PRE-MARKET ENVIRONMENTAL RISK ASSESSMENT OF TRANSGENIC PLANTS

A CASE-STUDY APPROACH UTILIZING MON 15985 COTTON

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INTRODUCTION

For more than 20 years, the application of modern biotechnology tools has enabled the production of plants with traits that might not have been possible through traditional breeding techniques. The use of recombinant-DNA (r-DNA) techniques theoretically affords the opportunity to move specific genes from any organism in nature into economically important plants. In the process, ultimately a few modified plants are selected from among hundreds to thousands of "events" based on desired properties for a particular use. Subsequently, the process of culling out undesirable plants and selecting the final plant for a desired use is the same regardless of the technique used to manufacture it. As such, scientific bodies reviewing the safety of r-DNA technology (modern biotechnology) have concluded that the potential environmental risks (*e.g.*, adverse impact on protected species and habitats) are the same as those associated with conventionally bred plants (NAS, 1987; NRC, 1989). Stated differently, there is no evidence that the method used to produce a new crop variety (be it a product of conventional breeding, genetic engineering or both[1]) is as an effective predictor of the plant's environmental impact. Today there is wide consensus that a science-based environmental safety evaluation focuses on the nature of the plant, the introduced trait, the likely receiving environment and the interactions among these.

However, as with many new technologies, potential benefits and risks of applying modern biotechnology, sometimes referred to as genetic engineering or genetic modification (GM), to the production of novel crops and foods are not viewed universally as being equivalent to the use of conventional breeding. People have different views on how biotechnology developments could affect humans, animals and the environment, and many of these views are based on matters beyond science. One of the greatest challenges confronting this technology and government decision makers is integrating a science-based assessment process into a socially acceptable decision making process.

In every country where biotechnology-derived (GM) crops (known in some regions of the world as living modified or LMOs) have been commercialized, these concerns and the potential for adverse environmental and human health consequences have been addressed through the development of regulatory regimes that are specifically applied to assessing the safety of these products and controlling their use. Public acceptance of new technologies, including products of biotechnology such as GM foods and fibers derived from GM crops, is often tied to confidence in the regulatory system and in government institutions. In large measure, this confidence is based on a perception that sound science is being applied to the assessment and management of risks to human and environmental health.

The plan of this document is to present an introduction to the concepts and principles of environmental risk assessment of transgenic plants, including a discussion of international harmonization of environmental risk assessment of crops derived through modern biotechnology. Harmonization of environmental risk assessment ensures that an

[1] Most of the genetically modified crops currently available were derived through a process that started with a single genetically engineered plant that was subsequently manipulated using conventional breeding techniques to produce the commercial crop.

appropriate scientific rigor is used consistently, that agreed upon principles govern the risk assessment, and that regulatory processes are understood by parties seeking and making regulatory decisions. Harmonization of environmental risk assessment principles and processes is an important goal for stakeholders interested in the safe and efficient introduction and international trade of GM crops. Many useful and appropriate models for the science-based environmental risk assessment have been developed (NRC, 1983; Tiedje *et al*., 1989; OECD, 1993) that are consistent with international treaties (*e.g*., Convention on Biological Diversity and International Plant Protection Convention). In addition to developing an appropriate science-based assessment, countries must address the societal interests including economics in decision-making and risk management.

Finally, this document presents a case study around insect-resistant cotton event MON15985 (Bollgard II®) as a means of practically illustrating the nature and types of data generally evaluated to assess potential environmental risks.

This document focuses solely on the environmental risk assessment of transgenic crops being developed for the production of foods, feeds and fibers. It specifically does not address questions pertinent to food safety evaluation, or the use of transgenic plants in the manufacture pharmaceuticals and industrial chemicals, as these normally fall under a different regulatory regime. The following discussion is designed to address the science and concerns that underpin transgenic crops intended for unconfined release into the environment.

ENVIRONMENTAL RISK ASSESSMENT

1. RISK ASSESSMENT PRINCIPLES

Scientific risk assessment is a cornerstone of biotechnology regulatory systems and public policy decisions related to the safety and acceptability of GM crops. Some countries have incorporated additional or other considerations such as social, economic and ethical issues in their risk assessment processes. Regardless, a strong scientific capacity and knowledge base is widely viewed as key to assessing risk; which entails identifying hazards, assessing their magnitude and duration, and estimating their likelihood of occurrence while recognizing the nature and importance of the attendant uncertainty in each phase.

Many models of risk assessment hold that it is a science-driven process that quantitatively evaluates the probability of risk, largely removing as much as possible personal biases and emotive factors that influence risk perception (NRC, 1983). Risk is most often perceived as negative or adverse, though it should be kept in mind that beneficial and neutral outcomes could be the focus and are also possible outcomes from a risk assessment. More commonly, risk is defined as the likelihood, chance or probability that some adverse effect will result from consumption or other exposure to man or the environment from the release of an agent (*e.g.*, a chemical or GM plant). In this definition, environment is used broadly to include domesticated and undomesticated animals, plants and microorganisms. Subsequently, risk assessment is a process for

determining "*the relationship between the predicted exposure and adverse effects in four major steps: hazard identification, dose-response assessment*[2]*, exposure assessment and risk characterization.*" (OECD, 1995). The objective of risk assessment is to produce scientifically based and transparent risk information to inform the decision-making process.

Risk = f (hazard x exposure)

As it is commonly expressed, risk is a function of both a hazard or adverse effect (*i.e.*, the magnitude and duration the hazard) and the likelihood that the adverse effect will be realized (exposure).

1.A HAZARD

In the context of risk assessment, "hazard" must be understood as a process used to identify and characterize the nature, magnitude and duration of a potential adverse effect. During risk assessment and prior to characterizing risk, a process often called "hazard assessment" is undertaken. This is a two-step process that, according to the International Program on Chemical Safety (IPCS, 2004), is defined as: "a process to determine possible adverse effects of an agent or situation when an organism, system or (sub)population is exposed to that agent". The process includes distinct steps of hazard identification and hazard characterization. As the names indicate, a risk assessor first identifies the "type and nature of adverse effects" that the agent (*e.g.*, a GM crop) has the capacity to cause in an organism, system or (sub)population of interest. Each potential hazard identified is then characterized in terms of its consequences in quantitative (*e.g.*, dose-response relationship) or qualitative terms. As such, hazard assessment is the process of broadly considering the possible hazards, their causal links and pathways, followed by selecting those harmful outcomes that matter using scientific criteria and expert judgment including reasonableness; and ultimately describing the possible harmful consequence(s) using quantitative and qualitative information. The potential consequences are examined in terms of their severity, spatial and temporal extent, as well as whether they may increase or decrease over time. One of the key challenges in environmental hazard assessment is clearly defining "adverse effects" in terms that can be examined using the scientific method.

1.B EXPOSURE

According to the IPCS (2004), exposure is the "concentration or amount of a particular agent that reaches a target organism, system, or (sub)population in a specific frequency for a defined duration". In the formulation above, exposure is the likelihood or probability component of risk. Risk assessors examine properties and proposed uses of the agent that create exposure. Exposure assessment reveals the organisms and populations that are likely to be exposed, and the mechanisms by which exposure will be created. Another goal of exposure assessment is to understand the fate including the potential movement of the agent throughout or away from an area of interest (*e.g.*, drift or pollen-mediated gene flow).

[2] Note that the IPCS (2004) also defines risk assessment as a four step process. However, the IPCS (2004) uses the term "hazard characterization" in place of "dose response" as used in the OECD (1995) definition.

1.C RISK CHARACTERIZATION

In the final step in the risk assessment, the information on the hazard and exposure are integrated to characterize or estimate the risk associated with the proposed activity. To some, risk characterization can be represented as a matrix where the separate characterizations of hazard (*e.g.*, negligible, low, moderate and high) and exposure (*e.g.*, highly unlikely, unlikely, likely and highly likely) are presented in a matrix format (see OGTR, 2005). The United States Environmental Protection Agency (US EPA, 1998) divides this final step into two steps: risk estimation, which is a scientific analysis of the data; and risk characterization, which is the creation of a description of the risk in terms of the "significance of adverse effects and the lines of evidence supporting their likelihood" (US EPA, 1998). An important element of risk characterization is to describe the uncertainties and assumptions used in the characterization of risk. Minimal risk situations occur when either the hazard is judged to be insignificant (negligible), or the probability of exposure to be very small (highly unlikely), or both. Risks can be deemed minimal if the effect examined is low magnitude (*e.g.*, low toxicity) and no meaningful hazard is identified, or if a reasonable causal link cannot be made between a hazard identified and an adverse outcome (low exposure or likelihood of occurrence) based on the evidence available.

2. RISK MANAGEMENT AND DECISION MAKING

Risk Management is an important concept meriting comment especially in preparation for a discussion of GM crops. Risk management is a decision-making process that is supported by risk assessment, but may also be informed by other issues (as permitted in regulations). According to the IPCS (2004), risk management is a *"decision-making process involving considerations of political, social, economic and technical factors with relevant risk assessment information relating to a hazard so as to analyze, and compare regulatory and non-regulatory options and to select and implement appropriate response to that hazard."* As implied above, 'sound science' alone cannot always tell us the right choices to make and actions to take when it comes to protecting the environment. For example, in its reassessment of dioxin risks, the US EPA targeted only one aspect – dioxin-induced cancer – and failed to address stakeholders' other concerns such as non-cancer health effects, the fairness of exposing a community that may already have an abundance of toxic chemical sites to yet another toxic site, the possibility that the local population has characteristics that make it unusually susceptible to damage from an additional body burden of dioxin, and the effects of the contemplated action on local property values. "*A risk characterization focused solely on scientific questions about the dose-response relationship of dioxin to cancer may be highly unsatisfactory to some people because it is only marginally relevant to their most serious concerns*" (NRC, 1996).

Risk management also allows a regulator to answer the question, "What can we do about the risks identified?" There can be cases where the evidence for increased risk is low, but the residual uncertainty prevents a regulator from characterizing the risk as negligible or acceptable. In these cases, the regulator can impose conditions of approval or registration such as monitoring that allow use of the agent under specific conditions. Some regulatory systems also have the ability to impose time limits on approvals that require

re-evaluation of the risks based on experience and new information acquired during commercial use to provide a margin of safety and caution in decision-making. Many regulatory systems have legal requirements for submitting new information that comes to light during commercial use and is relevant to the risk conclusions.

As such, risk management includes the political dimensions that take into account societal values around acceptable levels of risk and scientific uncertainty to act in the public interest. Very often, it involves balancing individual rights (developers, industry, organizations) with the need to protect human health and the environment, including animal and plant health, from the adverse effects of unacceptable risks. Ideally, the political, social, economic, legal, and ethical bounds within which risk management decisions are made are properly defined and transparent.

Historically, benefits broadly have not been incorporated into regulatory decision-making for chemical agents. It follows that the decision-making process should consider potential benefits arising from the adoption of a new product or technology. For example, the development of pesticides raises concerns about residues in food and environmental pollution; while, on the other hand, the effective control of pests afforded by the pesticide increases food production per unit of land, which can result in lower food costs and reduced pressure on the environment to use more land for food production. Society is clearly values both, the former from the perspective of preventing an adverse effect and the latter from the viewpoint of a benefit. However, as noted below, decision-making surrounding GM crops has broadened to include other considerations including benefits.

3. ENVIRONMENTAL RISK ASSESSMENT APPLIED TO TRANSGENIC CROPS

In a well-constructed regulatory system, the general principles of environmental risk assessment described above are integrated. Because transgenic crops raise unique issues and considerations that are different from many of those associated with chemical agents such as synthetic pesticides used in agricultural production systems many details of their risk assessments are not relevant. For certain transgenic crops such as insect and virus protected plants, risk assessment applied to biocontrol agents used in protecting crops against pests provides a good frame of reference. However, given the diversity of transgenic crops being developed, no single, historically-based model exists, and successful environmental risk assessments have been developed using basic principles.

The purpose of environmental risk assessment of transgenic crops is to identify and evaluate the risks associated with the release and cultivation of these plants in comparison with a conventional counterpart that typically has a history of safe use. Amongst those countries with established regulatory programs for environmental risk assessment of transgenic plants, there are common safety concerns that must be addressed on a case-by-case basis prior to commercialization of a novel plant (OECD, 1993). In addition to information on the molecular characterization and stability of the genetic modification (which are not directly addressed here), data must be available for: the host organism; the donor organism; the impact of gene transfer to related plants; gene transfer to unrelated organisms and its consequence; altered weediness potential; and secondary and non-target adverse effects.

As noted earlier, evaluating the potential environmental risks associated with transgenic crops includes a consideration of the nature of the trait, the nature of the crop, the nature of the likely receiving environment and the interactions between these. Assessing the environmental safety of a transgenic crop requires comparative knowledge of the biology of the non-modified crop plant itself and the agricultural practices employed in its cultivation. This knowledge provides the basis of familiarity, which increases with experience *i.e.*, experimentation and use. The concept of familiarity is a key approach used in identifying and evaluating environmental risks and in informing practices that may be needed to manage recognized risks (OECD, 1993; Hokanson *et al*., 1999; EFSA, 2006). For example, knowledge about the biology of the plant can help in hazard identification by identifying species-specific characteristics that may be affected by the introduced trait, thus permitting the transgenic plant to become "weedy," invasive of natural habitats, or otherwise harmful to the environment. Similarly, scientific information on the pollination and dispersal biology of the plant informs the risk assessment on critical elements that affect exposure to the environment. Furthermore, knowledge of accepted production practices provide a baseline to assess how the introduction of a new trait might result in changed agricultural practices that could affect the environment.

Science-based hazard identification considers both direct and indirect effects. Direct effects that must be considered include the potential adverse impact of an introduced trait after gene flow into related plant species through outcrossing, the potential build-up of resistance in insect populations to engineered insecticidal traits, unintended adverse effects on non-target organisms such as toxicity, and potential effects on biodiversity such as a plant becoming and invasive weed. Indirect effects often associated with transgenic crops include effects on insect community structure due to trophic mechanisms and changes in agricultural practice made possible by the new crop variety. Often, indirect effects present a greater challenge to regulators because they may be low probability with uncertain consequences (*e.g.*, horizontal gene transfer), or evidence for their existence may be solely hypothetical (*e.g.*, expanded host range of a plant virus due to recombination). Furthermore, because knowledge of the importance of community structure and ecological function in agriculture is very limited, detected effects may not necessarily be adverse.

By convention, the agro-ecosystem context is the initial focus of the environmental risk assessment process for transgenic crops because this is the likely receiving environment. Other environments are also given consideration based on the properties of the transgenic crop, the circumstances of its release and its propensity for gene flow, the movement of introduced genes through seed, pollen and other viable plant materials. Importantly, the environmental risk assessment is designed to evaluate the incremental risks associated with replacing a conventional crop variety with a genetically modified one. Defining those biodiversity impacts that matter in the context of crop production is an important foundation for hazard identification and defining what direct and indirect effects might be adverse.

The quality of pre-market environmental risk assessment is limited by the quality of the information available to a risk assessor. Ecological and agricultural sciences serve as the foundation for examining the perturbations (either positive or negative) resulting from the potential introduction of a transgenic crop can be measured and evaluated. Existing

knowledge of ecosystems and understanding of the biology of the unmodified host organism, including its potential interactions within ecosystems available for the risk assessment varies in terms of its quality and relevance to environmental risk. In Australia, the Office of Gene Regulator (OGTR) has recognized this in their Risk Analysis Framework (OGTR, 2005) stating that some evidence is stronger than other for evaluating risk. For example, evidence from validated studies conducted according to internationally recognized protocols is stronger than single, stand alone peer reviewed reports or publications, which are stronger than unsubstantiated statements (see OGTR, 2005). The OGTR also notes that in hazard identification a test for reasonableness should be applied: *"Although it is important to identify all potential hazards, it is also important to apply a test of reasonableness"* (OGTR, 2005). While all information is considered an environmental risk assessment of a GM crop, the quality of the information is an important consideration, and how valuations of information were made must be explained by a risk assessor in a decision document.

The discussion that follows presents three examples of international standards for environmental risk assessment based on consensus guidance from the Organization for Economic Cooperation and Development (OECD), the Cartagena Protocol on Biosafety (Protocol), and the International Plant Protection Convention of the United Nations Food and Agriculture Organization (FAO). These standards all embody the concept of relative risk. That is to say, the potential hazards posed by the introduction of a new organism (or practice) should be evaluated relative to some existing product or practice, and within the context of the receiving environment (*e.g.*, agricultural ecosystems *vs*. unmanaged ecosystems). Furthermore, they reaffirm that appropriate risk assessment is science based, uses all the information available as well as expert opinion when it is available. These standards, while consistent in their approach to evaluating the science-based concerns differ in their handling of other considerations.

INTERNATIONAL STANDARDS FOR ENVIRONMENTAL RISK ASSESSMENT OF TRANSGENIC PLANTS

4. THE ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT

In 1993, the Organization for Economic Cooperation and Development (OECD) published general principles that its member states concluded should be applied to larger-scale production and commercialization of transgenic crops.

According to the OECD:

> "*Safety in biotechnology is achieved by the appropriate application of risk/safety analysis and risk management. Risk/safety analysis comprises hazard identification and, if a hazard has been identified, risk assessment. Risk/safety analysis is based on the characteristics of the organism, the introduced trait, the environment into which the organism is introduced, the interaction between these,*

> *and the intended application. Risk/safety analysis is conducted prior to an intended action and is typically a routine component of research, development and testing of new organisms, whether performed in a laboratory or a field setting. Risk/safety analysis is a scientific procedure, which does not imply or exclude regulatory oversight or imply that every case will necessarily be reviewed by a national or other authority.*" (OECD, 1993).

Safety assessment under the OECD model embodies two important concepts: familiarity and comparative evaluation.

Assessing the environmental safety of a transgenic crop requires familiarity with the biology of the crop plant itself and the agricultural practices employed in its cultivation. This concept of "familiarity" was jointly developed by different groups (NRC, 1989; Tiedje *et al*., 1989) and is a key approach used in identifying and evaluating environmental risks and in informing practices that may be needed to manage recognized risks. Underlying this concept were three important assumptions:

(1) the process of genetic engineering was not inherently more risky than conventional plant breeding and introduced transgenes behaved in essentially the same manner as any other gene within the plant genome;

(2) there is a significant history of introducing new traits into crop plants and in evaluating these new varieties in agriculture; and

(3) with time and experience familiarity increases.

In order to arrive at the conclusion of "familiarity", sufficient knowledge of the biology of the plant, the introduced trait and the receiving environment is required. For example, knowledge about the biology of the plant can help identify species-specific characteristics that may be affected by the introduced trait, thus permitting the transgenic crop to become "weedy," invasive of natural habitats, or otherwise harmful to the environment. Likewise, the introduction of a new trait may result in changed agricultural practices that affect the environment. A conclusion of "familiarity" does not imply a particular level of risk, but that there is sufficient information upon which to characterize potential hazards and arrive at an assessment of risk.

Coupled with the concept of "familiarity" is the idea of comparative risk assessment and that environmental risk is not determined by the novelty of the genetic elements introduced into a plant species, but by the novelty of the resultant plant. The objective is to determine if the new organism presents any new or greater risks in comparison with its traditional counterpart, or whether it can be used interchangeably with its traditional counterpart without negatively affecting the environment in which it is grown, or human or animal health. The goal is not to establish an absolute level of safety, but rather a relative level of safety, so that there is a reasonable certainty that no undue risk to the environment or health will result from the anticipated use of the new transgenic plant. Useful information can be gained from evaluating whether a trait expressed in an engineered plant is similar to traits already introduced into that species. For example, the potential impact of introducing a transgenic herbicide-tolerant plant into the environment may be addressed relative to the impacts that were observed when conventionally bred varieties with the same, or similar, herbicide-tolerance trait were released. Applying this

comparative approach requires the availability of sufficient analytical data, either existing or generated *de novo*, to allow effective comparison between the new organism and its traditional counterpart. This suggests a basic limitation of the approach when confronted with a lack of sufficient baseline data against which the perturbations (either positive or negative) resulting from the potential introduction of a transgenic plant can be measured and evaluated.

For transgenic plant species, one useful reference tool when conducting an environmental safety assessment is a detailed monograph describing the biology of the species under review. In addition to identifying species-specific characteristics, it can provide details on significant interactions between the plant and other life forms, which must be evaluated during the risk assessment. Typically, such a document includes the following:

- taxonomic description;
- consumption and uses of the crop plant;
- regional/national breeding, seed production, and agronomic practices;
- reproductive biology of the crop plant, including details on pollination, mechanisms for dispersal of pollen and seed, and any other means of gene escape;
- occurrence and viability of intraspecific, interspecific, and intergeneric hybrids;
- details on the centres of origin and genetic diversity for the plant species;
- details on the ploidy of the cultivated crop, its progenitors and any sexually compatible species;
- distribution and ecology of related species or feral biotypes, including any evidence of weediness;
- common diseases and pests;
- potential interactions with other organisms such as pollinators, mycorrhizal fungi, animal browsers, birds, soil microbes and soil insects.

Detailed consensus documents or monographs about the biology of specific crop plant species have been prepared by the OECD as well as national regulatory authorities. These consensus documents are not perfect but a good starting point.

4.A CATEGORIES OF INFORMATION

Since it was established in 1995, the OECD Working Group on Harmonisation of Regulatory Oversight in Biotechnology has worked on achieving a consensus on the information used in risk assessment as well as the methods of analysis. Although organized somewhat differently, much of what follows is based on their most recent report (OECD, 2000).

4.A-1 Background Information

The following categories of information provide the necessary contextual basis for environmental risk assessment by focusing on the basic biology of the organism and its interaction with receiving environment:

- **The host organism** (as described above);
- **The donor organism:** Information about the natural history of the donor organism is required, particularly if the donor or other members of its genus normally exhibit characteristics of pathogenicity or environmental toxicity, or have other traits that affect environmental safety and/or human health.
- **The likely receiving environment:** Information on the agro-ecosystem, including knowledge of existing agricultural practices for the plant species (*e.g.*, methods of pest and weed control, soil fumigation and crop rotation). These provide important baseline data against which to evaluate the impact of the environmental introduction or changes in agricultural practice on the environment.

4.A-2 Product Characterization

- **Molecular-genetic characterization:** OECD has not published a standard for molecular characterization except to indicate that genetic stability of the introduced trait should be demonstrated over multiple generations. This usually also includes a demonstration of within-generation segregation of the introduced trait according to Mendelian rules of inheritance. In many cases, information on trait stability has no impact on the environmental risk assessment, however, in some cases it may. For example, continued high levels of expression of *Bt* insecticidal proteins are integral to the high dose-refuge strategy currently employed for managing the potential development of resistant insect populations.

  In addition, many countries have further requirements that may include: information on 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. These requirements are generally based on the belief that knowledge of the introduced, or modified, genes, their regulation, and site of integration in the case of transgenic plants, may provide information on possible direct and indirect consequences of the genetic modification.

- **Expressed material:** Hazard identification through product characterization 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 possibility of post-translational modification (*e.g.*, glycosylation) in eukaryotic systems should also be taken into account, as this may affect allergenic potential.
- **Phenotypic characterization:** New crop plants are routinely evaluated for agronomic performance and phenotypic characteristics in comparison with a conventional counterpart. In addition to yield and performance data, other parameters may include seed dormancy and germination rates, time to flowering or maturity, plant height and vigor, time to pollen shed and susceptibility to disease. These data are used when evaluating potential environmental

consequences of introduction, particularly in assessing any increased tendency to weediness, competitiveness or invasiveness.

4.A-3 Environmental Consequences

- **Potential for gene transfer to related plants:** The potential for introgression of genetic material from one plant to another is significant when certain conditions are met such as the two plants are sexually compatible, in sufficiently close proximity and 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 usually presupposes 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 (*i.e.*, the potential environmental hazard) varies with each plant/trait combination, requiring case-by-case assessment. It is the potential consequence arising from outcrossing to other plant cultivars that must be considered and assessed for environmental risk (EFSA, 2006). Dispersal and gene flow *per se* are not hazards and the focus of risk assessment and monitoring should be on any unanticipated consequences of the cultivation of the transgenic crop, such as enhanced weediness, invasiveness or changes in plant population dynamics or populations of biota associated with the transgenic crops.

- **Potential for gene transfer to unrelated organisms:** This type of gene transfer is termed horizontal gene transfer (HGT), which is the non-sexual exchange of genetic material between organisms belonging to the same, or different, species. It is a naturally occurring phenomenon that was first demonstrated to occur between bacteria, and whose importance in prokaryotic genome evolution has been inferred from phylogenetic analysis. The significance of this concern as a risk depends on the likelihood of HGT occurring and the magnitude of associated adverse outcome. For the antibiotic resistance markers that have been used to date, the probability of HGT to bacteria is exceedingly small and, should it occur, the consequences would not be significant. Caution must be tempered by the realization that HGT is a natural process of cross-species gene movement responsible for effecting genetic change, and transgenes that are stably integrated into the nuclear genome are generally no more likely to be transferred to other organisms than are other nuclear genes. There is no scientific rationale for treating possible gene transfer events involving transgenic organisms differently from those involving naturally occurring organisms (Salyers, 1997).

- **Potential for establishment and persistence – weediness in the agricultural setting:** Weeds are considered a subset of plants that may be considered pests. The term weed is used to describe a plant that is a nuisance in managed ecosystems such as farms or forest plantations. Weediness potential is a measure of a plant's ability to successfully colonize an ecosystem, especially when it may also lead to the displacement of other species. Generally, weediness depends on

the selective advantage of many genes functioning in combination, which are unrelated to the genes usually introduced for agronomic reasons. However, traits that enhance tolerance to environmental stresses such as drought, cold or dormancy have the potential to increase the survival and distribution of the plant in managed and unmanaged ecosystems. Additionally, traits which provide for resistance to biotic stresses that play a significant role in the ecology of the plant (*e.g.*, insect or pathogen resistance) could permit the plant to become persistent and/or invasive within and outside of the agricultural ecosystem.

For many of the major crop species cultivated around the world today, the process of domestication (human selection, breeding and cultivation) has reduced their inherent fitness and competitiveness relative to their wild progenitors. For example, crops such as cotton, maize and soybean are not generally considered to be "weedy" or to persist in the environment without human intervention. Experience with these crops in the environment provides important information in assessing the potential weediness of the modified crop (*i.e.*, familiarity).

- **Potential secondary and non-target adverse effects:** Risk assessment must consider the unintended consequences of the environmental release of a transgenic plant, particularly as this may impact on existing agricultural practices and the agro-ecosystem. Information on the nature of the introduced trait is used to determine the likelihood of non-target effects, and if indicated, the range of non-target organisms that are appropriate for ecotoxicity testing. Generally, the range of test species selected includes the following functional groups found in agricultural fields and other habitats: birds, freshwater fish, predators and parasitoids of crop pests, soil invertebrates and pollinators. If detrimental effects are observed under laboratory conditions, field studies are required to assess the actual abundance of non-target species under test and control conditions. In the field, insects, for example, are usually exposed to smaller amounts of toxin than the laboratory test dose because of diet choice and other environmental factors within the field setting. The choice of appropriate indicator organisms is based on the potential for field exposure to the novel protein expressed in transgenic plants, which is dependent on the tissue specificity of expression.

The information from above is brought together to integrate hazard identification and exposure estimation to characterize the potential risk.

Other important concerns must be assessed, including the potential buildup of resistance in insect populations to engineered insecticidal traits in the case of plants expressing insecticidal proteins, and potential effects on biodiversity. In this regard, it is important to focus the assessment within the context of the likely receiving environment, which is firstly an agricultural field, and distinguish between the biodiversity of natural populations and that of crops and other organisms within the agro-ecosystem.

Within the OECD model for risk assessment, and also agreed by the EFSA guidance document on risk assessment (EFSA, 2006), there is no explicit consideration of issues such as ethical and socio-economic factors. These considerations are presumed to be dealt with elsewhere and to be factored into the decision-making process to the extent permitted within the regulatory and legal frameworks of individual countries.

5. THE CARTAGENA PROTOCOL ON BIOSAFETY

Internationally, the need to ensure biosafety was recognized as a priority within the 1992 Convention on Biological Diversity (CBD), signed in Rio de Janeiro at the UN Conference on Environment and Development, and more explicitly within Chapter 16 of Agenda 21. Agenda 21 is a blueprint for sustainable development in the 21st century, and Chapter 16 requires governments to consider international cooperation on the "Environmentally Sound Management of Biotechnology" and to act appropriately to ensure that developing countries have effective participation in biotechnology research activities as well as priority access to the results and benefits of biotechnology on a fair and equitable basis.

One result of the Convention has been the Cartagena Protocol on Biosafety (Protocol), which was adopted in Montreal on January 29, 2000 and addresses the safe transfer, handling, and use of living modified organisms (LMOs).[3] The Protocol, which has been ratified, or acceded to, by 141 countries as of August 10, 2007, came into force on September 11, 2003.

The objective of the Cartagena Protocol is to:

> *"…contribute to ensuring an adequate level of protection in the field of the safe transfer, handling, and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements."* (CBD, 2000)

Article 15 of the Protocol requires that scientifically sound risk assessment be performed for decisions on import of LMOs to be released into the environment, and that the purpose of such assessments shall be "to identify and evaluate the possible adverse effects of living modified organisms on the conservation and sustainable use of biological diversity, taking also into account risks to human health."

The Protocol stresses that "recognized risk assessment techniques" shall be taken into account, including guidelines developed by relevant international organizations. The Protocol's principles of risk assessment are consistent with the use of familiarity as a general principle of comparative risk assessment. For example, the Cartagena Protocol includes the following general principle for risk assessment of LMOs:

> "*Risks associated with living modified organisms or products thereof, namely, processed materials that are of living modified organism origin, containing detectable novel combinations of replicable genetic material obtained through the use of modern biotechnology, should be* ***considered in the context of the risks posed by the nonmodified recipients or parental organisms*** *in the likely receiving environment.*"[4] (Emphasis added) (CBD, 2000)

[3] Living modified organism means any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology. From the Cartagena Protocol on Biosafety, Article 3 (g).
[4] Cartagena Protocol on Biosafety, Appendix III.

The Protocol describes a method in Annex III that encompasses the safety considerations much like those outlined by OECD. Under the Protocol, risk assessment should entail the following steps:

- an identification of any novel genotypic or phenotypic traits that could have an adverse effect on biological diversity in the receiving environment, including adverse impacts on human health;
- an estimation of the likelihood of adverse effects being realized;
- an evaluation of the consequences of any adverse effects, should they be realized;
- an estimation of overall risk (based on the above);
- a recommendation on the acceptability or not of identified risks, including risk mitigation strategies; and
- in cases where there remains uncertainty about the level of risk, additional research or risk mitigation measures or monitoring of the LMO in the receiving environment may be required.

With respect to the specific safety considerations that must be evaluated, the Cartagena Protocol is quite consistent with similar guidance published by OECD (Table 1).

Table 1 Comparison of information requirements under the Cartagena Protocol risk assessment procedure and under OECD guidelines

Cartagena Protocol on Biosafety	OECD Guidelines
Recipient (host) organism – biology, taxonomy, center(s) of origin, center(s) of diversity, common name, habitat **Intended use** – including changes in use or practice compared with parental organism	**Host organism** – reproductive biology, taxonomy, center(s) of origin, consumption/uses, interactions with other organisms, occurrence and viability of interspecific hybrids and anticipated changes in agronomic practices.
Donor organism(s) – biological characteristics, taxonomic status, common name and source	**Donor organism(s)** – known toxicological or pathogenicity concerns
Vector – identity, source, host range **Insert and characteristics of modification** – genetic characteristics of inserted DNA and function **LMO** – identity of the LMO noting any differences between the biological characteristics of the LMO and the host organism	**Molecular genetic characterization** – identity and source of genes and/or vectors; modification method; composition and integrity of introduced DNA; multigenerational stability (expression) and inheritance of the introduced trait; levels and tissue, or temporal, specificity of expression
Receiving environment – geographical, climatic and ecological considerations, including biological diversity **Detection and identification** – suggested detection methods and their specificity, sensitivity and reliability	**Establishment/persistence (weediness)** – seed dissemination, dormancy, germination, competitiveness, disease resistance and stress tolerance **Gene transfer** – both to other sexually compatible organisms and to unrelated species

Significantly, since regulatory decision-making always occurs within an environment of some uncertainty, the Protocol leaves the decision as to what constitutes an acceptable or unacceptable degree of scientific uncertainty to the Party taking the decision. On this matter, Article 10.6 states: *"Lack of scientific certainty due to insufficient relevant*

scientific information and knowledge regarding the extent of the potential adverse effects of a living modified organism on the conservation and sustainable use of biodiversity, taking into account risks to human health, shall not prevent that Party from taking a decision, as appropriate, with regard to the import of the living modified organism in question…in order to avoid or minimize such potential adverse effects." In addition, Annex III.8(f) affords insights into options that Parties might take in the face of uncertainty: *"Where there is uncertainty regarding the level of risk, it may be addressed by requesting further information on the specific issues of concern or by implementing appropriate risk management strategies and/or monitoring the living modified organism in the receiving environment."*

The precautionary approach described in the Protocol (Article 1) has been subject to wide interpretation among Parties. Importantly for environmental risk assessment, Annex III.4 clarifies that the notion that a lack of scientific information is simply that, and does not imply any "particular level of risk, an absence of risk, or an acceptable risk." Hill *et al.* (2004) concluded that precaution and risk assessment as described in the Protocol are consistent. They note that some of the confusion results from extreme views, which hold that precaution is tantamount to absolute certainty; and that risk assessment is of no use when the standard is absolute certainty.

Risk management is also explicitly addressed in the Protocol (Article 16) and as noted in Annex III.8(f) (see above). Some of the elements of risk management described in Article 16 are consistent with the principles described in Section 2. Most notable is that risk management measures should be based on risk assessment (Article 16.2).

In carrying out risk assessments, the Protocol also provides for the option to consider socio-economic factors when it states that in making decisions, the Parties "*may take into account, consistent with their international obligations, socio-economic considerations arising from the impact of living modified organisms on the conservation and sustainable use of biological diversity, especially with regard to the value of biological diversity to indigenous and local communities*." (Article 26).

Article 26 of the Protocol lacks specificity on matters of socio-economic considerations in decision making, and provides little guidance on how these concerns should be handled. However, it appears that Article 26 also recognizes that Parties may have obligations under other agreements that must be considered in decision making (*e.g.*, SPS agreements). It is reasonable to expect further discussion because the WTO does not accept socio-economic concerns in considering the risk associated with exports of traditional crops that could undermine local cultures and traditions in importing countries.

One interpretation of the socio-economic provisions within the Protocol is that they may only be taken into account within the context of considering the direct or indirect economic consequences of an environmental introduction of an LMO. For example, the economic consequences, if any, of lost biodiversity or costs associated with environmental rehabilitation or increased monitoring, could be considered but not the economic impacts on trade or employment generally.

6. INTERNATIONAL PLANT PROTECTION CONVENTION

The International Plant Protection Convention (IPPC) under the FAO is recognized in the World Trade Organization (WTO) Agreement on Sanitary and Phytosanitary Measures (SPS) as a multilateral instrument aimed at preventing the introduction and spread of plant pests[5] and diseases through setting harmonized phytosanitary standards and control measures. The IPPC is a legally binding international agreement, but the standards developed and adopted by the Convention are not legally binding under the IPPC. However, WTO members are required to base their phytosanitary measures on international standards developed within the framework of the IPPC and phytosanitary measures that conform to an International Standard for Phytosanitary Measures (ISPM) are presumed to be consistent with the relevant provisions of the SPS Agreement. The full range of pests covered by the IPPC extends beyond pests directly affecting cultivated plants and includes weeds and other species that have indirect effects on plants, including wild flora. In common with the CBD, the goals of the IPPC are to protect the environment and biodiversity against introductions of alien species. Current provisions and standards of the IPPC provide a strong basis and actively support the implementation of international standards for protection against the introduction of alien species pursuant to Articles 8(h) of the CBD.[6] In reference to the Protocol, it has been recognized that LMOs also have the potential to be invasive species and may be considered to be plant pests under certain circumstances.

More recently, the Interim Commission on Phytosanitary Measures (ICPM)[7] endorsed statements regarding the role of the IPPC with respect to LMOs.[8] Of importance, the ICPM endorsed the IPPC mandate in protecting plant health and plant pest concerns including considerations pertaining to the introduction of LMOs. It also noted that the IPPC has harmonized approaches to phytosanitary measures, risk analysis and risk management systems, and that these tools were appropriate for assessing and managing risks presented by the introduction of LMOs. With respect to the role of the IPPC regarding LMOs, the ICPM decided that an Open Ended Working Group would provide detailed guidance on pest risk analysis (PRA) for LMOs governing trade of LMOs and their associated potential phytosanitary risks.

In April 2004, the ICPM endorsed a supplement on pest risk analysis for LMOs and agreed that it should be integrated into ISPM 11 (FAO, 2004). The supplement includes details a three step process involving initiation, pest risk assessment and risk management. The scope of ISPM 11 covers analysis of risks of plant pests to the environment and biological diversity, including those risks affecting uncultivated/unmanaged plants, wild flora, habitats and ecosystems contained in the PRA

[5] Any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products. FAO, 1990; revised FAO, 1995; IPPC, 1997.
[6] Report of the Consultation on IPPC – CBD Cooperation. Interim Commission of Phytosanitary Measures Feb. 6-8, 2001. Bangkok.
[7] The 1997 amendments to the IPPC included a provision for a Commission on Phytosanitary Measures to promote the full implementation of the objectives of the Convention. Until the 1997 revision comes into force, the proposed commission is preceded by the Interim Commission on Phytosanitary Measures (ICPM). Currently the ICPM's membership is open to all FAO members and contracting parties.
[8] Report of the ICPM Open-ended Working Group on Specifications for an International Standard for Phytosanitary Measures on Living Modified Organisms. Interim Commission of Phytosanitary Measures, Sept. 2001.

area.[9] The supplement does not alter the scope of ISMP No. 11 but is intended to clarify issues related to the PRA for LMOs.

It is also recognized that while many LMOs will not be characterized as pests, some may present phytosanitary risks and therefore warrant a PRA. Hence, the initiation stage of the PRA includes identifying whether the LMO has the characteristics of a potential pest, requiring further assessment. For example, changes in adaptive characteristics such as tolerance to drought or other abiotic stresses, pesticide tolerance, disease resistance, or alterations in reproductive biology or seed dispersal, could increase the potential for introduction or spread, including invasiveness, of the plant. In the case of LMOs, the proposed risk assessment phase of the PRA includes an evaluation of many of the same risk factors as previously described for risk assessment under the Cartagena Protocol or following guidelines published by OECD.

Where the PRA process under the IPPC departs significantly from other risk assessment processes is in its explicit evaluation of potential economic consequences. The economic consequences that can be considered can be both direct pest effects and indirect effects. Direct effects can include such things as crop losses, the cost of implementing pest control measures, effects on existing production practices, or the reduction, displacement, or elimination of other plant species. Indirect economic consequences can include effects on domestic and export markets (including effects on market access for imports and exports), resources needed for additional research and advice, or social and other effects (*e.g.*, tourism).

In the case of environmental risks, examples of indirect pest effects that could be considered include:

- significant effects on plant communities;
- significant effects on designated environmentally sensitive or protected areas;
- significant change in ecological processes and the structure or stability of ecosystems (including further effects on plant species, erosion, water table changes, increased fire hazard, nutrient cycling, *etc*.);
- effects on human use (*e.g.*, water quality, recreational uses, tourism, animal grazing, hunting, fishing); and
- costs of environmental restoration.

In the case of LMOs, the economic impact should relate to the pest nature of the LMO *(i.e.*, injurious to plants and plant products), thus a consideration of direct effects on human or animal health are outside the scope of this standard.[10]

The IPPC has historically maintained that the adverse consequences of plant pests, including those concerning uncultivated/unmanaged plant species, wild flora, habitats

[9] The PRA area can be an officially defined country, part of a country or all or parts of several countries. FAO, 1995; CEPM, 1999; based on the WTO SPS Agreement.

[10] Under the Sanitary and Phytosanitary (SPS) Agreement, the Codex Alimentarius Commission is responsible for maintaining international standards relevant to food safety that should be recognized by the WTO, and for determining whether national measures are sufficiently "based on scientific principles" to comply with WTO rules.

and ecosystems, are measured in economic terms.[11] In addition to providing for the consideration of both qualitative and quantitative economic harms, the IPPC's PRA process also allows for an evaluation of the potential economic benefits. Costs and benefits can be counted whether they occur as a direct or indirect result of a pest introduction or if a chain of causation is required before the costs are incurred or the benefits realized.

The final stage of the IPPC PRA process is an evaluation of the acceptability of risk (both phytosanitary and economic) and the identification of appropriate risk management options. In addition to the prohibitive measures available for plant pests, additional measures for LMOs could include pest resistance management schemes, control of novel trait expression, control of reproductive ability (*e.g.*, male sterility), or implementation of post approval monitoring and/or surveillance. Under IPPC, the principle of "modification" states: "*As conditions change, and as new facts become available, phytosanitary measures shall be modified promptly, either by inclusion of prohibitions, restrictions or requirements necessary for their success, or by removal of those found to be unnecessary.*"[12] Thus the imposition of any particular prohibition or condition should not, *a priori*, be considered permanent but rather an interim measure pending future research, monitoring, or other change in circumstance.

[11] Economic impact is described in ISPM No. 5: Glossary of phytosanitary terms, Supplement No. 2: Guidelines on the understanding of potential economic importance and related terms. FAO, Rome.
[12] ISPM No. 1: Principles of plant quarantine as related to international trade. FAO, Rome.

COTTON EVENT MON 15985 CASE STUDY

7. 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 2).

Table 2 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 has been organized to closely follow the categories of information discussed in section 4.A. As previously stated, these data 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.

8. 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.

8.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.

8.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.

8.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).

8.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*.

8.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 3.

Table 3 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

8.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.

8.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 4).

Table 4 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.

8.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 5). 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 5 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.

8.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.

8.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).

8.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.

8.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.

8.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 6). 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 6 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.

8.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.

8.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.

8.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.

9. PRODUCT CHARACTERIZATION

9.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 9.A-2) and MON 15985 (section 9.A-3).

9.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).

9.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.

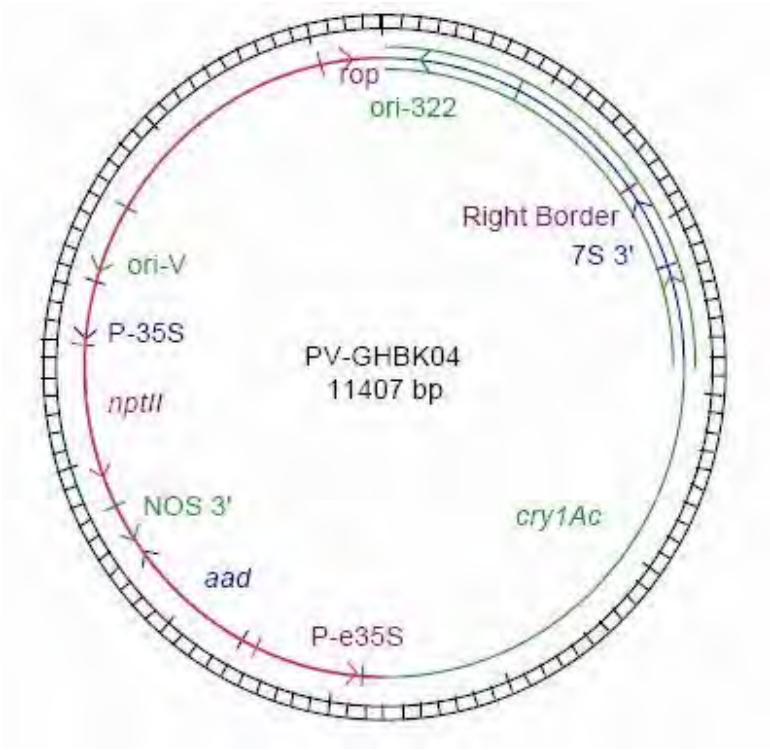

Figure 1 Plasmid map of PV-GHBK04

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 7.

Table 7 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.

9.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 8). 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).

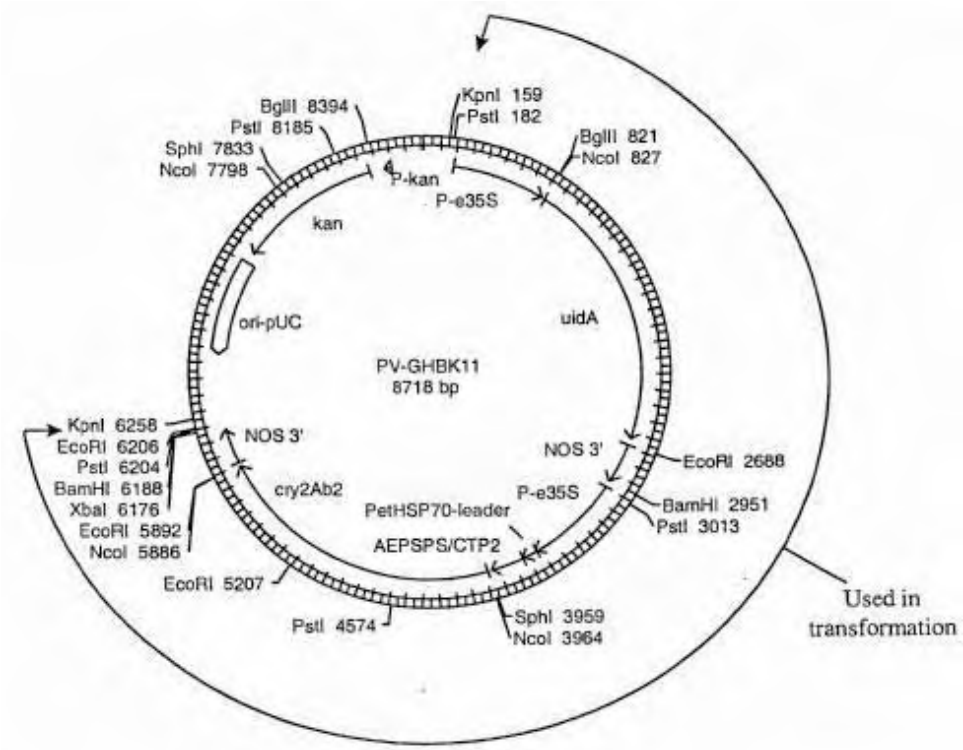

Figure 2 Plasmid map of PV-GHBK11
The region used for transformation, between the two *Kpn*I sites and containing the *cry2Ab* and *uidA* gene cassettes, is illustrated. The remaining portion is the plasmid backbone.

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 8 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

9.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.

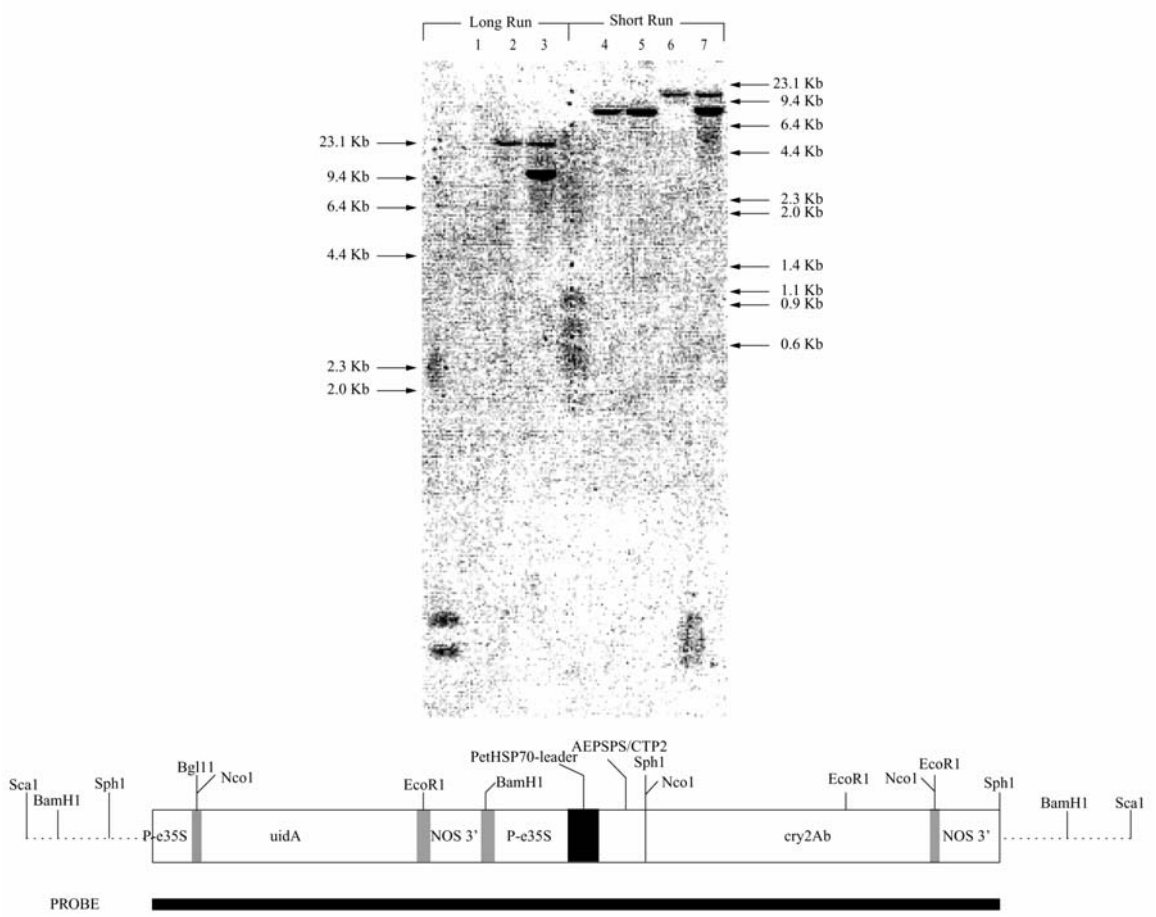

Figure 5 Southern blot analysis of MON 15985: Insertion site determination
Ten µg of DP50, DP50B and MON15985 genomic DNA isolated from leaf tissue were digested with *Sca*I. Samples were separated by alkaline gel electrophoresis, blotted onto nylon membranes and probed with the ^{32}P-labelled PV-GHBK11 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).

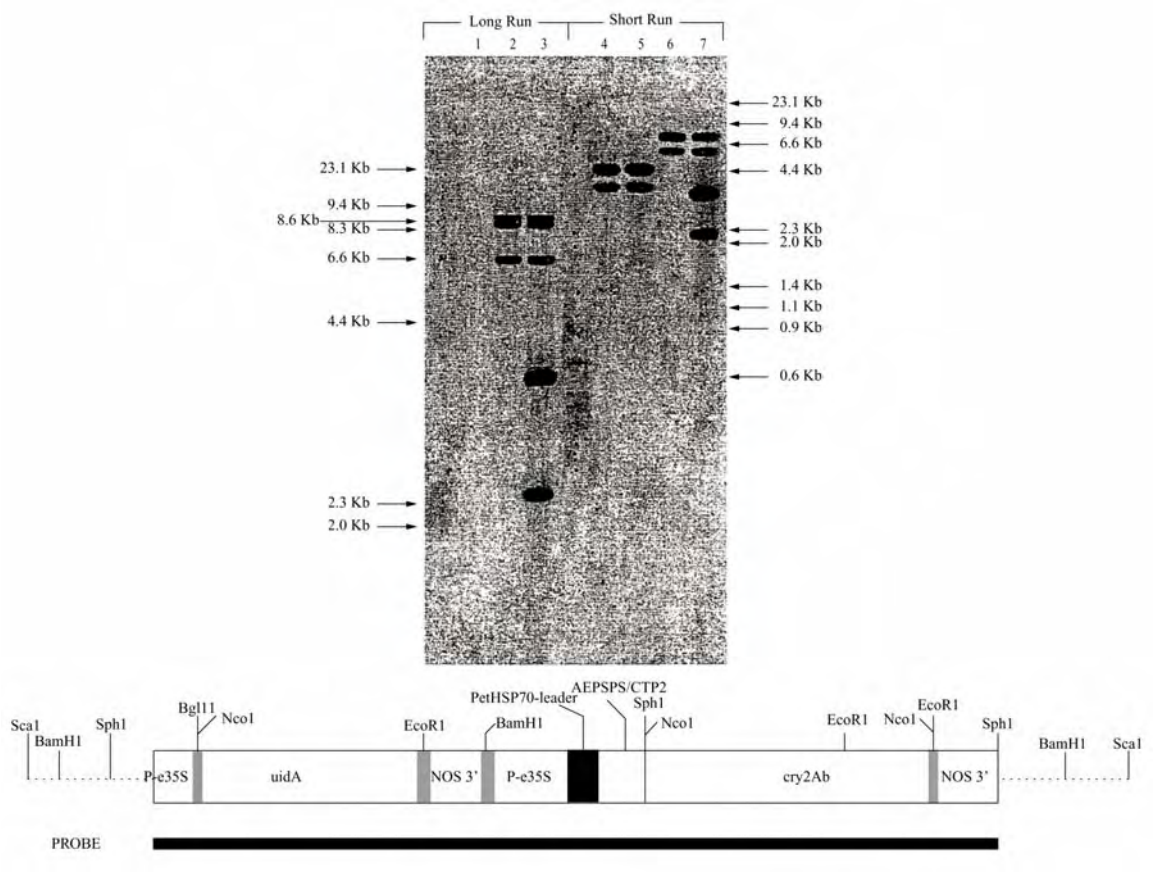

Figure 6 Southern blot analysis of MON 15985: Copy number analysis
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *SphI*. The blot was probed with the ^{32}P-labeled PV-GHBK11 fragment shown. Lane designation: 1) DP50, Long run; 2) DP50B, Long run; 3) MON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

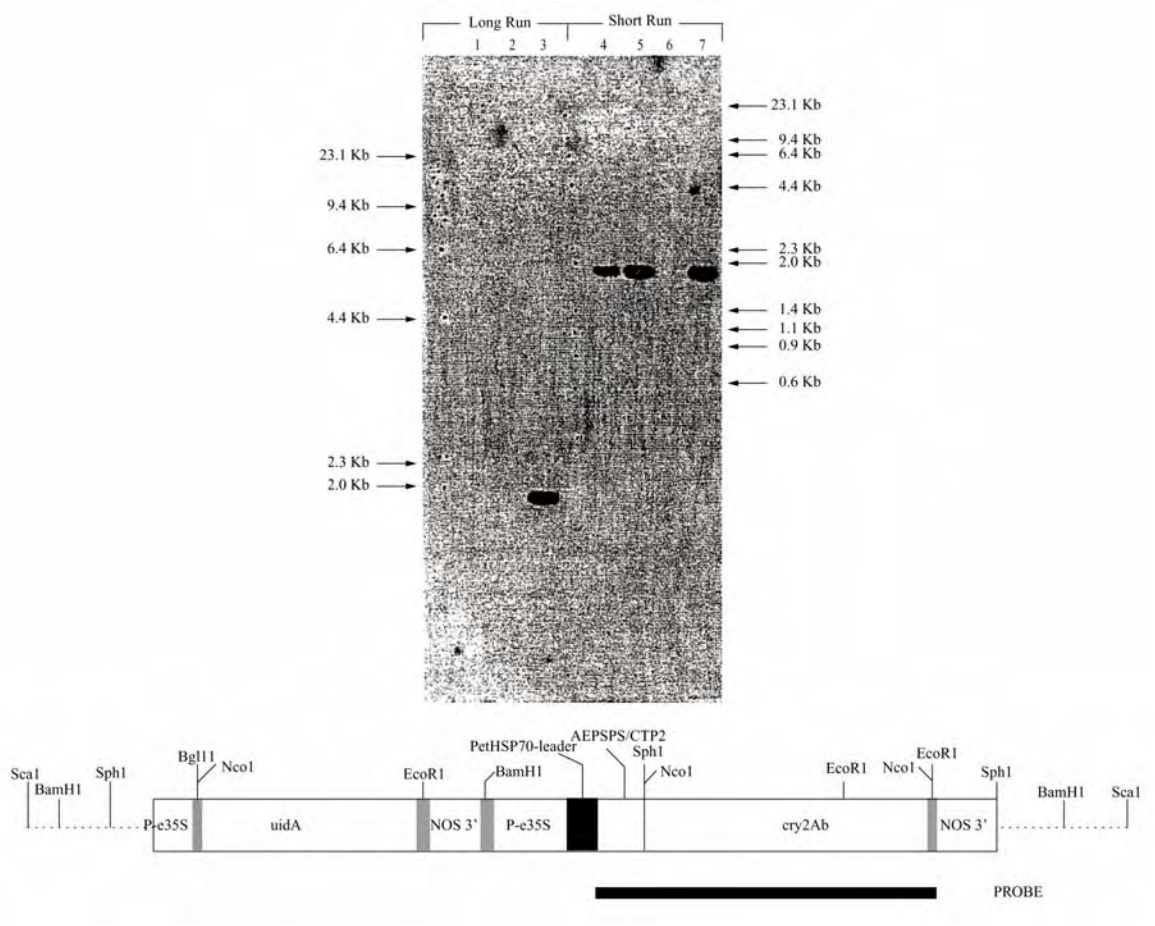

Figure 7 Southern blot analysis of MON 15985: Integrity of *cry2Ab* coding region
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Nco*I. The blot was probed with the ^{32}P-labeled *cry2Ab* coding region shown on the plasmid diagram. Lane designation: 1) 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).

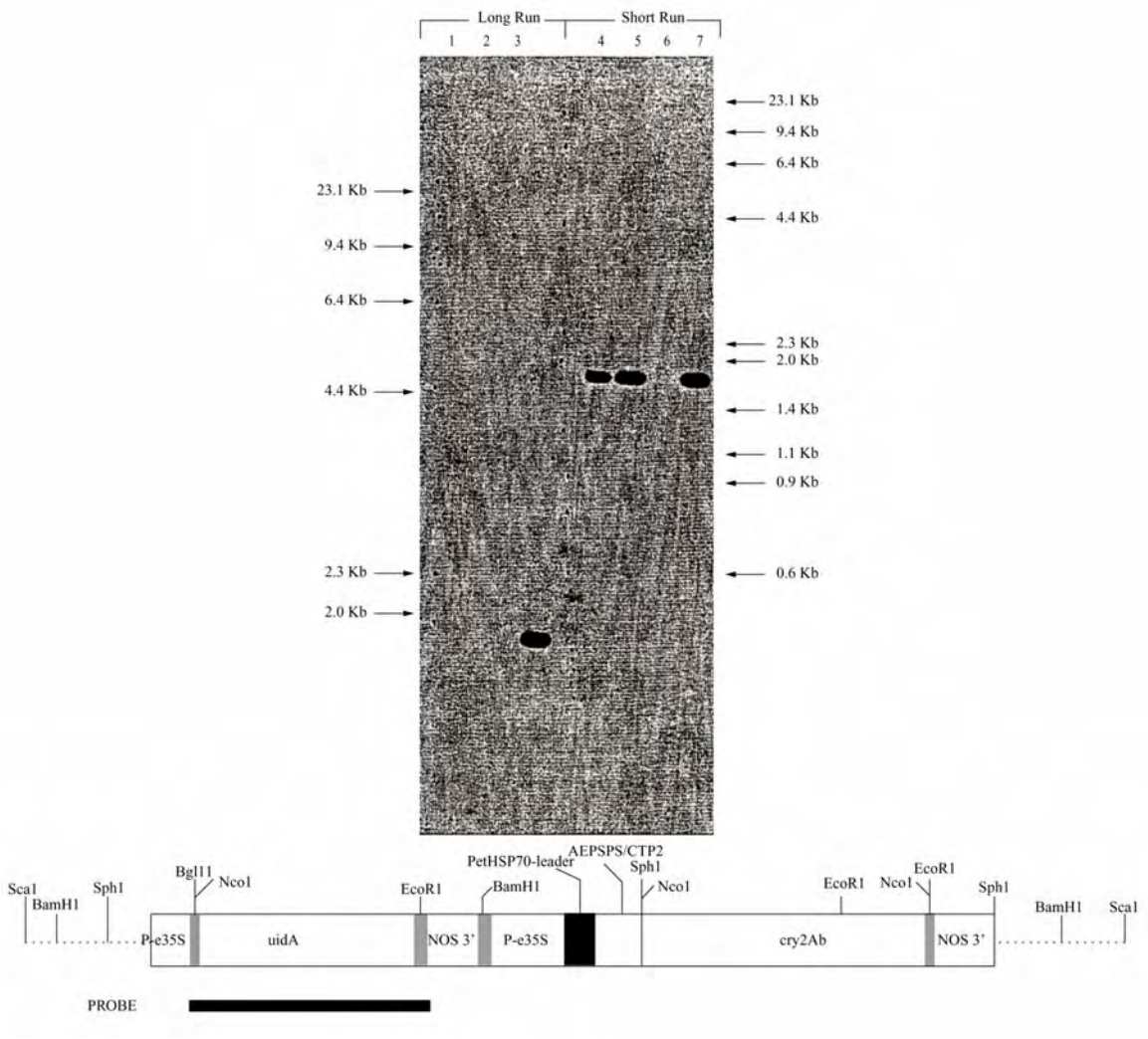

Figure 8 Southern blot analysis of MON 15985: Integrity of the *uidA* coding region
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Eco*RI and *Bgl*II. The blot was probed with the ^{32}P-labeled *uidA* coding region shown. Lane designation: 1) DP50, Long 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.

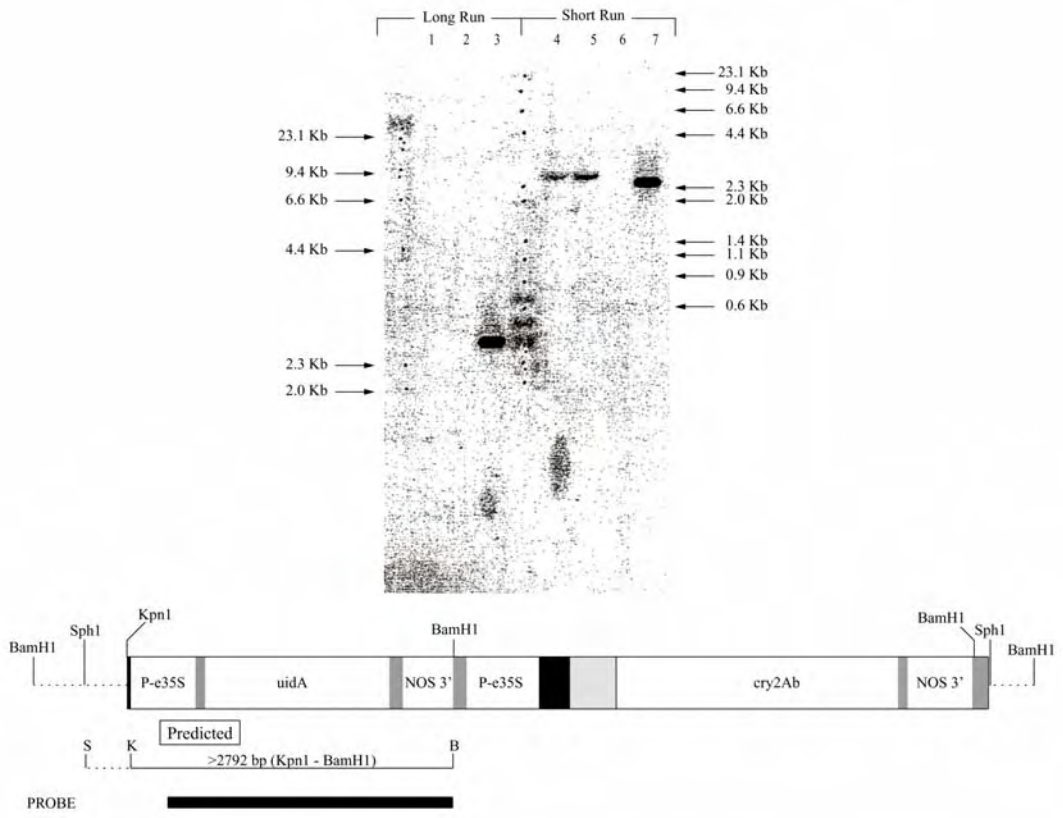

Figure 9 Southern blot analysis of MON 15985: Integrity of the *uidA* expression cassette – uidA probe
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Bam*HI and *Sph*I. PV-GHBK11 DNA was digested with *Pst*1 and added to the DP50 DNA samples prior to precipitation. 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 (**Error! Reference source not found.**).

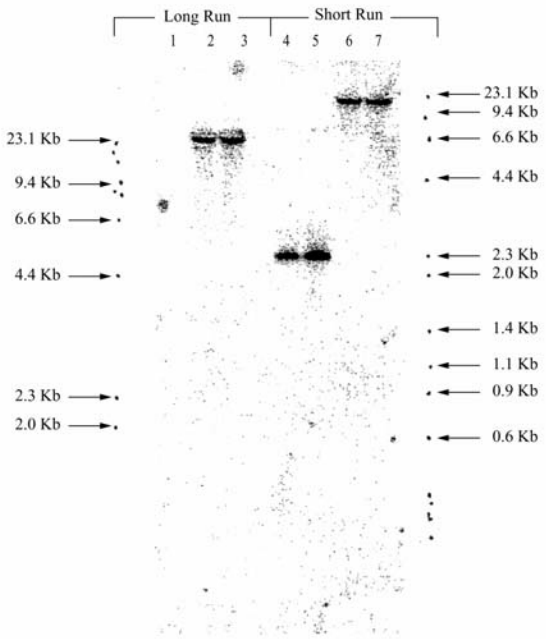

Figure 10 Southern blot analysis of MON 15985: Analysis for backbone sequences
Ten µg of DP50, DP50B and MON 15985 genomic DNA isolated from leaf tissue were digested with *Kpn*I. The blot was probed with the ^{32}P-labeled PV-GHBK11 backbone sequence (not shown). Lane designation: 1) DP50, Long run; 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 (**Error! Reference source not found.**, 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.

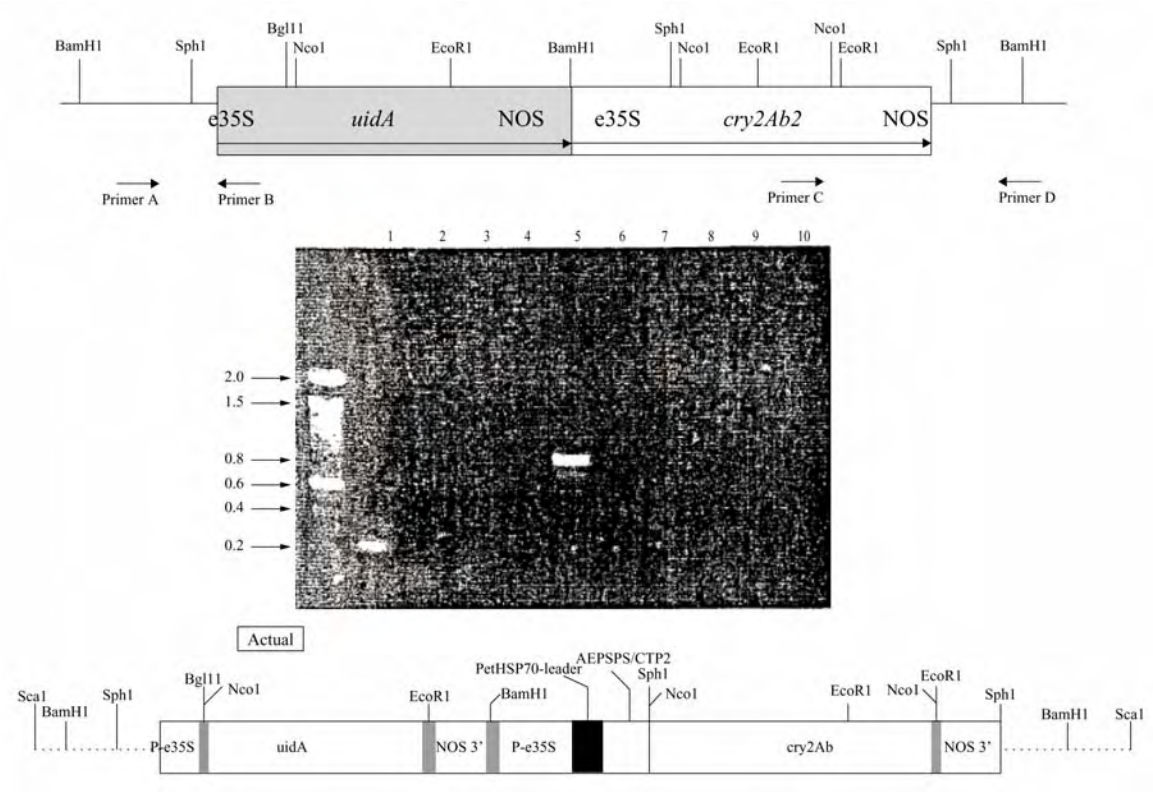

Figure 11 PCR confirmation of the 5' and 3' border sequences of the MON 15985 insert
PCR was performed using primers specific to the 5' and 3' border sequences for MON 15985 on genomic DNA isolated from leaf tissue for DP50 (non-transgenic control), DP50B (cry1Ac control), an alternate *cry2Ab* event 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 9.

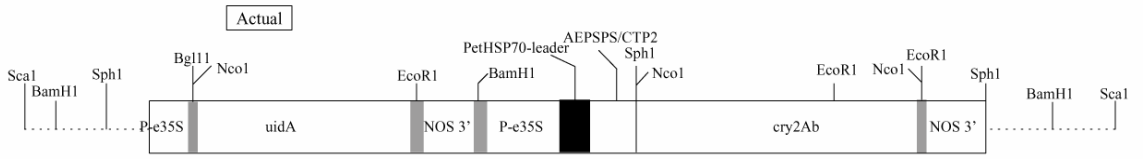

Figure 12 A restriction map of the insert in MON 15985

Table 9 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

9.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 10. 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.

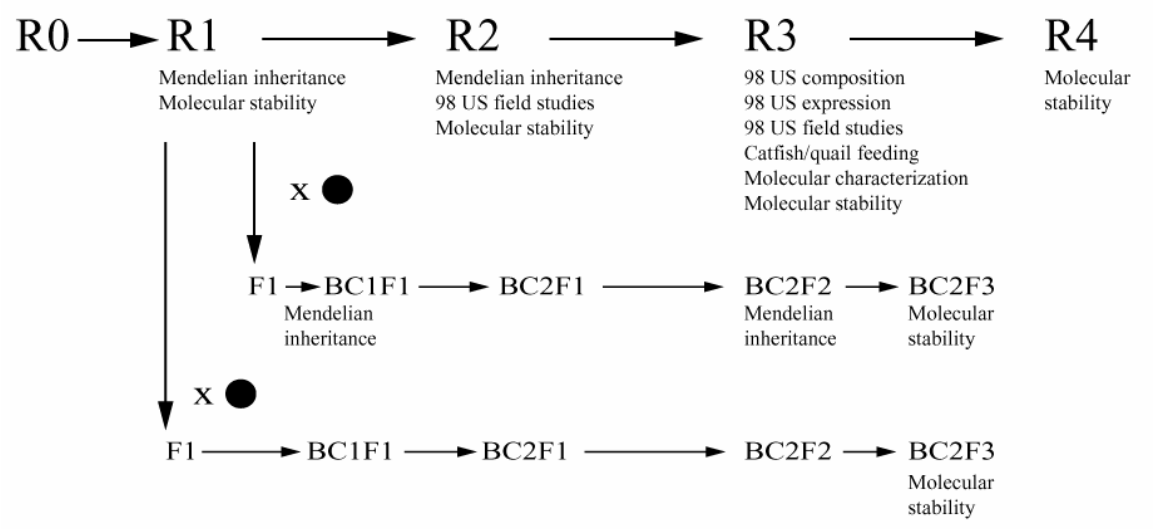

Figure 13 Progeny map of cotton MON 15985 generations used for specified testing

Table 10 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).

9.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).

9.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 11 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]				
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 12 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 13 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 14 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 15 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 11). The mean levels and ranges of Cry2Ab2 protein in leaf tissue for each location are summarized in Table 12. 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 13). 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 11). 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 14.

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 11). 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 15.

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 11, Table 16-17).

Table 16 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 17 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 16. 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 11). 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 17. 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 11). 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.

9.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 18.

Table 18 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.

9.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.

9.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 19.

Table 19 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).

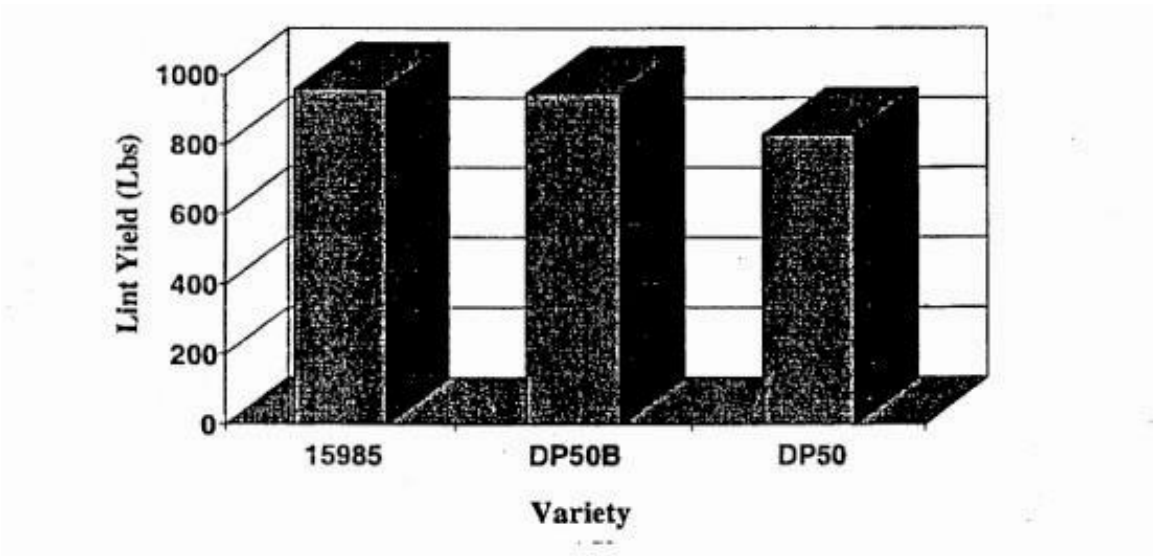

Figure 14 Lint yield in pounds per acre averaged across locations in the 1998 and 1999 field trials
Notes for figure: 15985 = MON 15985; DP50B = MON 531; DP50 = conventional cotton

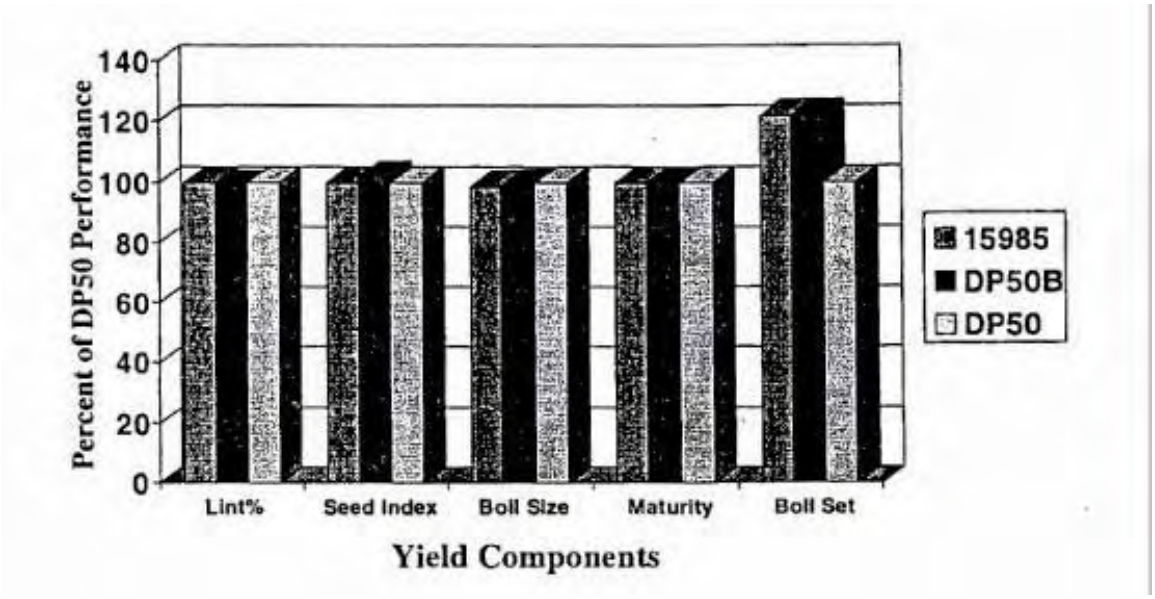

Figure 15 Yield characteristics as a percentage of DP50 performance in the 1998 and 1999 field trials
Notes for figure: 15985 = MON 15985; DP50B = MON 531; DP50 = conventional cotton

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.

9.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.

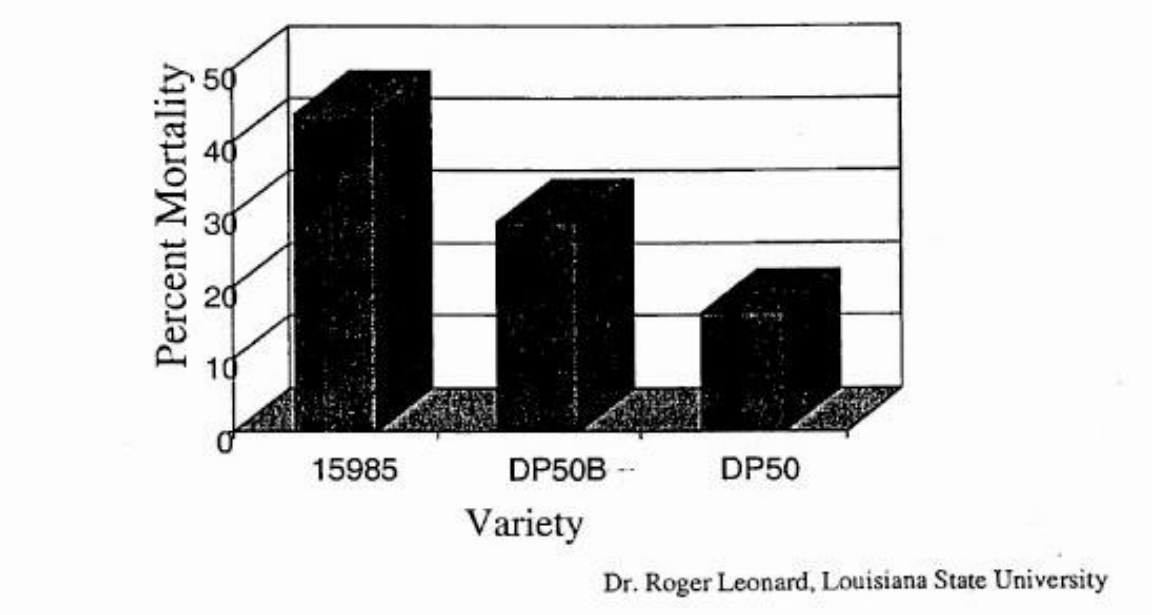

Figure 16 Per cent mortality of cotton bollworm 72 hours after infestation on field generated leaf tissue
Notes for figure: 15985 = MON 15985; DP50B = MON 531; DP50 = conventional cotton

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.

10. 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.

10.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 20summarizes the introgression rates for MON 15985 measured at several distances from the pollen source during field trials in Burkina Faso.

Table 20 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.

10.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 21). 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 22).

Table 21 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 $P \leq 0.05$.

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

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 22 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 21 and Table 22). 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).

10.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).

10.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.

10.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 23). The doses were set to exceed the predicted environmental exposure.

Table 23 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 23). In the remaining studies, the No Observed Effect Concentration exceeded the maximum predicted environmental concentration by more than 10 to 100 fold (Table 23), 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 23).

10.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.

10.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 24).

Table 24 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.

10.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.

11. OTHER CONSIDERATIONS

Biosafety risk assessment focuses on the safety issues related to the impact of a transgenic organism on its receiving environment, including impact on food and feed safety. However, during the risk assessment of transgenic organisms, reviewers sometimes raise concerns that are relevant to the deployment of the technology, but are not safety issues. These non-safety concerns are largely social and economic in nature and may be considered outside of the biosafety assessment when national regulations allow for the consideration of non-safety in biosafety decision making. Examples of social issues include ethics, impact on traditional heritage and cultural dietary requirements. Examples of economic issues include access to new technology, impact on trade and impact on other existing technologies, such as through the development of insect resistance to conventional pest control measures. Some countries have national policy that requires the consideration of national imperatives at all levels of government decision making. National imperatives include reducing poverty, creating jobs, environmental protection, improved health care, food security and sustainable development. Critical to the review of such non-safety considerations is the determination of parameters against which the impact of the introduction of transgenic organisms can be measured. Clear definition of end points (the particular factor which is assumed to be affected, such as impact on exports or rural incomes) allows for evaluation of any impacts and enables the review to be conducted in a transparent manner.

11.A RESIDUAL UNCERTAINTIES

Much attention has been paid to the idea that genetic modification via recombinant-DNA technology has the potential to result in unintended or unanticipated consequences, which could result in environmental harm. However, all methods of genetic modification, including traditional cross-breeding, have the potential for unintended consequences and there are no data indicating that breeding methods employing modern biotechnology have any greater potential for unintended consequences than more traditional methods. Unintended consequences can generally be of two types, those that are predictable and those that are not. Some unintended consequences may be predictable based on knowledge of the biological activity of the newly expressed protein, including its involvement in metabolic pathways. Unpredictable consequences can be due to the action of extraneous genes linked to the target trait, genomic modifications and rearrangements, and pleiotropic effects caused by the new trait.

The accumulation of potentially toxic glycoalkaloids in potato tubers is a recognized food safety issue. During traditional potato breeding there is the potential for the complementation of genes derived from parental lines to result in unintended increases in glycoalkaloids, particularly when introgressions from wild species are present. For example, the potato cultivars, Lenape (U.S. and Canada) and Magnum Bonum (Sweden), were both withdrawn due to excessive glycoalkaloid content in their tubers (Zitnak and Johnston, 1970; Hellenäs *et al*., 1995). This highlights the importance of routinely

testing the glycoalkaloid content of new potato cultivars resulting from both traditional breeding and genetic engineering. It is reasonable to assume that unintended consequences, such as the down-regulation or interruption of a gene encoding a metabolically important enzyme, that are meaningful in regards to the safety of a crop will have a phenotypic manifestation that becomes apparent during rigorous phenotypic, agronomic, and compositional analysis of the new product in just the same way that unanticipated deleterious changes are noted and eliminated in conventional plant breeding. One of the features of the method of creating transgenic plants and animals is that truly deleterious metabolic effects will not result in a viable organism being produced and by the time a transgenic event is considered for field trials, gross changes in phenotype will already have been eliminated.

In some cases, the regulatory attention focused on unintended consequences (unknown effects) has been a driving force behind increasingly complex data requirements for the molecular-genetic characterization of transgenic crops. The result has been a "molecular arms race", in which regulators with access to state of the art molecular diagnostic technologies have developed costly data requirements which afford no greater certainty regarding a product's safety. With regard to environmental risk evaluation and approval of field trials in particular, requirements for excessive molecular data serve only to increase the time and expense to develop transgenic crops. Less developed nations suffer the most when data requirements are excessive, unnecessary and cost prohibitive as the investment required to comply with these data requirements force developers to focus on crops which offer the highest financial returns.

Related to this is the recent emphasis on developing non-targeted, or profiling techniques that would allow for the screening of potential changes in the physiology of the modified plant at different levels, including: at the genome level; at the level of gene transcription (transcriptome) and translation (proteome); and at the level of metabolic pathways (metabalome). The sensitivity of these various "-omics-based" profiling methods in detecting even subtle changes is also a limitation to determining the biological significance of any observed differences. Any changes due to the specific genetic modification may be masked by differences arising from a host of other factors, including: genetic characteristics (cultivar, individual, isogenic lines, heterosis); agronomic factors (soil, fertilizers, pesticide application); environmental influences (location effect, weather, time of day, abiotic stress); plant-microbe interactions (disease status); maturity stage; and post-harvest effects (storage conditions, adventitious mixing). Notwithstanding the fact that these methods are early in their development and lack practical application in environmental risk assessment, the concern is that they may unduly focus risk assessment on a "search for differences" without understanding the biological and environmental significance of any observed differences. The result is likely to be a greater uncertainty for a regulator to address because of the gap between the information collected and its relevance to safety.

Successful adoption of regulatory policies recognizing the sources of residual uncertainty for transgenic crops will require a more enlightened approach to the issue of pleiotropy and unintended effects. Delays created when regulators must address data with no known relevance to safety create a different risk; the risk of not adopting a new technology that could reduce the risks associated with existing agricultural practices. The decision to

require certain information for risk assessment must be made carefully recognizing that it is not always appropriate or valuable to society to simply ask for more.

11.B INSECT RESISTANCE MANAGEMENT AND PRODUCT STEWARDSHIP

In the U.S. the use of a natural refuge for MON 15985 (as opposed to the cultivated refuge, planted with non-transgenic cotton) was requested after collecting scientific data to show that a sufficient number of cotton bollworms and tobacco budworms are present on non-cotton crops and other plants in the eligible growing areas. The natural presence of these pests outside of cotton, combined with the dual efficacy of MON 15985 cotton, greatly reduces the chance that these pests will develop resistance to MON 15985 cotton.

In June 2007 the U.S. Environmental Protection Agency (EPA) approved a natural refuge option for MON 15985 insect-protected cotton planted in clearly defined growing regions in the United States. This option allows cotton producers in eligible regions to count non-cotton crops and other plants as a refuge for certain pests. Where sufficient natural refuge is available, the farmers will not be required to plant a non-Bt cotton refuge for MON 15985 cotton.

Eligible regions for the natural refuge option for MON 15985 cotton are plantings in the states of Alabama, Arkansas, Florida, Georgia, Kansas, Kentucky, Louisiana, Maryland, Missouri, Mississippi, North Carolina, Oklahoma, South Carolina, Tennessee, Texas (excluding the following counties: Brewster, Crane, Crockett, Culberson, El Paso, Hudspeth, Jeff Davis, Loving, Pecos, Presidio, Reeves, Terrell, Val Verde, Ward, and Winkler), and Virginia.

States and counties where the natural refuge option is not approved are areas where pink bollworm is a significant pest. A structured, non-Bt cotton refuge continues to be required as part of an insect resistance management (IRM) program for MON 531 in all states, and for MON 15985 planted outside eligible areas.

A similar insect resistance management dispensation could be used for MON 15985 in Burkina Faso, where target pests are known to feed on cotton, maize and wild vegetation (Section 8.A-8.).

11.C IMPACTS ON AGRICULTURE, INCLUDING BENEFITS OF ADOPTION

Significantly expanding populations and rapid urbanisation have increased regional demand for agricultural products in West Africa. In areas of the Sahel, cotton is one of the only viable cash crops. It is one of the success stories in Sahelian agriculture where cotton has contributed to the improvement of incomes, livelihoods, and access to social facilities (education, health centres and pharmacies, *etc*.). Cotton production also appears to be correlated with rapid increases in cereal production thanks to the cotton production support systems (maintained by the state and national cotton companies) and the promotion of innovation processes. In most zones that have benefited from the cotton production support systems, the cotton boom has been accompanied by an agricultural boom that has contributed to increased cereal production (Hussein *et al*., 2005).

Some one to two million West African households cultivate cotton, with up to 16 million people estimated to benefit directly or indirectly from cotton production. These family farms generate 30-50% of national GDP in West Africa – depending upon the country – and in some countries they generate the largest export revenues. These family farms also produce almost all of the region's staple food crops, oilseeds and cash crops, in addition to being important consumers of diverse imported fruit, vegetables and processed foods (Hussein *et al*., 2005).

As with other technologies that require 'learning by doing', it may take time to achieve the higher yield associated with a better technology. In addition, some areas that are currently planted to non-transgenic cotton may not be conducive to transgenic insect resistant cotton resulting in a lower yield advantage (*e.g.*, 10 to 20 %). The rate of adoption is not certain for the region. Adaptation of transgenic insect resistant cotton inevitably requires the involvement of the developer of the technology, and this has important institutional ramifications that ultimately affect adoption rate (Sanders *et al*., 2005).

Table 25 summarizes the aggregate benefits both at the national and regional levels, from the introduction of transgenic insect resistant cotton at three adoption rates, and using three yield advantage assumptions. The result depended on how benefits for each interest group in the cotton sector were affected. Sanders *et al.* (2005) estimates of benefits were those that accrue directly to the farmers. Benefits to the cotton company (the sole supplier of production inputs, and the single buyer of cotton), and the owner of the technology were not explicitly covered in their analysis.

Table 25 Aggregate benefits from transgenic insect resistant cotton at varying rates of adoption (Sanders *et al*., 2005)

Country	Cotton area [1] (1000ha)	Total benefits [2] (million US$)		
		100% adoption	50% adoption	30% adoption
45% yield advantage				
Mali	493	67	34	20
Burkina Faso	300	41	20	12
Benin	383	52	26	16
Côte d'Ivoire	281	38	19	11
Senegal	55	7	4	2
Total	1512	205	103	61
30% yield advantage				
Mali		45	23	14
Burkina Faso		28	14	8
Benin		35	18	10
Côte d'Ivoire		26	13	8
Senegal		5	2	1
Total		139	70	41
10% yield advantage				
Mali		23	11	7
Burkina Faso		14	7	4
Benin		18	9	5
Côte d'Ivoire		13	6	4
Senegal		3	1	1
Total		71	34	21

1 Two-year average area (1998-2000) data provided by IER.
2 Derived by multiplying land area by the difference in per hectare profits from cotton between Bt and non-Bt.

An important factor in adoption rate may be the increased cost of transgenic seed or technology fees that may be levied on the cultivation of these crops. In their ex-ante analysis of the benefits and costs of introducing transgenic insect resistant cotton in West Africa, Sanders *et al*. (2005) reviewed the impact of a technology fee on the crop mix and farm profit for West African cotton farmers (Table 26). Their analysis indicated that higher technology fees will result in lower adoption rates for transgenic insect resistant cotton and reduced farm profit for West African farmers.

Table 26 Effect of technology fee for transgenic insect resistant cotton on crop mix and farm profit (Sanders *et al*., 2005)

	Alternative technology fee (US dollars per hectare				
Particulars	**$15**	**$50**	**$60**	**$70**	**$80**
			Area (hectares)		
Cereals					
Sorghum	2.7	2.4	2.3	2.2	2.4
Millet	3.9	4.2	4.6	4.9	3.9
Maize	2.9	3.2	3.3	3.4	3.2
Cash crops					
Groundnut	-	0.3	0.3	0.3	0.3
Non-Bt	-	-	-	-	3.1
Bt-cotton	5.5	4.9	4.6	4.2	2.1
			Farm profit (US$)		
	1,593	1,213	1,080	967	890

Note: Yield advantage of 45% was used in the estimation procedure.

Nonetheless, Sanders *et al*. (2005) estimates of benefits were significant for countries in West Africa. Their estimates range from a low of $7 million to a high of $67 million in Mali; $4 million to $41 million in Burkina Faso; $5 million to $52 million in Benin; $4 million to $38 million in Cote d'Ivoire; and, $1 million to $7 million in Senegal. The reduction in insecticide use was an added environmental benefit. Assuming a 100% adoption rate, and a 3-litre per hectare reduction in insecticide application, Mali would be applying 1,500 tons less insecticides, Burkina Faso 900 tons, Benin 1,100 tons, Cote d'Ivoire 843 tons and Senegal 165 tons.

In their review of the impact of transgenic crops on trade, Brookes and Barfoot (2006) calculated that in 2005/06 about 26% of global production of cotton was traded internationally. Of the leading exporting nations, the transgenic cotton growing countries of the US and Australia were prominent exporters accounting for 54% of global trade. Based on the proportion of production in the countries that was transgenic in 2005, it is estimated that 47% of globally traded cotton was transgenic. However, if it is assumed that there is no active segregation of exported cotton from these countries into transgenic versus non-transgenic product (*i.e.*, exported cotton is likely to comprise a mix of both transgenic and non-transgenic cotton) then the transgenic share of global exports can reasonably be expected to have been 57% in 2005 (Brookes and Barfoot, 2006). In terms of cottonseed meal the transgenic share of global trade is about 37% (Brookes and Barfoot, 2006).

In their review of the benefits of adoption of transgenic cotton in South Africa, Gouse *et al*., (2004) found that besides yield benefits, the adoption of transgenic insect resistant cotton decreased the volume of insecticides sprayed. Spraying less insecticide meant lower application costs. For large-scale farmers this is reflected in lower diesel costs and fewer tractor hours. For small-scale farmers the benefit is largely in labour savings, as a

farmer has to walk a distance of 10–20 km to apply pesticide to one hectare of cotton manually - with a knapsack sprayer on his back. Reduced spraying usually means more time for weeding and other management practices. In addition, for the small-scale farmer, water to mix insecticides has to be carried by hand from communal water sources, and in dry areas clean water is scarce. Illness due to exposure to pesticides is not uncommon among small-scale farmers in South Africa (Gouse *et al*., 2004).

A high percentage of large-scale farmers indicated that peace of mind about bollworms is an important benefit of transgenic insect resistant cotton in South Africa (Gouse *et al*., 2004). Peace of mind about cotton bollworm infestations gave large farmers managerial freedom to devote time to other crops or general farming activities— the value of peace of mind might then be represented by the value of increased production of other crops. Large-scale farmers also noticed increased populations of beneficial insects (such as ladybirds and lacewings) in transgenic insect resistant cotton fields, indicating a further possible benefit to the environment due to reduced insecticide applications.

Using only estimates for the value of yield, the cost of pesticides, seed costs, and technology fees, Table 27 shows that both large- and small-scale farmers realize increases in per-hectare income despite higher seed costs and the additional technology fee. The income advantage listed at the bottom of Table 27 could be considered a conservative estimate, because it does not include an application cost benefit or any value for peace of mind or managerial freedom. To the extent that reduced application costs benefit small farmers more than large ones, and managerial freedom impacts large farmers more than small ones, the differences between yield advantages in Table 27 could be greater or smaller if these factors were included. The figures reported in Table 27 indicate that savings on chemical insecticides alone are not enough to offset the additional seed cost of transgenic insect resistant cotton seed. The size of the yield increase, linked to more efficient pest management using transgenic insect resistant varieties, is thus very important.

Table 27 Summary of income benefit to large and small-scale farmers in South Africa following adoption of transgenic insect resistant cotton in U.S.$/hectare in 2004. (Adapted from Gouse *et al*., 2004)

	Small-scale farmer	Large-scale farmer	
	Dryland	Dryland	Irrigation
Yield benefits per hectare @US $0.39/kg	70.52	44.46	246.53
Reduced pesticides benefit	4.53	16.14	41.49
Increased seed and technology fee detriment	-23.08	-33.13	-59.33
Income advantage	51.97	27.47	228.68

Yield benefits might vary substantially between seasons, depending on rainfall and insect pressure, but irrigated cotton that shows the highest income advantage has a more stable insect pest problem than dryland. There are seasons for dryland cotton when bollworm infestation levels are low; in those years it is expected that the yield benefit of transgenic insect resistant cotton will be marginal. Similarly, Gouse *et al*. (2004) data for the 1999/ 2000 season showed a yield advantage for small-scale farmers in excess of 40%, but initial analysis of data collected on the Makhatini Flats during the dry 2002/03 season by the French research institute CIRAD in collaboration with the University of Pretoria showed no statistical significant yield difference (Gouse *et al*., 2004).

11.D SURVEILLANCE AND MONITORING

The need for surveillance and monitoring is determined on a case-by-case basis. Not all transgenic crops require monitoring after approval, and where monitoring has been a condition of an approval this can be for safety, non-safety, or socio-economic reasons. For example, permission to produce insect resistant cotton in South Africa was linked with a condition to monitor for the development of insect resistance, which could have an economic impact on the usefulness of the technology. The developers contracted a public research institute to undertake this surveillance. They established a baseline for resistance to the *cry1Ac* gene in local stem borer populations and monitored fields for resistance for five years. No resistance was detected in this study or reported by farmers growing the crop. The requirement for the monitoring was then withdrawn. The U.S. Department of Agriculture has an ongoing surveillance program on the use of pesticides in relation to the adoption of transgenic crops. These data are published regularly and indicate how the adoption of the new technology changed the types of pesticides used by farmers and increased or decreased their use depending on the traits in transgenic crops.

Monitoring procedures vary from qualitative to quantitative and from simple to complex. The objectives of the monitoring plan determine the measurement endpoints and these plans should consider: available knowledge of the organism and release environment; identified potential risks; and regulatory requirements, such as risk management conditions linked to release permits. A monitoring plan should determine appropriate levels of monitoring intensity, specific testing procedures and provide a mechanism to enable on-going evaluation of the effectiveness of the monitoring. This will enable modifications to be made in response to changing conditions and unanticipated problems with the methodology (Traynor *et al*., 2002).

When predetermined sampling regimes are impractical, surveillance provides post-release observation of the environmental impact of approved transgenic organisms. Devising a meaningful surveillance program presents difficulties when the environmental effects following the release of a transgenic organism are only speculative (Traynor *et al*., 2002). Monitoring large areas over long time periods with many sampling sites may present both resource and technical difficulties. When the U.S. EPA first granted a permit for the sale of insect-resistant Bt cotton, the agency required the developer to implement a surveillance program to monitor for insect pest resistance to the cry protein. After the agency had evaluated the methods used by the developers it called for the use of more sensitive methods to improve early detection of resistance development in local pest populations (EPA, 2001).

Biosafety assessors need to anticipate and avoid potential pitfalls in any monitoring methodology. They need to understand the objectives and have some assurance that useful data will be obtained by the proposed methodology. Regulators should ensure that there is a strategy for addressing any unwanted environmental impact identified during monitoring or surveillance. If monitoring is intended as an early-warning mechanism for unwanted environmental impact, then there should be a mechanism in place to respond in a timely fashion (Traynor *et al*., 2002).

These examples raise some important points about monitoring. Firstly, not all crops will need monitoring after obtaining an approval for unconfined use. Secondly, the need for

monitoring is not always a safety need and thirdly, the monitoring is not always carried out by the developer. In some cases governments fund monitoring programmes and in others, the monitoring fits the mandate of public research in the country or region of release. Fourthly, monitoring can only be required by regulators when they have a clear understanding of what they are looking for. A vague request to monitor for 'unintended effects' following the introduction of a new transgenic crop is not practical. It is simply not feasible to design a monitoring program unless you are clear about what is being investigated. Finally, the monitoring program needs to address the consequences of an action, rather than the likelihood. If one estimates there is a very small likelihood that DNA will pass from transgenic plant waste to soil microbes, it is of little additional value to know through experimentation that this value is in the order of 10^{-27}. The risk assessors will want to determine what impact the uptake of DNA by soil microbes will have on the environment.

CONCLUDING COMMENTS

Many useful and appropriate models for the science-based risk assessment have been developed (Tiedje *et al*., 1989; OECD, 1993, EFSA, 2006) that are consistent with international treaties (CBD and IPPC). In addition to developing an appropriate science-based assessment, countries face the challenge of how to address the societal interests (including micro and macro economic interests) in decision-making and risk management. No single model exists to do this.

Internationally, the standards and practice of environmental risk assessment of transgenic crops are generally consistent with respect to the safety concerns that must be addressed. It is accepted broadly that the potential hazards posed by the introduction of a new organism or production practice should be evaluated relative to an existing product or practice, and should consider the characteristics of the introduced trait, the phenotypic expression of the new trait and the interaction of the modified plant in the receiving environment. The quality of pre-market environmental risk assessment is realistically limited by our knowledge of existing ecosystems and by our understanding of the biology of the unmodified host organism, including its potential interactions within ecosystems. In this context, there is a need for increased public sector research to increase our understanding of both natural and managed ecosystems, both to strengthen pre-market risk assessment and to provide baseline data to support systematic hypothesis-driven post-market monitoring. However, the new molecular and "omics" technologies currently being examined have more uncertainty associated with them since they have not been validated yet for use in mainstream risk assessment. It is equally important to recognize that delays in adopting some technologies while waiting for new knowledge is not without risk.

Experience from over 10 years of producing transgenic crops has increased familiarity with certain products from the perspective of field testing, importation and production. It is reasonable to use this experience in a retrospective analysis and differentiate among risks. Trait and crop combinations with multiple years of safe production should be viewed differently from experimental transgenic materials. Likewise, the risks associated

with importation of transgenic food and feed commodities should be evaluated in an appropriate manner based on the level of exposure; while for field trials the focus should be on adequate risk management (confinement). Lastly, post-market monitoring/surveillance can be a valuable and appropriate exercise when based on the results of the risk assessment. Poorly defined goals and criteria for monitoring could lead to a mischaracterization of the risk or false sense of security.

Perhaps the greatest uncertainty regarding the future of regulatory decision making for transgenic crops can be seen when contrasting the environmental risk assessment standards under OECD, the Cartagena Protocol, and the IPPC. Specifically, there are distinct differences in how other considerations are handled. Under OECD recommendations, these are not dealt with within the risk assessment process *per se* although they are presumed to play some role in ultimate decision-making. The Cartagena Protocol formally introduces the notion of socio-economic concerns being an important consideration, but to date has not provided specific guidance on how and to what extent they may be incorporated in biosafety decisions. Finally, under the IPPC we see a more formalized incorporation of economic (not social) analysis as an integral part of the pest risk analysis.

As evidenced by the public debate in Europe, rigorous science-based risk assessment is a necessary but not sufficient condition for gaining social acceptance of agricultural biotechnology. In decision making processes, non-safety concerns may be an important consideration in the public's acceptance that affects regulatory decision-making. As such, a challenge for governments is how best to address these within their legal and regulatory framework, while also meeting their obligations under other international agreements. Any movement towards a more complete cost-benefit risk assessment for the introduction of transgenic crops will take time and require additional research on defining and using relevant economic indicators of environmental impact. Finally, if one is to include potential socio-economic impacts as part of the environmental risk assessment, then intellectual honesty would require one to consider equally the potentially positive and negative impacts.

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