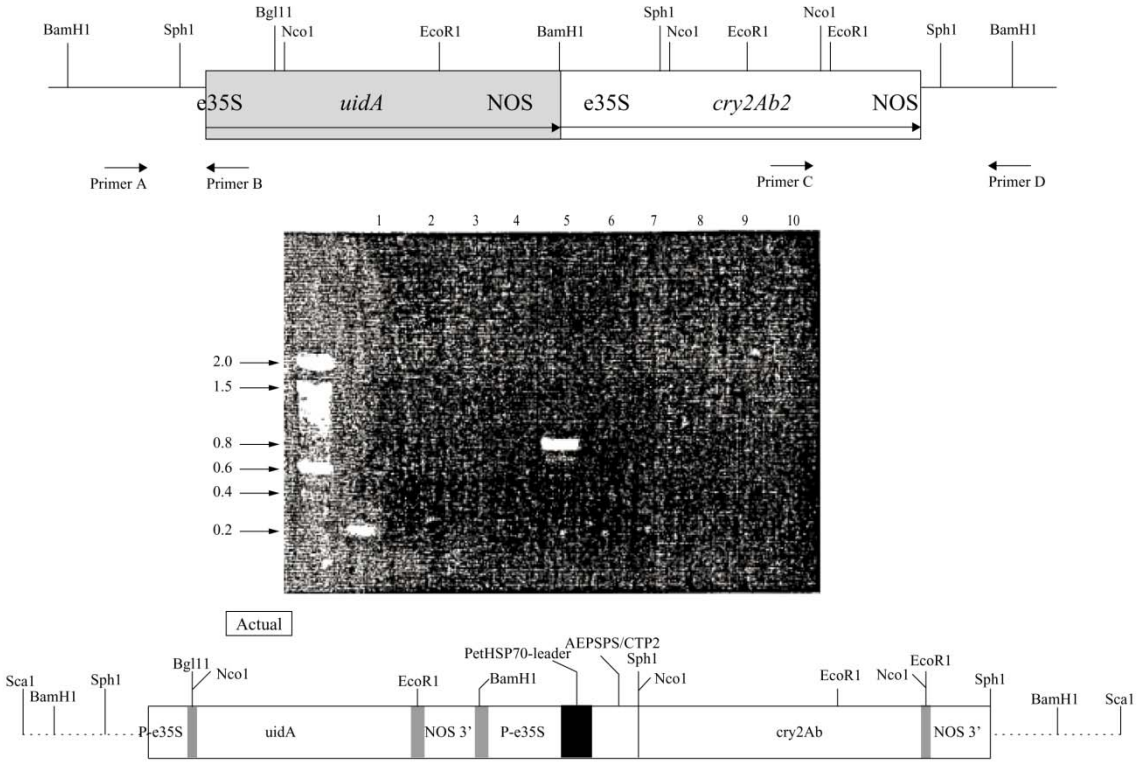

Figure 11 PCR confirmation of the 5' and 3' border sequences of the MON 15985 insert
PCR was performed using primers specific to the 5' and 3' border sequences for MON 15985 on genomic DNA isolated from leaf tissue for DP50 (non-transgenic control), DP50B (cry1Ac control), an alternate *cry2Ab* event and

MON 15985. DNA was amplified with primers A and B from the 5' end of MON 15985 and Primers C and D from the 3' end of MON 15985. Lane designations: 1) 10 µl of 5' MON 15985 reaction product; 2) 10 µl of 5' alternate *cry2Ab* reaction product; 3) 10 µl of 5' DP50 (non-transgenic) negative control reaction product; 4) 10 µl of 5' DP50B (*cry1Ac*) negative control reaction product; 5) 10 µl of 3' MON 15985 reaction product; 6) 10 µl of 3' alternate *cry2Ab* reaction product; 7) 10 µl of 3' DP50 (non-transgenic) negative control reaction product; 8) 10 µl of 3' DP50B (*cry1Ac*) negative control reaction product; 9) 10 µl of 5' no template negative control reaction product; 10) 10 µl of 3' no template negative control reaction product.

→ symbol denotes size of DNA in kb, obtained with MW markers.

Results: The non-transgenic samples did not yield a PCR product when either the 5' and 3' primer set was used (Figure 12, lanes 3 and 7). The DP50B sample (*cry1Ac* event) did not yield products with either primer set (lanes 4 and 8) and the alternate cry2Ab event, 15813, also did not yield products with either primer set (lanes 2 and 6). MON 15985 yielded a 230 bp product at the 5' end with the A and B primers (lane1) and an 869 bp product for the 3' end using primers C and D (lane 5). These products were of the sizes expected to contain sequences flanking the 5' and 3' ends of the *cry2Ab* insert in MON 15985 generated with the primer pairs (Figure 11). This study confirmed the border sequences for the insert in MON 15985 and these primers can be used to distinguish this event from other Bt cotton events.

Summary of the molecular analysis of MON 15985: The cotton event MON 15985 was produced by particle acceleration technology using a *Kpn*I DNA segment from plasmid PV-GHBK11, containing the genes *cry2Ab* and *uidA*. MON 15985 contained one new DNA insert that was located on a 9.3 kb *Sca*I segment. The insert contained one complete copy of the cry2Ab expression cassette linked to one copy of the uidA expression cassette. The latter was missing approximately 260 bp at the 5' end of the enhanced CaMV 35S promoter, but was still fully functional. MON 15985 did not contain any detectable plasmid backbone sequences from PV-GHBK11 and the 5' and 3' junction sequences of the insert with the plant genome were verified with PCR. A restriction map of the insert in MON 15985 is shown in Figure 13. A summary of the molecular characterisation of MON 15985 is given in Table 8.

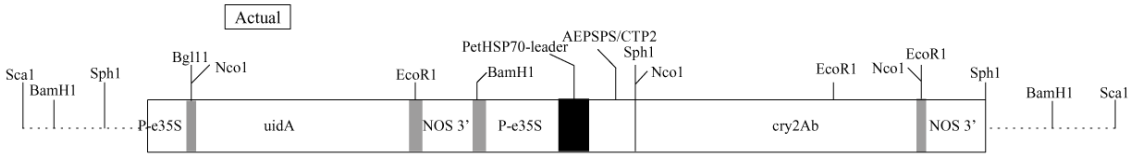

Figure 12 A restriction map of the insert in MON 15985

Table 8 A summary of the molecular characterisation of MON 15985

No. of insertions	1
No. of copies of the *cry2Ab* and *uidA* expression cassettes	1 of each
Genetic elements:	
enhanced CaMV 35S promoter for *uidA*	Intact with 260 bp missing from 5' end
uidA coding region	Intact
NOS 3' polyadenylation for *uidA*	Intact (data not shown)
enhanced CaMV 35S promoter for *cry2Ab*	Intact (data not shown)
NOS 3' polyadenylation for *cry2Ab*	Intact (data not shown)
Backbone DNA	None detected

3.A-5 Genetic Stability and Inheritance

The inheritance and stability of each introduced gene that is functional in the transformed plant must be determined. For each novel gene, the pattern and stability of inheritance must be demonstrated as well as the level of expression of the trait which is produced by the gene. Inheritance can be determined using DNA-based methods, by analysis of gene products or the phenotype produced by the gene, such as insect resistance. Serological techniques are generally used to measure protein expression either qualitatively [*e.g.*, Western immunoblotting, enzyme linked immunosorbent assay (ELISA), *etc*.] or quantitatively (*e.g.*, ELISA, radioimmunoassay, *etc*.). If the new trait is one that does not result from the expression of a new or modified protein (*e.g.*, transgenic plants containing inserted antisense sequences, such as the Flavr Savr™ tomato, which contains an antisense sequence corresponding to the polygalacturonase encoding gene) then its inheritance can be determined by examining the DNA insert directly or by measuring RNA transcript production.

To determine the inheritance pattern of the *cry2Ab* gene in MON 15985 a qualitative Cry2Ab2 enzyme-linked immunosorbent assay (ELISA) was performed on segregating populations from four generations produced as shown in Figure 14. The results are reported in Table 9. Statistical significance for the segregation data was determined using Chi square analysis. In addition, genomic DNA from plants of the R1, R2, R3 and R4 generations and two backcrossed lines (BC2F3, Figure 14) was digested, blotted and probed with the entire *cry2Ab* coding region to assess the stability of the inserted DNA. The *Sph*I restriction enzyme was used because it generated a unique Southern blot banding pattern for MON 15985 when probed with the full *cry2Ab* coding region. The stability of the inheritance of the functional insert in MON 531 was determined in a similar manner prior to its approved use and has been confirmed through generations of commercial use around the world.

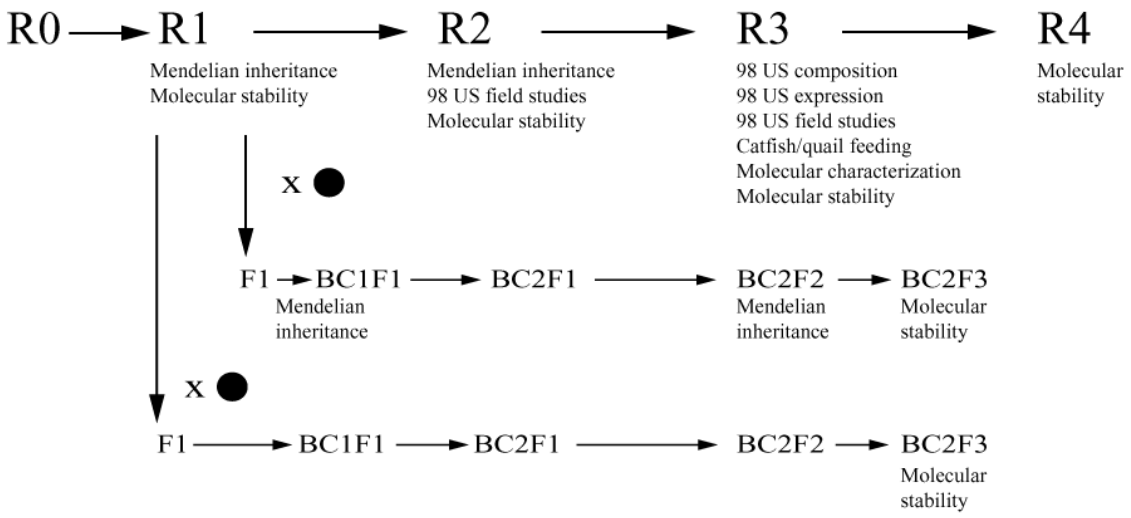

Figure 13 Progeny map of cotton MON 15985 generations used for specified testing

Table 9 Segregation data and analysis of progeny of MON 15985 cotton event

	Expected		Observed [1]		
Generation [2]	**Positive**	**Negative**	**Positive**	**Negative**	**ChiSq**
R1 (3:1)	202.5	67.5	210	60	1.11 ns
R2 (3:1)	45	15	43	17	0.356 ns
BC1F1 (1:1)	199	199	213	185	1.970 ns
BC2F2 (3:1)	568	189	549	208	2.477 ns

1. Data expressed as number of positive and negative plants based on Cry2Ab qualitative ELISA.
2. R1 seed was from initial R0 transformant in a DP50B background.
   R2 seed was pooled from heterozygous R1 plants in a DP50B background.
   BC1F1 and BC2F2 plants were pooled from five different elite cultivar backgrounds.

ns = Not significant at p=0.05 (chi square = 3.84, 1 df).

All generations segregated as expected for a single insertion site. The R1 progeny of MON 15985 yielded the expected segregation ratio of 3:1 with respect to the detection of the Cry2Ab2 protein. Progenies of MON 15985 backcrossed to commercial cotton cultivars yielded the expected segregation ratio of approximately 1:1 with respect to the Cry2Ab2 protein. The Chi square analysis of the segregation results showed that the segregation pattern was consistent with a single active site of insertion into the genomic cotton DNA and segregated according to Mendelian genetics. These data confirmed that MON 15985 contained a single DNA insert which segregated according to Mendelian genetics and remained stably integrated into the plant genome over successive selfed and backcrossed generations.

The stability of the DNA insert in MON 15985 and expression of the foreign proteins across five generations was confirmed by data from Southern blot, ELISA and Western blot analysis (Bookout *et al*., 2001, Pineda *et al*., 2002). These studies were carried out on multiple generations. The non-transgenic control DNA and the parental control DNA produced no hybridization to *cry2Ab*. The hybridization banding patterns for DNA extracted from the five plant breeding generations were the same and showed no

differences (data not provided). This demonstrated that the insert was stable in the successive generations.

Based on this information, there is no evidence for genetic instability of the inserted DNA in MON15985. Other data (not provided) demonstrated that *cry1Ac* and *cry2Ab* segregate independently of one another and are inserted at different positions on the plant genome. The data collected over many generations of crossing and backcrossing, with no significant variation from expected segregation rations for the two insecticidal genes confirms that the *cry1Ac* and *cry2Ab* genes are maintained as single dominant Mendelian traits over (Shappley, 2002).

3.B EXPRESSED MATERIAL

Hazard identification requires knowledge of which introduced genes are expressed, the characteristics, concentration and localization of expressed products, and the consequences of expression. Where the result of the modification is the expression of a novel protein, or polypeptide, this material must be characterized with respect to: identity; functionality; and, where appropriate, similarity to products from traditional sources. The expression data help determine the level of environmental exposure to the new proteins and are useful for determining the efficacy of the product and the susceptibility of the crop to the development of insect resistance.

The concentration of novel protein expressed in transgenic plant tissues can be very low, often times much less than 0.1% on a dry weight basis. Studies, such as acute toxicity testing, that require relatively large amounts of material are often not feasible using the protein purified from plant tissue. Instead, these studies normally make use of protein purified from bacterial expression systems. In such cases, it is necessary to demonstrate the functional equivalence (*i.e.*, equivalent physiochemical properties and biological activities) of proteins purified from the two sources. When equivalence is demonstrated based on serological cross-reactivity, it is important to use antisera (either polyclonal or monoclonal) that have been well characterized with respect to their specificity.

The possibility of post-translational modification (*e.g.*, glycosylation) in eukaryotic systems should also be taken into account, as this may affect allergenic potential.

In cases where the modification results in the expression of a novel non-translatable RNA transcript, the sensitivity and specificity of the desired action should be established. Examples of this include the production of antisense mRNA or other RNA species resulting in the reduced production of an endogenous protein (*e.g.*, transgenic plants containing inserted antisense sequences).

3.B-1 Protein Expression Levels in Different Plant Tissues

Levels of Cry2Ab2 and GUS proteins were determined in samples collected from eight field trial locations in the U.S. in 1998, which represented the major U.S. cotton producing regions and a variety of environmental conditions. Locations in Texas and Arizona represented ‘plains’ type cotton culture, while locations in Mississippi, South Carolina, Louisiana and Alabama represented typical southern and south-eastern growing

areas and conditions. MON 15985 and control cotton lines were successfully grown and harvested under conditions typical for each region.

The trials were planted in a single block with two 15-foot row plots at Louisiana, South Carolina and Texas sites; four replicate blocks of 15-foot at Mississippi, Alabama, Louisiana and Arizona. At the Starkville, Mississippi site, the test and control events were planted in a single block in plots consisting of one 30-foot row. Sampling was carried out as follows:

Young leaf: At each site the first newly-expanded leaves of approximately 25 cm^2 size from six plants per plot were collected from each plot at 28 days after planting (DAP). Sub samples were ground on dry ice prior to analysis.

Cottonseed: Bulk seed cotton (2 kg) was collected from each location. The cottonseed was ginned and acid delinted at Monsanto research facilities in St. Louis. Sub samples were ground on dry ice prior to analysis.

Over-season leaf: Young terminal, fully-expanded leaves were collected from six plants per plot approximately every four weeks until just prior to application of the defoliant at the Alabama and one Mississippi site. Sub samples were ground on dry ice prior to analysis.

Whole plant: Four whole plants, including leaves, roots, stem, but not bolls, were collected from the test and control plots at the Alabama and one Mississippi site just prior to application of the defoliant. Whole plants were cut into pieces of 2-3 inches. Sub samples were ground on dry ice prior to analysis.

Pollen: Samples of pollen were collected at the Louisiana and one Mississippi site. Pollen was collected from approximately 80 plants, placed in labelled graduated tubes and pooled across replicates at each site to obtain sufficient material for analysis.

Samples collected for MON 15985 and the parental control line, DP50B, were stored under conditions to preserve the integrity of the sample. Samples were analysed using ELISA to estimate the protein levels. (The methods and assay validation were provided to regulators, but are confidential business information.) From the raw data, the mean, the range and the standard deviation (SD) were calculated. In addition, the percentage coefficient of variation (%CV) was calculated. This is essentially the standard deviation expressed as a percentage of the mean and helps in a comparison of the variability irrespective of the absolute values.

Cry2Ab protein production. Cry2Ab protein was detected in MON 15985 at low levels in various plant tissues (Tables 11 -15).

Table 10 Summary of levels of Cry2Ab2 and GUS protein in different tissues collected at locations in the U.S. during the 1998 field season

	Young Leaf MPL[1]±SD[2] (Range)[3]	**Seed** MPL[1]±SD[2] (Range)[3]	**Whole Plant** MPL[1]±SD[2] (Range)[3]	**Pollen** MPL[1]±SD[2] (Range)[3]
Cry2Ab2[4]				

	Young Leaf MPL[1]±SD[2] (Range)[3]	Seed MPL[1]±SD[2] (Range)[3]	Whole Plant MPL[1]±SD[2] (Range)[3]	Pollen MPL[1]±SD[2] (Range)[3]
MON 15985	23.8 ± 6.3 (10.1-33.3)	43.2 ± 5.7 (31.8-50.7)	8.80 ± 1.20 (7.3-10.5)	<0.25
DP50B	<2.65	<2.31	<1.24	<0.25
DP50	<2.65	<2.31	<1.24	<0.25
Gus[5]				
MON 15985	106 ± 32 (51.7-176)	58.8 ± 13.0 (37.2-82.3)	NA	NA
DP50B	<0.91	<4.42	NA	NA
DP50	<0.91	<4.42	NA	NA

NA = Not Analyzed

1. Mean Protein Levels (µg/g fwt). Protein levels are reported as microgram of protein per gram fresh weight of tissue and have been corrected for overall assay bias.
2. Standard Deviation. The mean and standard deviation were calculated from the analyses of plant samples, one from each of eight field sites except for tissues collected from a single site.
3. Range. Minimum and maximum values from the analyses of samples across sites.
4. The Limit of Detection for the Cry2Ab2 assay is 2.65 µg/g in leaf tissue and 2.31 µg/g in seed tissue. The Limit of Quantification for the Cry2Ab2 assay is 1.24 µg/g in whole plant tissue and 0.25 µg/g in pollen tissue.
5. The Limit of Detection for the GUS assay is 0.91 µg/g in leaf tissue and 4.42 µg/g in seed tissue.

Table 11 Levels of Cry2Ab2 protein in young leaf samples from MON 15985 collected at locations in the U.S. during the 1998 field season

Site	Mean Cry2Ab2 (µg/g fwt)	% CV	Range (µg/g fwt)	Standard Deviation
Winnsboro, LA[1]	20.2	NA	NA	NA
Florence, SC[1]	14.0	NA	NA	NA
Corpus Christi, TX[1]	33.3	NA	NA	NA
Leland, MS[2]	15.9	19.7	12.4-20.0	3.1
Loxley, AL[3]	21.0	23.4	15.5-24.9	4.9
Bossier City, LA[2]	14.8	14.2	12.2-16.7	2.1
Maricopa, AZ[2]	10.7	5.7	10.1-11.3	0.6
Starkville, MS[1]	27.3	NA	NA	NA

NA = Not Analyzed

1. The % CV, range or standard deviation are not reported since there was only one plot.
2. The % CV, range and standard deviation for this site are from four replicate plots.
3. The % CV, range and standard deviation for this site are from three replicate plots.

Table 12 Levels of Cry2Ab2 protein in leaf samples collected through the season from MON 15985 at locations in the U.S. during 1998

	28 DAP* MPL[1]±SD[2] (Range)[3]	55 DAP MPL[1]±SD[2] (Range)[3]	85 DAP MPL[1]±SD[2] (Range)[3]	108 DAP MPL[1]±SD[2] (Range)[3]
MON 15985	21.0 ± 4.9 (15.5 - 24.9)	40.1 ± 6.5 (34.6 - 49.4)	19.7 ± 2.7 (15.9 - 21.8)	16.7 ± 0.6 (15.8 - 17.3)
DP50B	<2.65	<2.65	<2.65	<2.65
DP50	<2.65	<2.65	<2.65	<2.65

1. Mean Cry2Ab2 protein levels (µg/g fwt). Protein levels are reported as microgram of protein per gram fresh weight of tissue and corrected for overall assay bias. The value was estimated from the analyses of four samples from Loxley, AL site. The Limit of Detection for the Cry2Ab2 assay is 2.65 µg/g in leaf tissue.
2. Standard Deviation. The mean and standard deviation were calculated from the analyses of plant samples, one from each of eight field sites except for tissues collected from a single site.
3. Range. Minimum and maximum values from the analyses of samples across eight sites.

* DAP = days after planting

Table 13 Levels of Cry2Ab2 protein in seed samples from MON 15985 at locations in the U.S. during the 1998 field season

Site	Mean Cry2Ab2 (µg/g fwt)	% CV	Range (µg/g fwt)	Standard Deviation
Winnsboro, LA[1]	46.7	NA	NA	NA
Florence, SC[1]	34.3	NA	NA	NA
Corpus Christi, TX[1]	48.9	NA	NA	NA
Leland, MS[2]	41.6	8.7	37.3 - 46.2	3.6
Loxley, AL[2]	42.6	20.0	31.8 - 50.5	8.5
Bossier City, LA[2]	42.3	11.2	36.7 - 47.9	4.7
Maricopa, AZ[2]	47.4	9.7	40.7 - 50.7	4.6
Starkville, MS[1]	39.3	NA	NA	NA

NA = Not Analyzed

1. The % CV, range or standard deviation are not reported since there was only one plot.
2. The % CV, range and standard deviation for this site are from four replicate plots.

Table 14 Levels of Cry2Ab2 protein in whole plant samples from MON 15985 at locations in the U.S. during the 1998 field season

Site	Cotton Event or Line	Mean Cry2Ab2 (µg/g fwt)	% CV	Range (µg/g fwt)	Standard Deviation
Leland, MS[1]	15985	8.89	14.8	7.27 - 10.5	1.31
	DP50B	<1.24	NA[2]	<1.24	NA[2]
	DP50	<1.24	NA[2]	<1.24	NA[2]
Loxley, AL[1]	15985	8.72	14.5	7.31 - 9.87	1.27
	DP50B	<1.24	NA[2]	<1.24	NA[2]
	DP50	<1.24	NA[2]	<1.24	NA[2]

1. The % CV, range and standard deviation for this site are from four replicate plots.
2. The % CV or standard deviation is not reported since levels were below the limit of detection.

The levels of Cry2Ab2 protein in young leaves was consistent across all plots and field locations with a range from 10.1 to 33.3 µg/g fwt (fresh weight), and a mean across all locations of 23.8 ±6.3 µg/g fwt (Table 10). The mean levels and ranges of Cry2Ab2 protein in leaf tissue for each location are summarized in Table 11. The mean level of Cry2Ab2 protein production in leaf samples peaked at 55 DAP and subsequently declined over the growing season to a mean of 16.7 µg/g fwt at 108 DAP (Table 12). No Cry2Ab2 protein was detected in leaf samples from the control line, DP50B, or the non-transgenic control, DP50, at any location (limit of quantification = 2.5 µg/g fwt).

Levels of Cry2Ab2 protein in cottonseed tissue were consistent across all locations, ranging from 31.8 to 50.7 µg/g fwt, with a mean of 43.2±5.7 µg/g fwt (Table 10). No Cry2Ab2 protein was detected in cottonseed samples from the control line, DP50B, or the non-transgenic control, DP50. The mean levels and ranges of Cry2Ab2 protein in cottonseed from the eight locations are summarized in Table 13.

In whole plant tissues, the mean levels of Cry2Ab2 protein were 8.80±1.20 µg/g fwt, with the range across locations of 7.28 to 10.45 µg/g fwt (Table 10). No Cry2Ab2 protein was detected in whole plant samples from the control line, DP50B, or the non-transgenic control, DP50. The mean levels and ranges of Cry2Ab2 protein in whole plant tissue from the two locations are summarized in Table 14.

In pollen, no Cry2Ab2 protein was detected above the limit of detection for the assay (0.25 µg/g fwt) at either location in either the test or control samples.

GUS protein production. Levels of the GUS protein were measured in newly expanded leaf and cottonseed using validated ELISA. GUS protein in MON 15985 was detected at low levels in these tissues (Table 10, Table 15-17).

Table 15 Levels of GUS protein in leaf samples from MON 15985 at locations in the U.S. in the 1998 field season

Site	Mean GUS (µg/g fwt)	% CV	Range (µg/g fwt)	Standard Deviation
Winnsboro, LA[1]	92.1	NA	NA	NA
Florence, SC[1]	101	NA	NA	NA
Corpus Christi, TX[1]	176	NA	NA	NA
Leland, MS[2]	119	12.3	101 - 135	15
Loxley, AL[3]	61.4	13.8	51.7 67.1	8.5
Bossier City, LA[2]	100	19.2	79.5 - 126	19
Maricopa, AZ[2]	103	10.5	92.0 - 116	11
Starkville, MS[1]	168	NA	NA	NA

NA = Not Analyzed

1. The % CV, range or standard deviation are not reported since there was only one plot from this site.
2. The % CV, range and standard deviation for this site are from four replicate plots.
3. The % CV, range and standard deviation for this site are from three replicate plots.

Table 16 Levels of GUS protein in cottonseed samples from MON 15985 at locations in the U.S. in the 1998 field season

Site	Mean GUS (µg/g fwt)	% CV	Range (µg/g fwt)	Standard Deviation
Winnsboro, LA[1]	50.6	NA	NA	NA
Florence, SC[1]	46.5	NA	NA	NA
Corpus Christi, TX[1]	71.3	NA	NA	NA
Leland, MS[2]	64.6	13.8	58.0 - 77.7	8.9
Loxley, AL[2]	51.8	19.8	37.2 - 60.6	10.3
Bossier City, LA[2]	54.5	23.7	44.2 - 73.4	12.9
Maricopa, AZ[2]	71.0	16.2	59.2 - 82.3	11.5
Starkville, MS[1]	39.6	NA	NA	NA

NA = Not Analyzed

1. The % CV, range or standard deviation are not reported since there was only one plot from this site.
2. The % CV, range and standard deviation for this site are from four replicate plots.
3. The % CV, range and standard deviation for this site are from three replicate plots.

The mean levels and ranges of GUS protein in leaf tissue are summarized in Table 15. The levels of GUS protein in young leaves ranged from 51.7 to 176 µg/g fwt, with a mean across all locations of 106 ± 32 µg/g fwt (Table 10). No GUS protein was detected in leaf samples from the control, DP50B, or the non-transgenic control, DP50, at any location.

The mean levels and ranges of GUS protein in cottonseed for each location are summarized in Table 16. Levels of GUS protein in cottonseed ranged from 37.2 to 82.3 µg/g fwt, with a mean of 58.8 ± 13.0 µg/g fwt (Table 10). No GUS protein was detected in cottonseed samples from the control, DP50B, or the non-transgenic control, DP50.

The levels of Cry2Ab2 and GUS proteins in the control samples, DP50 and DP50B, were below the level of detection in all sampled tissues. The levels of Cry2Ab2 and GUS proteins expressed in the tissues of MON 15985 are low compared to total protein content. Cry2Ab2 in leaves represents 0.014% of total protein and GUS in the leaves represents 0.072% of total protein.

Cry1Ac, NPTII and AAD protein levels in MON 531: The levels of Cry1Ac, NPTII and AAD proteins were analyzed in MON 531, the parent plant for MON 15985. These analyses were not repeated on MON 15985, but data submitted for the evaluation of MON 531 are summarised below. Analysis of MON 531 indicated that Cry1Ac and NPTII proteins were expressed constitutively in the plants at low levels in leaves, roots, flowers, pollen and seed and that levels declined from 46 daDAP. The mean level of the Cry1Ac protein in raw cottonseed obtained from trials over eight years and multiple sites ranged from approximately 1 to 9 µg/gram fresh weight. In raw cottonseed, the mean level of the NPTII protein ranged from 2.0 to 15 µg/gram fresh weight over the same period.

The mean levels of the Cry1Ac protein determined from field trial material grown in 1992 were 1.56 and 0.86 µg/gram fresh weight in leaf and raw cottonseed, respectively. The mean levels of the NPTII protein in the same material were 3.15 and 2.45 µg/gram fresh weight, respectively for leaf and raw cottonseed. The Cry1Ac protein was not detected in MON 531 nectar using an assay with a limit of detection of 1.6 ng/g fresh weight of the nectar. The Cry1Ac protein was present in pollen at levels just above the limit of detection of the assay: 11.5 ng/g fresh weight of the pollen.

Primary MON 531 fruiting structures showed an average Cry1Ac concentration of 259 µg/g dry weight at 46 DAP. This level declined in an exponential manner to 43 µg/g at 116 DAP. Expression in terminal foliage declined from 370 mg/g dry weight at 46 DAP to 144 mg/g at 116 DAP. However, the Cry1Ac protein levels remained sufficiently high for effective control of the targeted insect pests throughout the season.

Mature MON 531 plants contained an estimated 0.08 µg Cry1Ac protein/g fresh weight and 3.3 µg NPTII protein/g fresh weight on a whole plant basis. This equates to approximately 10 µg Cry1Ac protein per plant. After processing, the levels of Cry1Ac protein were reduced to non-detectable levels in the major cottonseed processed products: refined oil, linter brown stock and cottonseed meal.

The AAD protein was not detected in the leaf or seed tissue from MON 531 cotton at the limit of detection of 0.008 and 0.005 µg/gram fresh weight for leaf and seed, respectively. This result was expected since the *aad* gene is driven by a bacterial promoter and is not expected to be expressed in the cotton plant.

3.B-2 Protein Equivalency Studies

An applicant has to investigate the physical and biochemical properties of new proteins produced by the genes that have been introduced into the transgenic plant. Often, the low level of protein production in transgenic plants requires the applicant to undertake these studies on proteins produced by bacterial fermentation, as the only feasible means of obtaining enough protein for the studies. Where this is necessary, it is important for the applicant to provide data that confirms the equivalence of the bacterial and plant proteins so that extrapolation can be made between the test data and the transgenic plant. In addition, these data can provide information for the comparison between the transgenic organism and its conventional counterparts or parental lines. These comparisons help to identify possible changes to plant performance that might be related to the new genes or

their products. These data are of relevance to both the environmental risk assessment and the food and feed safety assessment of the transgenic plant and so this section is reviewed by both the environmental and food and feed safety review experts.

The very low levels of the Cry2Ab2 protein produced in MON 15985 plant tissues made it necessary to produce purified Cry2Ab protein by bacterial fermentation. This protein was used for characterization studies and ecotoxicity testing. As such, it was necessary to compare the bacterial Cry2Ab protein to Cry2Ab2 produced by MON 15985 to ensure that the studies on one could be extrapolated to the other with an acceptable level of scientific certainty.

Size determination. Solutions of the bacterial Cry2Ab protein were applied to a polyacrylamide gel (4 to >20%) run under reducing conditions. Molecular weight markers were used to determine the weight of the bacterial Cry2Ab and contaminant proteins. Densitometric analysis was used to determine the ratio of bacterial Cry2Ab protein to contaminant proteins. Protein molecular weight was estimated by comparison to marker proteins (data not provided). The bacterial Cry2Ab protein had the expected molecular weight of 63 kDa with a purity of 65.5 %.

Immunoreativity. Immunoblots were prepared and developed separately with either polyclonal anti-Cry2Ab rabbit antibody or monoclonal anti-Cry2Aa mouse antibody. One major protein (~63kDa) was recognized by both polyclonal anti-Cry2Ab antibody and monoclonal antibodies raised against Cry2Aa. An additional immuno-reactive protein (~53 kDa) was observed and was most likely a degradation product of the 63 kDa protein.

Bioactivity. The EC_{50} (half maximal effective concentration) and LC_{50} (lethal concentration at which half the test organisms die) using the pest insect *Helicoverpa zea* were determined on the bacterial Cry2Ab protein.

N-terminal amino acid sequence. The N-terminus of the major polypeptide in the bacterial Cry2Ab protein sample was determined to coincide to a large extent with the predicted sequence. A "ragged N-terminus" resulted in the identification of a major and minor sequence. This result may have been caused by "protease-sensitive" sites at the N-terminus of the protein. Further, the cysteine at position 13 was not observed in either determined sequence, which was consistent with the Edman degradation chemistry used in this method in which cysteine residues are chemically unstable.

Stability. The stability of the bacterial Cry2Ab protein in purified water was determined at storage temperatures of 4, -20 and -80 °C over a period of 87 days. Aliquots were removed at 0, 11, 41, 52, and 87 days and analyzed using SDS-PAGE (data not provided). Densitometric analysis was also performed on SDS-PAGE gels (data not provided). Based upon these gels, the protein was stable for at least 87 days stored at -80, -20 and 4 °C in purified water. Only the samples stored at 4 °C showed a small decrease in optical density, the samples stored at -80 and -20 °C did not show significant degradation.

The results of the bacterial Cry2Ab protein characterization are summarized in Table 17.

Table 17 Summary of Cry2Ab protein characteristics

Criteria	Method	Result
Identity and molecular weight	a) N-terminal sequence analysis b) Immunoblot	a) confirmed b) confirmed
Concentration	Protein assay and amino acids compositional analysis	correction factor of 1.7 was established
Strength	CEW bioassay (corrected for purity and amino acid compositional analysis)	EC_{50} of 0.24 g/ml LC_{50} of 52.4 g/ml
Purity	Densitometry	65.5%
Stability	SDS-PAGE and immunoblot analysis of solutions stored at 4, -20 and -80°C	≥87 days at - 20 and - 80°C; at least 52 days at 4°C
Heat stability	SDS-PAGE/Western Blot analysis of samples	no bands seen after treatment at 121°C for 30 mins

These data confirm that the characteristics of the Cry2Ab protein produced by bacterial fermentation were equivalent to those of the plant-produced protein and that studies with the bacterial protein could be used to study the safety of the plant protein.

Cry1Ac protein characterization. Cry1Ac protein analysis data was submitted by the developer for the approval of MON 531. These data showed that the microbial expressed and purified Cry1Ac delta-endotoxin was sufficiently similar to that expressed in the plant to be used for mammalian toxicological purposes. The plant and microbial produced Cry1Ac delta-endotoxins had similar molecular weights and immunoreactivity (SDS-PAGE and Western blots), lacked detectable post-translational modification (glycosylation tests), had identical amino acid sequences in the N-terminal region and similar results in bioassays against *Heliothis virescens* and *Helicoverpa zea*. While it is difficult to prove that two proteins are identical, the combined results of the studies indicated a high probability that these two sources produce proteins that were essentially identical.

Characterization and history of safe consumption of Cry2Ab2 and Cry1Ac. There is a history of safe use of Cry proteins in the long term use of microbial *B. thuringiensis*-based approved products (U.S. EPA, 1998; IPCS, 1999). Strains of *B. thuringiensis* have been used safely as commercial microbial pesticides for over 40 years. The naturally occurring Cry proteins produced in *B. thuringiensis* have been shown to have no deleterious effects to fish, avian species, mammals and other non-target organisms (US EPA, 1988; Betz *et al.*, 2000). The safety of the Cry proteins to non-target species is attributed to their highly specific mode of action, and rapid digestibility. The EPA and WHO have concluded that the potential dietary exposure to Cry proteins from use of microbial sprays on food crops does not raise any concerns: "The use patterns for *B. thuringiensis* may result in dietary exposure with possible residues of the bacterial spores on raw agricultural commodities. However, in the absence of any toxicological concerns, risk from the consumption of treated commodities is not expected for both the general

population and infants and children" (U.S. EPA, 1998) and "*B.t*. has not been reported to cause adverse effects on human health when present in drinking-water or food." (IPCS, 1999).

The amino acid sequence of the Cry2Ab2 protein produced in MON 15985 was predicted based on the nucleotide sequence of the coding sequence. The Cry2Aa protein exhibited a high degree of amino acid similarity (97%; 88% identical amino acids) with the Cry2Ab2 protein produced in MON 15985. Thus, safety studies conducted with microbial *B. thuringiensis* products containing Cry2A proteins were relevant to the safety assessment of the Cry2Ab protein present in MON 15985. The Cry2A protein as a component of *B. thuringiensis* microbial products has been shown to have no deleterious effects on fish, avian species, mammals, and other non-target organisms (US EPA, 1998; Betz *et al*., 2000).

The Cry1Ac protein was produced as an insoluble crystal in *B. thuringiensis* for safety testing. The crystal protein was the pro-toxin form of the protein. The amino acid sequence of the Cry1Ac protein expressed in MON 531 was predicted based on analysis of the coding nucleotide sequence. The Cry1Ac protein produced in MON 531 cotton was >99.4% identical to the protein produced by the *B. thuringiensis* bacterial strain.

Insecticidal activity of the Cry1Ac protein requires that the protein be ingested. In the insect gut, the protein is solubilized due to the high pH of the insect gut and is proteolytically cleaved to the active core of the protein, which is resistant to further degradation by the insect gut proteases. The core protein binds to specific receptors on the mid-gut of lepidopteran insects, inserts into the membrane and forms ion-specific pores (English and Slatin, 1992). These events disrupt the digestive processes and cause the death of the insect. The lack of acute toxicity of the Cry proteins to non-target species is attributed to their highly specific mode of action and rapid digestibility.

Characterization and history of safe consumption of NPTII. The NPTII protein expressed in MON 531 is chemically and functionally similar to the naturally occurring NPTII protein (Fuchs *et al.,* 1993). This gene has been integrated into a number of transgenic crops and its gene product has been consumed safely over the last decade.

Characterization and history of safe consumption of GUS. The GUS protein produced in MON 15985 has an extensive history of safe use. Exposure of humans to the GUS protein is common, because GUS is present in intestinal epithelial cells, intestinal microflora bacteria, and numerous foods, and no harmful effects have been reported (Gilissen *et al*., 1998). GUS activity has been detected in over 50 plant species (Hu *et al.,* 1990). These species include a number of human food sources, including potato, apple, almond, rye, rhubarb, and sugar beet (Schulz and Weissenbock, 1987; Hodal *et al.,* 1992; Wozniak and Owens, 1994). GUS is also present in beef and in a number of invertebrate species, including nematodes, molluscs, snails, and insects (Gilissen *et al*., 1998). Even when ingested in raw foods such as shellfish or apples, GUS is not known to cause harmful effects (Gilissen *et al.,* 1998). Likewise, the metabolites of *E. coli*-derived GUS are non-toxic (Gilissen *et al*., 1998). The *E. coli*-derived GUS enzyme produced by

MON 15985 was 99.8% homologous and functionally equivalent to the GUS enzyme from *E. coli* naturally present in the human gut (data not provided).

These data indicated that the new proteins in MON 15985 all have a history of safe use and consumption and are not expected to result in adverse impact if the cotton event is cultivated, processed and consumed.

Digestion in simulated gastric and intestinal fluids. Rapidly digested proteins represent a minimal risk of conferring novel toxicity or allergy, comparable to other safe dietary proteins (Astwood and Fuchs, 1996; Astwood and Fuchs, 2000). The rate of degradation of the proteins was evaluated separately in simulated gastric (pepsin, pH 1.2) and intestinal (pancreatin, pH 7.5) fluids. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopoeia (1995). For the Cry1Ac and NPTII proteins, the absence of toxic effects in humans and other mammals was supported by data submitted for the approval of MON 531that showed the rapid degradation of these proteins in gastric digestion studies.

In vitro, simulated mammalian gastric and intestinal digestive mixtures were used to assess the susceptibility of the Cry2Ab2 protein to proteolytic digestion. The rate of degradation of the Cry2Ab2 was evaluated separately in simulated gastric (pepsin, pH 1.2) and intestinal (pancreatin, pH 7.5) fluids. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopoeia (1995).

The degradation of the Cry2Ab2 protein was assessed by SDS-PAGE, Western blot analysis and insect bioassay. SDS-PAGE analysis of simulated gastric fluid (SGF) demonstrated that greater than 98% of the Cry2Ab2 protein was digested within 15 seconds and that no fragments of the parent protein larger than 2kDa were resolved. The acid conditions of the stomach denature the native conformation of the Cry2Ab2 protein, facilitating its rapid digestion. Western blot analysis of simulated intestinal fluid (SIF) showed that within one minute the Cry2Ab2 protein was degraded to a relatively stable protein fragment (≈50kDa) that was bioactive for at least 24 hours. This result was expected because protease-resistant core proteins of *B. thuringiensis* insecticidal proteins are known to be resistant to further trypsin digestion (Lilley *et al*., 1980). *In vivo*, the Cry2Ab2 protein would be exposed to gastric conditions prior to entering the intestinal lumen. The low pH and pepsin in the stomach would be expected to either fully digest the protein or cause it to become susceptible to intestinal digestion.

In vitro, simulated mammalian gastric and intestinal digestive mixtures were used to assess the susceptibility of the GUS protein to proteolytic digestion. The rate of degradation of the GUS protein was evaluated separately in simulated gastric (pepsin, pH 1.2) and intestinal (pancreatin, pH 7.5) fluids.

The degradation of the GUS protein was assessed by Western blot analysis and enzymatic activity assays. Within 15 seconds of exposure to simulated gastric fluid, there was no detectable GUS protein in either assay. After two hours in simulated intestinal fluid, 91% of the original GUS activity was lost in the enzyme assay, with only

a faint band detected in the Western blot analysis. Based on these results, it was concluded that any GUS protein ingested by humans would be readily degraded in the digestive tract (Fuchs and Astwood, 1996).

The Cry2Ab2, Cry1Ac, NPTII and GUS proteins all degraded rapidly in simulated gastric fluids, indicating that they will be rapidly degraded in the stomachs of mammals and so are unlikely to be mammalian toxins. Digestion of NPTII and GUS in intestinal fluids was also rapid, but Cry2Ab2 and Cry1Ac degraded less and remained more stable in these fluids. This is consistent with the protease stable nature of the core Bt protein unit and is unlikely to result in mammalian toxicity because of initial degradation in gastric fluids before reaching the intestine, and the absence of receptors in mammalian intestines for attachment of the core Bt protein. The protein safety assessments of MON 15985 showed characteristics that were indicative of a high level of safety to consumers.

3.C PHENOTYPIC CHARACTERIZATION

Growth, morphology, yield and other agronomic characteristics are used by plant breeders to identify possible unintended genetic changes in new varieties that would be undesirable for any future product. For this reason, the growth and performance of transgenic events in representative environments is compared to that of known, acceptable varieties. The effect of the insertion of new genes on the phenotypic characteristics of MON 15985 was assessed by collection of agronomic data during field trials. This section compares the agronomic performance of the transgenic plant with the performance of closely related conventional cotton lines and the parent line, transgenic MON 531. Specific information and data are included in descriptive and tabular formats.

The U.S. field trials with MON 15985 were undertaken at eight locations in 1998, 90 locations in 1999 and 87 locations in 2000. The qualitative and quantitative assessments of agronomic performance were obtained through cooperation with academics, crop consultants and state variety trials. Most of the trials were randomized complete block arrangement of four rows from 30 to 60 feet in length. Detailed monitoring for growth and development characteristics and disease incidence in MON 15985 compared to control cotton plants was undertaken at least monthly during the growing season.

3.C-1 Agronomic and Morphological Characteristics

Weather conditions were typical for the growing regions during the field trial seasons, with the exception of hurricane conditions in one state in 1998 that produced higher wind and rainfall.

Agronomic criteria were measured at multiple locations each year across all fifteen major growing states to ensure equivalence to the parental cultivar. The measured criteria were yield, morphology and maturity, pest and disease susceptibility, and fibre quality. Yield and morphology and maturity were determined using a number of different observations common in cotton breeding assessment, and fibre quality was determined using high-volume instrument (HVI) classing, including measurements for fibre length, strength and micronaire (data not provided). Agronomic data collected from the trials was published (Mahaffey, *et al*., 2000)

Growth habit. Several criteria were measured to determine morphology and maturity: general plant appearance, days to emergence, seedling vigour, plant stand counts, height-to-node ratio, days to first white flower, days to first cracked boll, days to 50% open bolls, fruit retention, plant mapping and days to harvest.

No significant differences were noted in the growth habit between MON 15985 and control plants, DP50 (conventional cotton line) and DP50B (MON531 parental line).

Lifespan. Observations of MON 15985 during four years of field trials in 8 diverse locations confirmed that this cotton event has the same lifespan as conventional cotton.

Vegetative vigour. Summary data on mean height:node ratio, number of days to peak bloom and total cracked boll counts are presented in Table 18.

Table 18 Summary of mean height:node ratio, number of days to peak bloom and total cracked boll counts at eight locations in the U.S. in 1998

Event or line #	Height:node ratio	Mean number of days to peak bloom	Mean total number of cracked bolls/plot
MON 15985	1.70	15.29	407
DP50B	1.77	15.03	431
DP50	1.72	15.77	284

No significant differences in vegetative vigour were noted between MON 15985, DP50B (MON 531) and DP50. This indicated that the vegetative growth patterns of the transgenic cotton are similar to those in conventional cotton.

Reproductive characteristics. Extensive observations recorded by field co-operators in 1998, 1999 and 2000 field trials at multiple locations in the United States demonstrated that the mode and rate of reproduction in MON 15985 is typical of other cotton varieties.

Yield characteristics. No statistical differences were observed between MON 15985, DP50 and DP50B for lint per cent (mass of lint as a percentage of lint plus seed), seed index (mass on grams of 100 seeds), or boll size (Figure 14 and 15) (Mahaffey, *et al*., 2000).

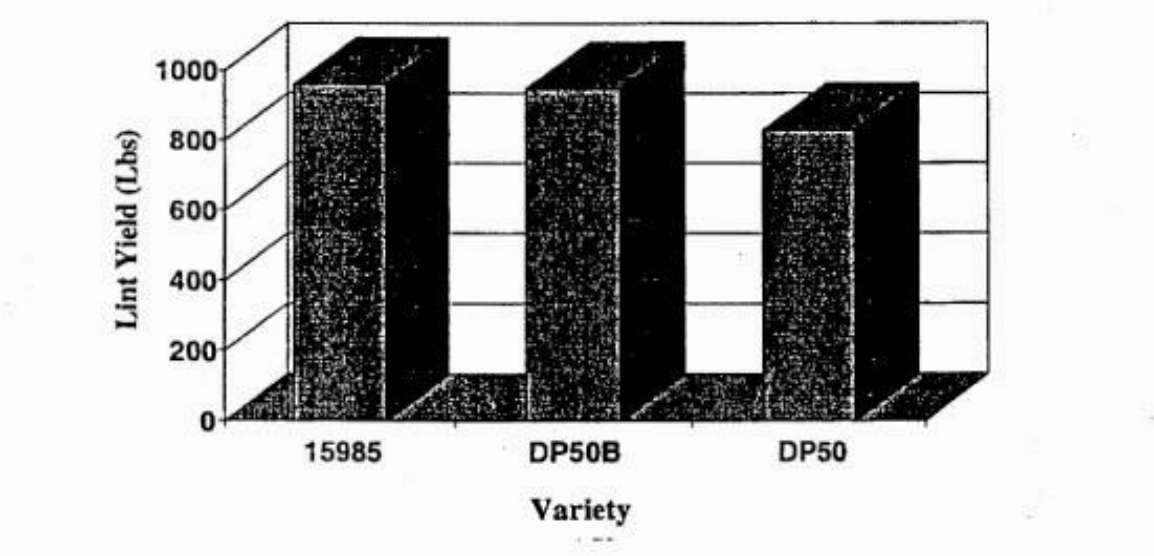

Figure 14 Lint yield in pounds per acre averaged across locations in the 1998 and 1999 field trials
Notes for figure: 15985 = MON 15985; DP50B = MON 531; DP50 = conventional cotton

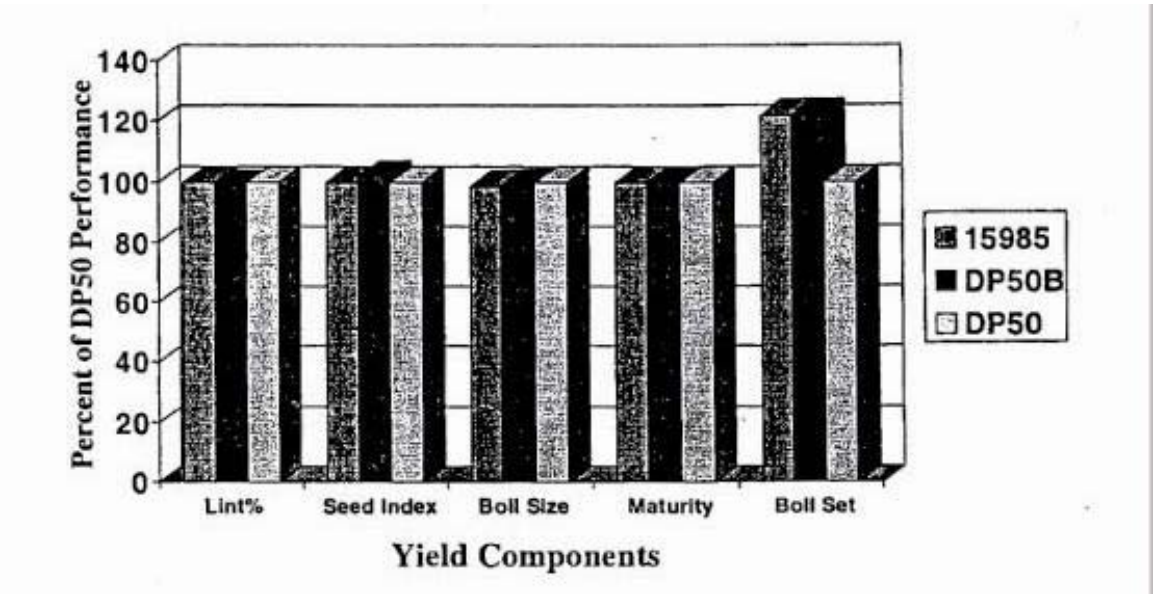

Figure 15 Yield characteristics as a percentage of DP50 performance in the 1998 and 1999 field trials
Notes for figure: 15985 = MON 15985; DP50B = MON 531; DP50 = conventional cotton

Pest and disease susceptibility. Disease symptoms were scouted once per month during the growing season at each location. Plots were visually inspected for the appearance of possible disease symptoms such as damping off, boll rot, spotted leaves, leaf necrosis, stunted or distorted plants and wilting. These symptoms are indicative of local cotton diseases.

Monitoring for the presence of insect infestation and disease was increased to weekly observations from the onset of lepidopteran larvae infestations. These observations followed routine protocols and the field trial reports were submitted with the application (300 pages, not included here).

Damage ratings were generated from both natural and artificial insect infestations. These were based on inspection of ten random plants per centre row (20 plants per site) from each test plot at identified periods of infestation in the conventional control plots (DP50). Data collected to determine damage ratings included some or all of the following:

- eggs and/or egg masses
- number of beet armyworm (*Spodoptera exigua*) 'hits'
- live larva identified by species and location on the plant
- damaged terminals and the suspected species causing this damage
- estimated % defoliation and the suspected species causing this damage
- damaged squares and the suspected species causing this damage
- damaged white blooms and the suspected species causing this damage
- damaged bolls and the suspected species causing this damage.

Approximately 13 per cent of the locations documented symptoms of disease and no difference was observed between incidence and severity of disease symptoms in MON 15985 compared to conventional cotton controls.

Based on extensive field observations during more than 250 field trials in the U.S. from 1998 to 2000, MON 15985 exhibited similar agronomic and morphological traits to conventional cotton controls. The development, agronomic performance and morphology of MON 15985 indicated that MON 15985 is typical of conventional cotton varieties in terms of growth and agronomic performance, was well suited to cotton production and did not contain any observed traits that were deemed undesirable for this purpose. These data support the conclusion that the combined effect of the gene inserts is improved insect protection only.

3.C-2 Efficacy

Efficacy of the Cry proteins expressed in MON15985 was evaluated during the field trials that were used to assess agronomic performance (Section 9.C.1). Insects were observed throughout the trials with qualitative assessments made over 530 times in the first 2 years of trials. Approximately 41 % of the field trial locations documented differences in target insect numbers between MON 15985 and conventional cotton controls. Efficacy data from these studies was published (Jackson *et al*., 2000).

Mortality of cotton bollworm is illustrated in Figure 16. For this study, leaf tissue was collected from a minimum of 100 plants per treatment and infested with one or two small larvae in laboratory containers. Mortality was visually assessed at 72 hours post-infestation.

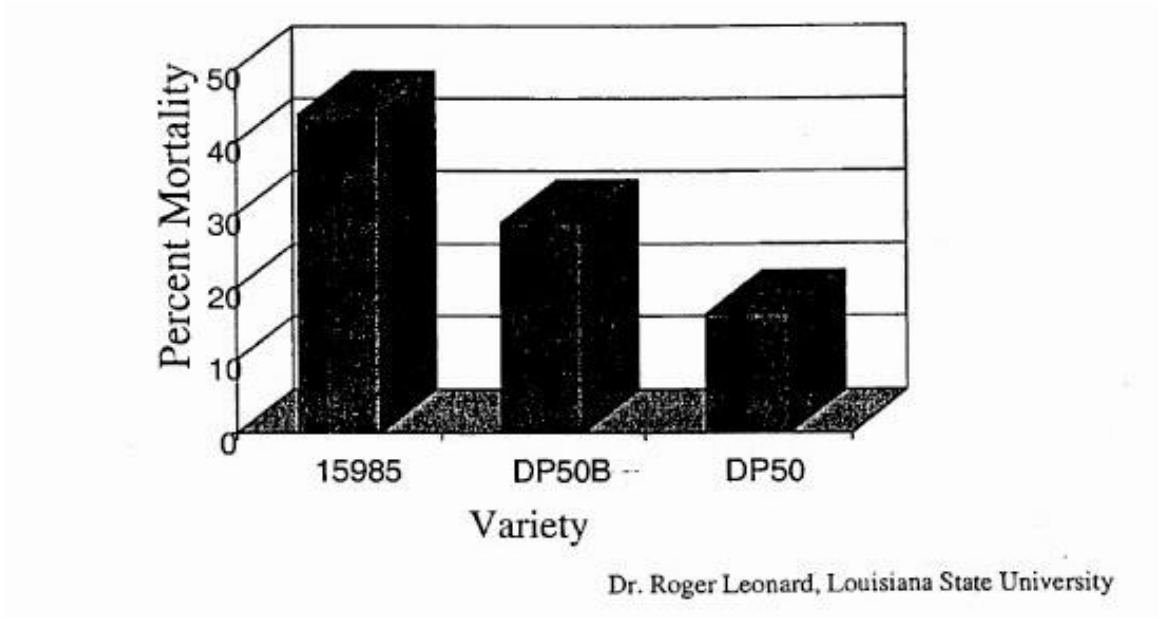

Figure 16 Per cent mortality of cotton bollworm 72 hours after infestation on field generated leaf tissue
Notes for figure: 15985 = MON 15985; DP50B = MON 531; DP50 = conventional cotton

The inserted genes improved the protection in MON 15985 against lepidopteran pests. Results from the damage rating observations indicated clearly that MON 15985 has improved efficacy against target insects (cotton bollworm, tobacco budworm, and pink bollworm) relative to MON 531 and conventional cotton, DP50.

MON 15985 consistently performed better against target insect infestations than the control cotton varieties. This confirmed that the *cry1Ac* and *cry2Ab* gene products provided effective protection against lepidopteran pests of commercial cotton.

4. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

The assessment of the interaction of MON 19985 with the environment has included studies on: gene transfer to other plants (outcrossing), gene transfer to other cultivated cotton (introgression), invasiveness, seed germination and dissemination, impact on non-target organisms and the plant's pest potential.

4.A CONSEQUENCES OF POTENTIAL GENE TRANSFER TO RELATED PLANTS

The introgression of genetic information from one plant to another is only significant if the two plants are sexually compatible and if their hybrid offspring are viable. In order to assess potential environmental risks associated with outcrossing from transgenic plants, the reproductive biology of the plant and distribution of sexually compatible relatives must be known, and the impact of the introduced trait, should it be introgressed into other plant species, must be understood. Information about the former may be obtained from reviews on the biology of the plant species, scientific literature including national or regional plant surveys, extension agronomists, and weed scientists.

A risk assessment should always presuppose that the transgenic plant under review is capable of outcrossing with sexually compatible species unless there is sound experimental evidence to indicate otherwise (*e.g.*, the transgenic plant has been rendered infertile). The environmental significance of trait introgression will vary with each plant/trait combination. For example, movement of a herbicide tolerant (HT) trait from *Brassica napus* (canola) to a weedy relative is considered a low environmental risk as herbicide tolerance does not increase the fitness of HT/non-HT hybrids or their progeny in absence of the selection pressure provided by the herbicide. Additionally, any herbicide tolerant hybrids that do arise can be effectively managed using alternative control strategies that are part of conventional canola cultivation. Conversely, the introgression of an insect resistance gene from transgenic cotton into populations of wild *Gossypium* could theoretically increase the fitness of the latter if the target insect was responsible for limiting population size or distribution of the wild relatives.

For gene flow to occur via normal sexual transmission, certain conditions must exist:

- the two parents must be sexually compatible;
- their fecundity must coincide;
- a suitable pollen vector must be present and capable of transferring pollen between the two parents; and
- resulting progeny must be fertile and ecologically fit for the environment in which they are situated.

Three potential routes for gene escape from cotton were considered: vegetative material, seed and pollen. Cotton does not commonly propagate by vegetative material and, if it does in the U.S., it would be unlikely to survive the freezing winters that occur throughout most of the cotton-growing regions. Cotton bolls, due to their size and general properties are unlikely to be dispersed by mechanisms such as wind, birds or terrestrial animals. This leaves pollen flow as the primary consideration for gene flow assessment from cultivated transgenic cotton.

Outcrossing to wild *Gossypium* species. Gene flow to wild species is possible only if pollen finds sexually compatible species. For cultivated cotton the recipient must be an allotetraploid with an AADD genome. In the U.S. there are only three *Gossypium* species which can serve as recipients for *G. hirsutum*. These are *G. hirsutum* itself, *G. barbadense* and *G. tomentosum*, which grows only in Hawaii. *G. barbadense* does not grow wild in the U.S. and is cultivated from seed produced in seed production fields that are isolated from commercial cotton fields. The harvest from this species is processed and not used for replanting. Thus, gene flow to commercial fields of *G. barbadense* plants would be short-lived.

The *Gossypium thurberi* native cotton indigenous to Arizona and Mexico is not sexually compatible with MON 15985, as it has a diploid, DD genome.

Feral *G. hirsutum* cotton has not been reported in Burkina Faso and so it is unlikely that cultivated cotton species (*Gossypium hirsutum*) will hybridise with feral *G. hirsutum*

cotton. No sexually compatible wild relatives of cotton exist in West Africa. A near relative, *Gossypium herbaceum* var. *africana*, occurs in the region, but this species is a diploid, preventing successful outcrossing with the allotetraploid *G. hirsutum* (sterile seeds would be generated if such a cross were to take place in nature).

Introgression with cultivated cotton. Although natural crossing can occur, cotton is normally considered to be a self-pollinated crop (Niles and Feaster, 1984). The pollen is heavy and sticky and transfer by wind in unlikely, however there are no morphological barriers to cross-pollination based on flower structure. Pollen is transferred by insects; in particular by various wild bees, bumble bees and honey bees in the U.S. The activity of honey bees has been studied in cotton fields in Burkina Faso (Sere, 2007) where they collect nectar.

The range over which natural crossing occurs is limited. McGregor (1976) traced movement of pollen by means of fluorescent particles and found that, even among flowers located only 150 to 200 feet from a cotton field that was surrounded by a large number of bee colonies, fluorescent particles were detected on only 1.6% of the flowers. For comparison, isolation distances for foundation seed are 1320 feet and for certified and registered cotton seed are 660 feet in the U.S. Based on information submitted for previous transgenic cotton events, the U.S. Department of Agriculture has stated in environmental assessments that the 'potential for gene introgression from genetically engineered cotton lines into wild or cultivated sexually compatible plants is very low" (USDA, 1995).

Low introgression rates to cultivated cotton were confirmed by studies conducted in Burkina Faso. Table 19summarizes the introgression rates for MON 15985 measured at several distances from the pollen source during field trials in Burkina Faso.

Table 19 Introgression frequencies for MON 15985 in Burkina Faso

Distance	Border unsprayed [1]	Border sprayed [2]
2 m	5.50%	8.30%
5 m	1.90%	4.20%
10 m	0.80%	5%
15 m	0.40%	0%

1. The border of the field was unsprayed with insecticide. N = 4140
2. The border of the field was sprayed with insecticide. N = 120

The data from Burkina Faso indicated that introgression occurred short distances (up to 15 m) within cultivated cotton and this suggested that pollen flow to neighbouring cultivated cotton could be managed with isolation distances, if necessary. In both the U.S. and Burkina Faso, outcrossing to wild relatives of cotton is not expected to occur because sexually compatible wild cotton varieties do not exist in these countries. Gene flow in Burkina Faso would be restricted to other cultivated cotton, which can be effectively controlled by short isolation distances of 15 m.

Based on these observations, the gene flow from commercial production of MON 15985 will pose minimal risk to the environment with no predicted effects on threatened or endangered species.

4.B POTENTIAL FOR ESTABLISHMENT AND PERSISTENCE

To evaluate if a transgenic plant has altered weediness potential in comparison with its conventional counterpart, the following may be examined:

- Dissemination of seed
- Dormancy of seed
- Germination of seed/survival
- Competitiveness
- Agronomic characteristics *e.g.*, time to maturity, disease and pest resistance
- Stress tolerance

As an annual plant grown in the United States, cotton is not considered to have weedy characteristics. It does not possess any of the attributes commonly associated with weeds, such as seed dormancy, long soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersal, or long distance dispersal of seeds. These characteristics of weeds are controlled by multiple, not single, genes. The only difference one would expect between MON 15985 and conventional cotton is that MON 15985 would better withstand damage from foliar-eating insects. This is confirmed by Eastick and Hearnden (2006) who demonstrated that the *cry1Ac* and *cry2Ab2* genes do not confer a meaningful change in fitness of cotton or increase its weediness. They concluded that no fitness advantage would be conferred to wild cotton relatives by the transfer of these genes.

In Burkina Faso, cotton is not considered to have weedy characteristics. As an annual plant it does not possess any of the attributes commonly associated with weeds, such as seed dormancy, long soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersal, or long distance dispersal of seeds. No MON 15985 plants emerged at Burkina Faso trial sites a year after the trials were harvested. Conventional seedbed preparation was sufficient to eliminate any seed that may have survived. To get cotton seed to germinate it must be ginned and planted in a shallow soil profile. In addition, the seed must be planted close together so that seedlings can assist each other to break through the soil crust for successful emergence. Cotton seedlings are weak and struggle to get through the soil surface on their own.

Dissemination of seed. *G. hirsutum* appears to be somewhat opportunistic towards disturbed land and appears not to be especially effective in invading established ecosystems. Cotton bolls, due to their size and general properties are unlikely to be dispersed by mechanisms such as wind, birds or terrestrial animals. In addition, the germination of cotton seed is inhibited by the attached fibres in natural seed distribution.

Germination of seed. In continental U.S., wild populations of *G. hirsutum* exist only in the southern tip of Florida, due in part to the freezing conditions in other growing areas regions that allow cotton to over winter. Germination and dormancy characteristics of MON 15985 seed were evaluated relative to the parental transgenic variety, DP50B, the

non-transgenic variety, DP50, and ten reference varieties. The study was conducted by BioDiagnostics, Inc. using standards established by the Association of Official Seed Analysis (AOSA 2000) using eight temperature regimes. Test and control seed samples were obtained from three geographically diverse 1999 field trial sites: Texas, South Carolina, Louisiana. Reference seed varieties were obtained from commercial seed stocks. The germinated and degenerated seeds were counted periodically throughout the 12-day study period. Seeds remaining on the final day were tested for viability using a tetrazolium test and characterised as hard or firm-swollen seed (Table 20). In addition, during standard seed sampling from 1998 field trial sites, 200 seeds per plot from two locations (a total of 1600 seeds/line) were tested for germination and vigour using the AOSA rules (Table 21).

Table 20 Germination and dormancy results for cotton event MON 15985 on seed harvested from three locations in 1999

Temp.	Variety[1]	Mean pvhs (Dormant)[2] (%)	Mean pgerm[2] (%)	Mean pfms[2] (%)	Mean pdegen[2] (%)
5º C	MON 15985	1.2	0.0	95.1	4.1
5º C	DP50B	0.0	0.0	95.2	5.4
5º C	Ref. Range	(0 - 41)	(0 - 1)	(53 - 99)	(1 - 20)
10º C	MON 15985	0.0	1.2	73.9*	26.4*
10º C	DP50B	0.0	1.3	78.5	21.7
10º C	Ref. Range	(0 - 28)	(0 - 3)	(38 - 91)	(9 - 62)
20º C	MON 15985	0.0	95.4	0.0	5.4*
20º C	DP50B	0.0	97.4	0.0	3.1
20º C	Ref. Range	(0 - 6)	(74 - 100)	(0 - 13)	(0 - 26)
30º C	MON 15985	0.0	93.9*	0.0	6.6*
30º C	DP50B	0.0	98.6	0.0	2.2
30º C	Ref. Range	(0 - 0)	(83 - 100)	(0 - 0)	(0 - 17)
40º C	MON 15985	0.0	85.9	0.0	14.9
40º C	DP50B	0.0	89.3	0.0	11.1
40º C	Ref. Range	(0 - 0)	(70 - 96)	(0 - 0)	(4 - 30)
5/20º C	MON 15985	0.0	NC	NC	NC
5/20º C	DP50B	0.1	NC	NC	NC
5/20º C	Ref. Range	(0 - 29)	NC	NC	NC
10/20º C	MON 15985	0.0	NC	1.9	7.5
10/20º C	DP50B	0.0	NC	1.2	5.8
10/20º C	Ref. Range	(0 - 18)	NC	(0 - 79)	(1 - 31)
20/30º C	MON 15985	0.0	NC	0.0	5.1
20/30º C	DP50B	0.0	NC	0.0	3.7
20/30º C	Ref. Range	(0 - 2)	NC	(0 - 1)	(0 - 17)

* Indicates level of significant difference from DP50B at P≤0.05.

NC = no comparison of combined means possible due to significant variety by site interaction at P≤0.05.

1. There were 12 observations for both event 15985 and DP50B, in addition to 44 observations for reference varieties in each temperature regime.
2. pvhs = percent viable hard seed, pgerm = percent germinated seed, pfms = percent viable firm-swollen seed, pdegen = percent degenerated seed.

Table 21 Germination and seedling vigour tests on seed harvested from two locations in 1998

Event or Line #	% Germination Day 4	% Germination Day 9	% Cool Germination at 18º C Day 7
MON 15985	76	77	72
DP50B	83	83	80
DP50	88	89	82

The results of the studies indicated that there were no differences in seed dormancy between MON 15985 and the control DP50B (Table 20 and Table 21). Five differences were identified for the other three parameters: percentage of germinated seed (pgerm), per cent viable firm-swollen seed (pfms) and the percent degenerated seed (pdegen). These differences revealed no observable trends and were within the range of values determined for the reference cottonseed.

G. hirsutum is ineffective as a weed in the U.S. and this appears to be the same in Burkina Faso. The USDA has determined that "cotton is not considered to be a serious, principal or common weed pest in the U.S." (USDA, 1995).

4.C POTENTIAL SECONDARY AND NON-TARGET ADVERSE EFFECTS

Environmental risk assessment must consider the potential secondary effects of the environmental release of a transgenic plant, such as effects on non-target organisms, particularly as this may impact on existing agricultural practices, the agro-ecosystem and biodiversity. This discussion of potential secondary effects on non-target organisms is illustrated using examples that address the U.S. Environmental Protection Agency's (EPA's) risk assessment methodology for determining adverse effects to non-target organisms. In the case of plant-pesticides, the intent of EPA's approach is to evaluate the potential hazard to terrestrial wildlife, aquatic animals, plants and beneficial insects. If detrimental effects are observed under laboratory conditions (Tier 0 studies), field studies (Tier 1 studies) are required to assess the actual abundance of non-target species under test and control conditions. For Bt crops where crop residue exposure is a possibility, EPA has required data on the toxicity of delta-endotoxins to birds (*e.g.*, quail), fish, honeybees, certain other beneficial insects (*e.g.*, lady beetles) and soil invertebrates (*e.g.*, Collembola, earthworm species).

4.C-1 Non-Target Test Organisms

A non-target organism is any plant, animal or microorganism that is unintentionally affected by cultivation of a transgenic plant. The following guidance on the selection of non-target test organisms has been adapted from U.S. EPA data requirements for protein plant-pesticides:

Avian test species: Young bobwhite quail or mallard ducks between 14 and 28 days of age at the beginning of the test period.

Aquatic animals: This is relevant to *Bt*-expressing aquatic plants that may have applications in forests, drainage ditches, riverbanks, and partially aquatic crops such as rice. It also applies in the case of field crops that are grown near bodies of water.

Freshwater fish species: EPA's guidelines provide that the species tested be selected from the list of species recommended with the exception of goldfish (warmwater species--bluegill sunfish, channel catfish, and fathead minnow; coldwater species--rainbow trout, brook trout, coho salmon). These species are desirable test organisms for several important reasons: they are frequently used to evaluate chemical and microbial pesticides; EPA has considerable background data on these species; standard methods for the care and handling of these species are available; and the species are widely distributed, are generally available, and have a variety of food habits and habitat requirements.

As appropriate, consideration should be given to testing species representative of the geographic region or ecosystem where the pesticidal plant will be cultivated. Fish species likely to scavenge intoxicated insects or the modified plant tissue (such as in farmed fish food) should be tested when appropriate. Unless there are other overriding considerations, the rainbow trout is recommended as the freshwater fish test species. It is a desirable test animal because it is partially insectivorous.

Aquatic invertebrate species: The most likely plant tissue to be tested is pollen. Due to the broad phylogenetic spectrum from which the investigator may choose, it is difficult to select the most appropriate aquatic invertebrate. Daphnia, a Cladoceran, has the advantage of having considerable background data for comparative purposes. In addition, Daphnia exhibits a bioconcentration effect. This results from the filter feeding habits of Daphnia and is a desirable feature in terms of assuring that the test animal ingests the toxin containing tissue. Both Daphnia and certain aquatic insects have the advantage of a short life cycle and are useful for assessment of reproductive effects.

Non-target insect testing: Selection of the predator/parasite species to be tested should take into account such factors as the likelihood of exposure to the plant protein, phylogenetic proximity of the test species to target pest species, and similar relationships.

Assessment of potential non-target insect hazard is complicated by a number of factors. Many plant-pesticides are expected to be specifically chosen for their ability to control pest insects. In most cases, it can be assumed that the non-target insect group most at risk will be closely related to the pest species. While there are few non-target insects that have been shown to be economically important to humans, there are many non-target insects which have an important role in ecological processes and may benefit humans indirectly.

The host range is an important factor in hazard evaluation for a protein plant-pesticide. A problem here is that extrapolation, even across species lines, is often not dependable. For this reason, tests should be conducted with representatives from a number of "beneficial insect" taxa. EPA recommends that testing be performed on pollinator species, such as honey bee, and three other species of insects, representing at least two of the following groups—parasitic dipterans, predaceous hemipterans, predaceous coleopterans, predaceous mites, predaceous neuropterans, parasitic hymenopterans.

The requirements for evaluating the potential toxic effects of protein plant-pesticides on representative soil organisms, such as Collembola and earthworms, were originally based on the possibility of long-term exposure of these organisms to crop residues incorporated or left upon the soil surface. (The US EPA does not require such testing for registration of conventional pesticides or spray *Bacillus thuringiensis* products.) One of EPA's reasons for requiring the non-target soil invertebrate tests was the concern that adverse effects on these species would cause a build up of plant detritus in cotton fields. The EPA has since discovered that the long term soil use of highly toxic chemical insecticides, such as aldicarb, terbufos, phorate and carbofuran, which have long term effects on soil invertebrate species, has not resulted in the build-up of plant detritus in soils based upon available information on current routine agronomic practices. Moreover, some of these chemicals have half-lives of 10 or more years. Thus protein plant-pesticide crops, which are expected to have less impact on these species than the highly toxic chemical pesticides, should not result in any increased build up of plant detritus. Supporting this conclusion are data which indicate that *Bt* toxin production in plant-pesticides ceases at plant senescence in the majority of registered *Bt* maize crops, allowing some time for protein degradation prior to harvest. Additionally, the environmental fate data indicate that for currently registered *Bt* maize crops only <1 to 90

grams of *Bt* protein per acre would enter the soil as a result of post harvest incorporation of *Bt* plants. Since proteins are known to degrade rapidly in the soil, the potential for significant soil build-up and hazard to non-target soil organisms is not anticipated from the growing of crops containing protein plant-pesticides.

4.C-2 Effects on non-target organisms

There is extensive information on the lack of non-target effects from microbial preparations of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) containing the Cry proteins. *Btk* Cry proteins are extremely selective for the lepidopteran insects (MacIntosh *et al*., 1990; Klausner, 1984; Aronson *et al*., 1986; Dulmage, 1981; Whitely and Schnepf, 1986), bind specifically to receptors on the mid-gut of lepidopteran insects (Wolfersberger *et al*., 1986; Hofmann *et al*., 1988a; Hofmann *et al*., 1988b; Van Rie *et al*., 1989; Van Rie *et al*., 1990) and have no deleterious effect on beneficial/non-target insects (Flexner *et al*., 1986; Krieg and Langenbruch, 1981; Cantwell *et al*., 1972; EPA, 1988; Vinson, 1989). The ecotoxicity of the Cry1Ac protein in MON 531 was assessed against a number of reference organisms (Mendelsohn *et al*., 2003) and no adverse effects were observed at concentrations significantly greater than the predicted environmental concentrations.

Cry2Ab2 protein. To confirm the environmental safety of Cry2Ab2 protein in MON 15985, thirteen studies were conducted on bird, fish and beneficial terrestrial invertebrate species. Details of the studies were provided in the application, but are not provided here because they are confidential business information.

Due to extremely low levels of Cry2Ab2 protein produced in cotton, it was necessary to produce sufficient quantities of the protein by bacterial fermentation, for the development of analytical methods (*e.g*., ELISA) and to conduct protein safety studies. The Cry2Ab2 protein was produced in and purified from *Bacillus thuringiensis* strain EG7699. To create *B. thuringiensis* strain EG7699, the *cry2Ab* gene for the wild-type Cry2Ab2 protein was cloned into a bacterial plasmid and introduced into a crystal-negative strain of *B. thuringiensis*. This strain was designated EG7699. The Cry2Ab2 protein produced by *B. thuringiensis* strain EG7699 was shown to have equivalent molecular weight and immunoreactivity to the Cry2Ab2 protein expressed in MON 15985. It lacked detectable post-translational modification (glycosylation), had equivalent electrophoretic mobility and detection with specific antibodies and functional activity (Section 9.B.2.; data provided to regulators, but not shown here, because it is designated confidential business information).

In summary, non-target organisms were exposed to leaf or seed tissue from MON 15985 cotton plants or to Cry2Ab2 protein incorporated into the diet for five days to eight weeks, depending on the study (Table 22). The doses were set to exceed the predicted environmental exposure.

Table 22 Summary of Cry2Ab2 protein studies on non-target organisms

Test Organism	Results	Test Substance	Conclusions[1]
Bobwhite Quail	No mortality or toxic effects in birds consuming Cry2Ab2 cottonseed at 10% of diet	MON 15985 cottonseed	MON 15985 cottonseed poses minimal risk
Channel Catfish	No effects on growth or survival in fish consuming MON 15985 cottonseed at 20% of diet	MON 15985 cottonseed	MON 15985 cottonseed can be used in catfish diet at up to 20%, the highest level tested, with no adverse effects
Adult Honey Bee	NOEC = 68 µg Cry2Ab2/ml diet	Cry2Ab2 protein	NOEC[2] > 56X predicted maximum Cry2Ab2 concentration in cotton
Larval Honey Bee	NOEC = 170 µg Cry2Ab2/ml , single dose	Cry2Ab2 protein	NOEC > 139X predicted maximum Cry2Ab2 concentration in cotton
Lady beetle	NOEC = 4500 µg Cry2Ab2/ml diet	Cry2Ab2 protein	NOEC > 88X predicted maximum Cry2Ab2 concentration in cotton leaf tissue
Collembola	NOEC = 69.5 µg Cry2Ab2/g diet	MON15985 leaf tissue	NOEC > 17X maximum predicted environmental exposure to Cry2Ab2 protein from cotton in soil
Green Lacewing Larvae	NOEC = 1100 µg Cry2Ab2/g diet	Cry2Ab2 protein	NOEC > 22X maximum predicted environmental exposure to Cry2Ab2 protein from cotton leaf tissue
Parasitic Hymenoptera (Wasp)	NOEC = 4500 µg Cry2Ab2/ml diet	Cry2Ab2 protein	NOEC > 3700X maximum environmental concentration predicted in cotton pollen
Earthworm	NOEC = 330 mg Cry2Ab2/kg dry soil	Cry2Ab2 protein	NOEC ≥ 83X maximum estimated environmental exposure from cotton in soil

1. Calculations were based upon the highest expression value determined from overseason cotton leaf tissue, pollen or soil, as appropriate to the test animal exposure.
2. NOEC = No observed effect concentration.

The quail study was conducted by Wildlife International Laboratories and the catfish study was conducted at the Thad Cochran National Warmwater Aquaculture Center at Mississippi State University. Studies conducted on earthworms and five invertebrates representing classes of insects that could be exposed to Cry2Ab2 protein from MON 15985 cotton (adult and larval honey bees, *Apis melifera*; collembola, *Folsomia candida*; green lacewing, *Chrysoperla carnea*; lady beetle, *Hippodamia convergens*; parasitic wasp, *Nasonia vitripennis*; and earthworm, *Eisenia fetida*) were conducted either at

Wildlife International Laboratories, California Agricultural Research INC, or Springborn Smithers Laboratories Inc.

Avian testing. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885.4050 Nontarget Avian Testing, Tier I. The study is scientifically sound and no treatment mortality or behaviour change was observed between the dosed and control replicates.

The dietary LC_{50} for Cry2Ab2 protein in cottonseed meal, when fed to juvenile northern bobwhite for 5 days, was determined to be greater than 100,000 ppm diet because no toxicity was observed at this level. Because 100,000 ppm was the highest dose tested, EPA has determined that the no observed effect concentration (NOEC) is also greater than 100,000 ppm. These data show that there will be no adverse effects on avian wildlife from incidental field exposure to Cry2Ab2 protein.

Freshwater fish testing. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This is a non-guideline study based on Nontarget Freshwater Fish Testing (OPPTS Series 885.4200), Tier I.

In an eight week feeding study, no toxicity was observed in channel catfish consuming a diet containing 20% cottonseed meal from MON 15985 with the Cry2Ab2 protein. Because 20% cottonseed meal containing Cry2Ab2 protein was the highest dose tested, EPA has determined that the dietary LC_{50} and the NOEC for Cry2Ab2 protein in cottonseed meal when fed to channel catfish for 8 weeks is greater than 20% of the diet. The data indicate that cottonseed meal derived from genetically modified cotton line, MON 15985 (Cry2Ab2), can be used as a feed ingredient in channel catfish diets up to levels of about 20% without adverse effects on fish growth, feed conversion efficiency, survival, behaviour, or body composition. The lack of adverse effects may be due in part to the significant reduction in the concentration of the Cry2Ab2 protein in the processed cottonseed meal as compared to raw cottonseed prior to commercial processing (toasting). However, a similar study performed with corn meal which contained Cry2Ab2 protein that was not denatured (MRID 450863-19) showed no adverse effects on catfish at 20%.

Non-target invertebrate - earthworm testing. The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 and 792; Organization for Economic Development (OECD) Principles of Good Laboratory Practice; and Japan Ministries of Agricultural Forestry and Fisheries (MAFF), with certain exceptions that did not affect the integrity of the test. The testing was conducted based on OPPTS Series 850.6200 Earthworm Subchronic Toxicity Test and OECD Guideline 207.

As there were no effects observed in the study, the 14-day LC_{50} for earthworms exposed to Cry2Ab2 protein in an artificial soil substrate was determined to be greater than 330 mg Cry2Ab2 mg protein/kg dry soil; the no observed effect concentration was determined to be greater than 330 mg Cry2Ab2 mg protein/kg dry soil, the highest concentration tested. This data indicated that no adverse effects to earthworms can be expected at Cry2Ab2 levels 12 and 83 times higher than the maximum expected environmental concentration for corn and for cotton respectively. Thus, an observable deleterious effect on earthworms is not expected to result from the growing of Cry2Ab protein-containing cotton plants.

Non-target arthropod testing - honey bee larvae. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. An acceptable study was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I.

It can be determined from this study that the no-observed-effect concentration (NOEC) for Cry2Ab2 protein fed to honey bee larvae (*Apis mellifera*) is greater than 100 µg/mL (ppm) (MRID 453371-02). The test was scored for survival to capping, adult emergence, and adult survival. The larvae developed into adult honey bees, normal in behaviour and appearance. A NOEC could not be determined from the results of an additional study submitted for review (MRID 450863-07). However, results from this study supplement results from MRID 453371-02 in demonstrating a lack of risk from larval honey bees feeding on Cry2Ab2 protein.

Adult honey bee testing. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I.

This study showed the no-observed-effect concentration (NOEC) for Cry2Ab2 protein fed to adult honey bees (*Apis mellifera*) is greater than 68 µg/mL Cry2Ab2 protein. Cry2Ab2 protein showed no measurable deleterious effects on honey bee larvae and adults up to the level tested.

Parasitic Hymenoptera larva testing. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Nontarget Insect Testing, Tier I.

The guidelines recommend terminating the test when 20% mortality is reached in the control group or after 30 days. This test should have been conducted until 20% mortality was achieved in the vehicle control group or for 30 days as described in OPPTS 885.4340. Due to the high rate of mortality in the assay control and 220 ppm Cry2Ab2 protein treatment group, the study was terminated prematurely and an LC_{50}

could not be determined. The high rate of morality in the assay control group; equal to the mortality in the 100 ppm potassium arsenate reference group suggested that there was a non-treatment related effect occurring.

On April 18, 2002, the developer submitted a letter to the EPA requesting a waiver from parasitic Hymenoptera toxicity testing. This waiver request was based on a lack of exposure of parasitic Hymenoptera to the Cry2Ab2 protein. In addition, parasitic Hymenoptera are not expected to be susceptible to Cry2Ab2 since it is highly specific against lepidopterans and dipterans. Due to the lack of exposure and susceptibility of parasitic Hymenoptera to the Cry2Ab2 protein expressed in cotton or corn, the EPA accepted the developer's request to waive this data requirement.

Green lacewing larva testing. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test.

This study was conducted based on OPPTS Series 885-4340 Nontarget Insect Testing, Tier I, except the test was terminated when 50% pupation was reached in the assay control group. The guidelines recommend terminating the test when 20% mortality is reached in the control group or after 30 days. However, it is known that younger larvae are more susceptible to *Bt* proteins than older larvae. It can be assumed that adverse effects related to green lacewing larvae feeding on Cry2Ab2 protein would be observed once 50% pupation occurred.

Based on this study, the no-observed-effect concentration (NOEC) for Cry2Ab2 protein fed to green lacewing larvae is greater than 1,100 ppm Cry2Ab2 protein and the LD_{50} is greater than 4,500 ppm. The NOEC represents 5.5 times the maximum concentration in corn plant material and 21.6 times the maximum concentration in cotton plant material. Based on these results it can be concluded that green lacewing will not be adversely affected when exposed to Cry2Ab2 in the field.

Lady beetle testing. This study was conducted in accordance with Good Laboratory Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Nontarget Insect Testing, Tier I.

The primary route of exposure to Cry2Ab2 protein by lady beetle adults and larvae would be from cotton pollen ingestion. Since some of beetles in the treatment and control groups were observed to be immobile/and or lethargic, a NOEC cannot be determined from this study. However, it can be concluded that the LC_{50} for adult lady beetles feeding on Cry2Ab2 protein is greater than 4,500 ppm which is a significantly higher level than would be encountered in the field.

Collembola testing. Although this study was not conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40CFR Part 160, the Agency has determined

that the study is scientifically valid. This study was conducted based on OPPTS Series 885-4340 Nontarget Insect Testing, Tier I.

This study determined that the presence of Cry2Ab2 protein was not toxic to Collembola. Cry2Ab2 protein also did not adversely affect the rate of Collembola reproduction. Mortality demonstrated in the positive control group and observations of green digestive tracts in the other groups verified that Collembola were ingesting the test cotton tissue material. Results of this study showed the no-observed-effect concentration (NOEC) of Collembola exposed to Cry2Ab2 protein from cotton leaf tissue in the diet was greater than 69.5 µg Cry2Ab2 protein/g diet. This study adequately addressed potential concerns for Cry2Ab protein expressed in transgenic cotton to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry2Ab proteins found in transgenic cotton pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial soil insects.

The Cry2Ab2 proteins derived from both the bacterial fermentation and plant sources were established to be physiochemically and functionally equivalent. The bird and fish feeding study data indicated that birds or fish exposed to Cry2Ab2 protein as part of their diet, will not be adversely affect. Bobwhite quail and catfish fish fed on MON 15985 cottonseeds at 10% and 20% of their diets, respectively, exhibited no mortality and no adverse effects on survival, growth or behaviour (Table 22). In the remaining studies, the No Observed Effect Concentration exceeded the maximum predicted environmental concentration by more than 10 to 100 fold (Table 22), demonstrating a wide margin of safety for these organisms. No adverse effects were observed at the maximum predicted environmental concentration to which the organisms world be exposed. These observations support the field observations on non-target populations conducted at numerous field trials (data not provided).

In summary, for Cry2Ab2 no adverse effects were observed at concentrations significantly greater than the predicted environmental concentrations (Table 22).

4.C-3 Endangered Species Considerations in the United States

Based on the submitted Cry1Ac and Cry2Ab2 protein toxicity and exposure data, a risk to endangered or threatened mammals, birds, plants and aquatic species to these Cry proteins is not anticipated. The non-target testing confirms the expectation that Cry1Ac and Cry2Ab2 protein toxicity is confined to Lepidoptera species larvae; therefore, non-lepidopteran endangered or threatened species will not be affected by these proteins. Cotton is insect pollinated and pollen containing the Cry proteins is not likely to drift out of fields. Nevertheless, relatively high Cry1Ac and Cry2Ab2 dosages were not toxic to the test species representative of organisms likely to be exposed to such pollen (***e.g.***, lady beetles, green lacewings, honeybees).

The potential for Cry1Ac and Cry2Ab2 proteins to affect non-target lepidopterans is understood from knowledge of the activity of these proteins on lepidopteran pests. However, hazard must combine with exposure to result in risk, and the only exposure to Cry2Ab2 proteins will be through pollen drift to the preferred host plants of non-target lepidopterans. Since cotton is not considered to be a wind pollinated plant, the deposition

of pollen on host plants is unlikely. In addition, the data indicate that Cry2Ab protein expression in pollen is very low and so substantial deposition and consumption would be required to have a negative impact.

As an example, a review of endangered Lepidoptera species in cotton growing counties (Quino Checkerspot butterfly, Riverside County CA; Saint Francis' Satyr butterfly, Cumberland and Hoke Counties, NC and Kern Primrose Sphinx moth, Kern County CA) determined that they are unlikely to be exposed to the Cry proteins because their habitats do not overlap with cotton fields. (For example, the Quino Checkerspot butterfly is found only in the coastal sage scrub habitat in southern California, the Kern Primrose Sphinx moth is found only on a privately owned ranch in Walker Basin, Kern County, California, and the only known populations of Saint Francis' Satyr butterfly are found in wetlands dominated by sages and grasses on Government property in North Carolina.) The larvae of these species do not feed on cotton and will not be exposed to Cry protein in pollen. The amount of pollen that would drift from these cotton plants onto plants eaten by endangered/threatened species, would be very small compared to the levels fed to the test species. Therefore, EPA does not expect potential risk to any endangered/threatened species and should any exposure occur, the levels of Cry1Ac and Cry2Ab2 protein would be too low to impact on these lepidopterans.

4.C-4 Combined effects of Cry1Ac and Cry2Ab2 proteins

MON 15985 contains both Cry1Ac and Cry2Ab2 proteins. Non-target testing with Cry1Ac and Cry2Ab2 proteins separately did not show any hazard to non-target species. Any unexpected synergistic effects from MON 15985 are not anticipated because no adverse effects were seen in several non-target tests (avian, earthworm and collembolla species) which were performed using tissue containing both Cry proteins. These studies supported field observations in the U.S. During the efficacy observations (Section 9.C.2) recordings of non-target insects were made on 24 % of the locations and showed no differences in thrips, aphids, stinkbugs, plant bugs, boll weevil and red spider mites. Thrips were the most commonly observed non-target insects. No substantial differences in non-target infestations or severity were noted between MON 15985 and control plants at any of the sites.

A confirmatory study was initiated at the research station in Burkina Faso (Farako-Bâ) in 2004 to assess the impact of MON 15985 on populations of non-target insect taxa, primarily predators, commonly found in cotton. The overall objectives of this ongoing study are to compare populations of these non-target insects between MON 15985 and conventional cotton over the entire growing season, and to contrast any potential effects relative to conventional production practices using selective and broad-spectrum insecticides. The results of this study show essentially no effects of MON 15985 on non-target insect populations and further show that in general there are minor reductions in density of non-target insects in plots treated by insecticides (Table 23).

Table 23 Study on the impact of MON 15985 on non-target insects in Farako-Bâ (2004-2005) Numbers of insects trapped in the fields

Test organisms	MON 15985 [a]	DP 50 [a]	FK 37 [a]	FK 37 [b]
Coccinellidae	9	4	2	3
Staphilinidae	6	1	6	8
Total Coleoptera	**15**	**5**	**8**	**11**
Apidae	40	22	30	16
Vespidae	11	1	12	0
Sphecidae	33 [a]	14 [b]	19 [a]	12 [b]
Formicidae	17	13	11	13
Braconidae	2	0	2	1
Ichneumonidae	1	1	1	1
Total Hymenoptera	**104 a**	**51 b**	**75 a**	**43 b**
Grillonidae	0	0	1	0
Total Orthoptera	**0**	**0**	**1**	**0**
Blattidae	3	1	2	1
Mantidae	0	0	0	1
Total Dyctioptera	**3**	**1**	**2**	**2**
Arachnidae	1	1	3	1

a Without pesticide treatment.
b Treated according to the guidelines adopted by cotton producers.

Overall, data provided in this submission and discussed above established the safety of the Cry1Ac and Cry2Ab2 proteins, as expressed in MON 15985, for beneficial and other non-target insects commonly found in cotton fields. The absence of toxic effects in the non-target organism studies even at Cry1Ac and Cry2Ab2 levels considerably above the maximum predicted environmental exposure demonstrated that the Cry1Ac and Cry2Ab2 proteins will not have adverse impacts on these and related non-target organisms, including endangered and threatened species.

GUS protein. The GUS protein has no insecticidal effect and there is no evidence of this protein producing environmental harm (Gilissen *et al*., 1998).

Plant pest potential. Data and information collected for this petition indicate that MON 15985 does not represent a unique plant pest risk in the U.S. Following extensive testing and field trials the MON 15985 event has been shown to be equivalent to the agronomic performance of traditional cotton varieties which are well established as having no plant pest risk in the U.S.

Considering all of the information available, the weight of evidence indicates no unreasonable adverse effects of Cry1Ac and Cry2Ab2 singularly or jointly expressed in cotton. The non-toxic nature of the GUS protein and the accepted safety of the NPTII protein together with the levels of environmental exposure, indicate that MON 15985 will not have an adverse impact on non-target organisms, including endangered and threatened species in the release environment. In conclusion, based on the history of safe use of the NPTII and GUS proteins and the well-characterised mode of action of the Cry proteins, the selectivity of the Cry1Ac and the Cry2Ab2 toxins for certain lepidopteran pests and the confirmation through single high dose and dose-response studies of no

adverse effects found, it is highly unlikely that MON 15985 would be hazardous to non-target organisms in Burkina Faso.

4.D ENVIRONMENTAL FATE OF AND EXPOSURE OF EXPRESSED PROTEINS

Soil organisms may be exposed to endotoxins from cultivated transgenic crops by exposure to roots, incorporation of above ground plant tissues into soil after harvest, or by pollen deposited on the soil. Root exposure may occur by feeding on living or dead roots or, theoretically, by ingestion or absorption after secretion of endotoxin into the soil. In addition, evidence suggests that some soil components, *e.g.*, clays and humic acids, bind endotoxins in a manner that makes them recalcitrant to degradation by soil microorganisms, but without eliminating their insect toxicity. Therefore, exposure to endotoxin bound to soil particles may be a route of exposure for some soil organisms.

The environmental fate of purified Cry proteins has been extensively studied. USDA has conducted environmental assessments of Cry proteins and has issued findings of no significant impact (FONSI) for the Cry1Ac protein (USDA, 1995). Cry protein crystals have been found to degrade readily in the field due to solar radiation and temperature (Palm *et al*., 1993, 1994, 1996). Cry protein adsorption to soil has been shown to be rapid and complete within 30 minutes (Venkateswerlu and Stotzky, 1992). From other studies of the biodegradation and binding of Cry proteins in soil (Tapp *et al.,* 1994; Tapp and Stotzky, 1995; 1998; Crecchio and Stotzky, 1998; Koskella and Stotzky, 1997) it has been shown that isolated Cry proteins bind to clay particles and humic acids in artificial soil mixes.

The Cry1Ac protein levels measured in whole mature MON 531 plants obtained from field tests in 1992 and 1993 indicated that the Cry1Ac protein load to the soil was estimated to be 1.44 and 0.6 g/acre, respectively.

Based upon these values, an *in vitro* soil degradation study was conducted using insecticidal activity to measure degradation of the protein in MON 15985. This DT_{50} (time to 50% degradation) study was completed for the Australian registration of MON 15985 cotton containing Cry2Ab2 and Cry1Ac proteins (data not provided).

According to these studies, Cry2Ab2 and Cry1Ac proteins degraded rapidly in sandy loam soil (typical soil type for cotton production). The DT_{50} was 2.3 days, DT_{90} was 15 days, and 75% of the protein degraded in the first week of incubation. However, the study with Cry2Ab2 used the cotton bollworm (*Helicoverpa zea*) as the indicator species in the insect bioassay. The cotton bollworm is not as sensitive to Cry2Ab2 as other lepidopterans and it is less sensitive to Cry2Ab2 than Cry1Ac. As such, an accurate degradation time (DT_{50_0}) could not be determined from this study.

The MON 531 study showed that the Cry1Ac protein was rapidly degraded in the soil in both the purified form of the protein and as part of the cotton plant tissue. The half-life of the Cry1Ac protein in plant tissue was calculated to be 41 days, which is comparable to the degradation rates reported for *B. thuringiensis* microbial formulations (Betz *et al.*, 2000). The half-life for the purified protein was less than 20 days. These values are similar to the degradation rates observed by Palm *et al.,* (1993, 1994, 1996) for transgenic

plants producing Cry proteins. However, the soil degradation study on Cry2Ab2 was not conducted using a highly sensitive indicator species and so gave an inconclusive result. In addition, the study was not performed using soils high in clay and humic acid and, since these constituents slow the rate of microbial degradation of these toxins, the results may not be fully comparable.

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