97-287-01p

Monsanto

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October 6, 1997

Coordination, Technical Assistance,
Biotechnology, Scientific Services,
Plant, Protection, and Quarantine,
Animal and Plant Health Inspection Service
U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Re: PETITION FOR DETERMINATION OF NON-REGULATED STATUS FOR INSECT RESISTANT TOMATO LINE 5345

Enclosed is a copy of a petition for determination on the regulatory status of *Lycopersicon esculentum* that has been modified to be resistant to feeding by certain lepidopteran tomato pests through the expression of the Cry1Ac protein. This insect resistant tomato line is currently deemed a "regulated article". Based on the data and information contained in the enclosed petition, we believe that there is no longer "reason to believe" that the modified tomato plants should be deemed to be regulated articles. The modified tomato plants do not present a plant pest risk and are not otherwise deleterious to human health or the environment. The enclosed petition does not contain confidential business information.

Sincerely,

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10/14/97

97-287-01p

Petition for Determination of Nonregulated Status

Insect Resistant Tomato Line 5345

(*Lycopersicon esculentum*)

Producing the Cry1Ac Insect Control Protein

of

Bacillus thuringiensis subsp. *kurstaki*

97-235U

Submitted By:

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CONTAINS NO CONFIDENTIAL INFORMATION

Petition for Determination of Nonregulated Status Insect Resistant Tomato Line 5345

(*Lycopersicon esculentum*) Producing the Cry1Ac Insect Control Protein

of *Bacillus thuringiensis* subsp. *kurstaki*

SUMMARY

Tomato, *Lycopersicon esculentum*, has been genetically engineered to be resistant to selected Lepidopteran insect pests (Lepidoptera). Resistance was accomplished by the stable insertion of a gene encoding the Cry1Ac protein from *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) HD-73 into the tomato genome. The Cry1Ac protein is insecticidal to Lepidoptera larvae, but is safe to mammals, birds, fish, and beneficial insects. Larvae of some Lepidopteran insect species are important pests impacting successful tomato production. Commercial tomato production typically requires numerous chemical insecticide applications for control of insect pests. The tomato varieties expressing the Cry1Ac protein are expected to significantly reduce chemical insecticide use in tomato production and, therefore, provide a major benefit to tomato growers and the environment. The Cry1Ac protein expressed in Insect Resistant (IR) Tomato Line 5345 is identical to that expressed by Bollgard® Cotton lines 531 and 757 which the USDA determined should no longer be considered regulated articles under 7 CFR Part 340 (Federal Register, 1995). The safety and efficacy of the Cry1Ac protein has been thoroughly evaluated in cotton and corn by both the EPA and FDA and has been granted an exemption from tolerance under Section 408 of the Federal Food Drug and Cosmetic Act (FFDCA)(EPA, 1994; EPA, 1997).

IR Tomato Line 5345 was developed by *Agrobacterium tumefaciens*-mediated transfer of the *cry1Ac* gene into the genome of a commercial tomato variety. Transgenic plants were generated by transformation with binary plasmid vector PV-LEBK04. This vector is identical to PV-GHBK04 used to produce Monsanto's Bollgard® Cotton lines 757 and 531. The vector contains a region of DNA, referred to as T-DNA (transferred DNA), that was transferred from *Agrobacterium tumefaciens* into the tomato genome. The T-DNA from plasmid PV-LEBK04 contains three genes: *cry1Ac*, *nptII* and *aad*. The *cry1Ac* gene encodes the insecticidally-active Cry1Ac protein. This protein is more than 99.8% identical to that found in nature and in commercial *B.t.k.* formulations registered as pesticides with the Environmental Protection Agency (EPA) for the past thirty years. The *nptII* gene encodes neomycin phosphotransferase II (NPTII), which allows for the selection of genetically improved plant cells on kanamycin during the plant transformation process. The *aad* gene encodes aminoglycoside adenylyltransferase (AAD), and is present in the vector to provide for selection in the laboratory. The *aad* gene, which is driven by a bacterial promoter, allows for selection of bacteria in media containing spectinomycin or streptomycin, in steps prior to plant transformation. The *aad* gene, lacking a plant promoter, is not expressed in IR Tomato Line 5345 plants.

Data and information for IR Tomato Line 5345 transformed with the plasmid vector PV-LEBK04 are provided to demonstrate that this tomato line and its progeny are no more likely to become a weed than traditional tomato varieties and are unlikely to increase the weediness potential of any cultivated plant or native wild species. In addition, these lines do not exhibit plant pathogenic properties and exhibit no toxicity to non-target organisms, including those organisms beneficial to agriculture.

Therefore, based on the data and information enclosed in this petition, Monsanto requests a determination from APHIS that IR Tomato Line 5345 and any progenies derived from crosses between IR Tomato Line 5345 and traditional tomato varieties no longer be considered regulated articles under regulations in 7 CFR part 340.6.

CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

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ABBREVIATIONS

AAD	aminoglycoside adenylyltransferase
aad	aminoglycoside adenylyltransferase gene
BC7F1	Backcrossed progeny
bp	base pairs
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
CFR	Code of Federal Regulations
Cry1Ac	Class I Bacillus thuringiensis crystal protein
cry1Ac	Class I Bacillus thuringiensis crystal protein gene
cv.	Cultivar
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
fwt	fresh weight
IR	Insect Resistant
kb	kilobase
kD	kilodalton
mg	milligram
NPTII	neomycin phosphotransferase II
nptII	neomycin phosphotransferase II gene
PV-LEBK04	Plasmid Vector LEBK04
PV-GHK04	Vector used to transform Bollgard cotton lines 531 and 757 and is identical to PV-LEBK04
spp.	Species
µg	microgram

I. RATIONALE FOR THE DEVELOPMENT OF IR TOMATO LINE 5345

Tomato, *Lycopersicon esculentum*, is an important and valuable crop for human consumption. It is also vulnerable to insect consumption, serving as host plant for more than 100 insect species worldwide (Berlinger, 1986). Economic damage to tomatoes by insects is considerable (Schwartz and Klassen, 1981). Consumer preferences and market quality standards leave little room for cosmetic damage or contamination of tomato fruit, so strict control of insects has depended heavily on the use of insecticides (Farrar and Kennedy, 1991).

Reliance on agricultural chemicals has become increasingly precarious, however, as a number of associated problems have been recognized (Tingey and Steffens, 1991; Stoner, 1992; Trumble *et al.*, 1994). These problems include detrimental consequences for human and animal health, environmental pollution, increased costs of crop production, adverse effects on natural enemies, the development of insecticide-resistant insect biotypes, and heightened regulatory constraints on the registration and use of insecticides. Widespread use of these chemical controls has also resulted in a negative public perception of agriculture. In recent decades, these difficulties have encouraged greater efforts in research and development of alternative methods of insect control, among them the utilization of biodegradable microbial insecticides with greater specificity and less non-target impact (Bauer, 1995; Kennedy and Whalon, 1995). One such group of microbial insecticides are the delta-endotoxins, obtained from the ubiquitous soil bacterium *Bacillus thuringiensis* (*B.t.*). Cry1Ac, one of a number of these different crystalline proteins, is synthesized by *B. thuringiensis* subsp. *kurstaki* (*B.t.k.*). It is toxic to certain lepidopteran insects, and is coded for by the gene *cry1Ac*.

Commercial formulations of Cry1Ac for foliar application have been used widely for many years. However, field efficacy has often been less than desired, because these materials are subject to weathering and deterioration by the elements, and must be regularly reapplied or supported by the use of other chemicals (Bohorova *et al.*, 1997). One approach to utilize the efficacy of Cry1Ac while avoiding this shortcoming has been the genetic engineering of plants containing the *cry1Ac* gene. In contrast to foliar application, these genetically modified plants produce the insect control protein Cry1Ac within the plant cells. This ensures that target insect pests are exposed to it whenever they feed on the plants. As a result, control may be more efficacious, and applications of other insecticides to control the target species may be reduced or eliminated.

BHN Research/Gargiulo Inc., a subsidiary of Monsanto Company, is developing insect-resistant tomato varieties containing the *cry1A(c)* gene for proprietary use, pending regulatory approval. IR Tomato Line 5345 tomatoes are derived from genetically modified plants originally produced at Monsanto Company (Fischhoff *et al.*, 1987). The Cry1Ac protein expressed in tomato is virtually identical to that produced by *B. thuringiensis* subsp. *kurstaki* in nature, and to that found in commercial formulations of *B.t.k.* used in agriculture, and is identical to that used in Bollgard® Cry1Ac cotton, developed by Monsanto (EPA Registration No. 524-478). Laboratory bioassays have shown this gene to be effective and specific in its control of the most important lepidopteran tomato pests, tomato pinworm (*Keiferia lycopersicella*) and tomato fruitworm (*Helicoverpa zea*), as well as others including potato tuber moth (*Phthorimaea operculella*), tomato hornworm (*Manduca sexta*) and cabbage looper (*Trichoplusia ni*), the latter two of which have exhibited extreme sensitivity to Cry1Ac (Delannay *et al.*, 1989; Hardee and Bryan, 1997). Results from four years of field experiments conducted in all the targeted growing regions of the U.S. under notifications (Table 1) acknowledged by the United States Department of Agriculture (USDA) have demonstrated that tomato producing the Cry1Ac protein provides protection from its primary caterpillar insect pests. At the same time, the protein has no effect on non-target organisms, such as beneficial insects, birds, fish, mammals and humans (EPA, 1997). When this advantage is coupled with the fact that tomato expressing the Cry1Ac protein will reduce chemical insecticide applications for control of these targeted caterpillar pests, alternative management strategies for nontarget tomato pests become easier to implement.

IR Tomato Line 5345 will represent an efficacious and environmentally compatible addition to the existing options for tomato insect pest management. In addition, it will provide significant benefits to growers, the general public and the environment, including:

1. A more reliable, economical and less labor intensive means to control lepidopteran insect pests.
2. Insect control without harming non-target species, including humans.
3. A means for growers to significantly reduce the amount of chemical insecticides currently applied to the crop while maintaining comparable yields. Therefore, lepidopteran insect control can be achieved in a more environmentally compatible manner than is currently available.
4. A reduction in the manufacturing, shipment and storage of chemical insecticides used on tomato.
5. A reduction in the exposure to workers to the pesticides and pesticide spray solutions.
6. A reduction in the number of empty pesticide containers and amount of pesticide spray solution that must be disposed of according to applicable environmental regulations.
7. An excellent fit with Integrated Pest Management Programs, IPM, and sustainable agricultural systems.

Monsanto intends to obtain the following regulatory approvals to support the commercialization of IR Tomato Line 5345:

1. This determination from USDA/APHIS that IR Tomato Line 5345 and all progenies derived from crosses between IR Tomato Line 5345 and other tomato cultivars are no longer regulated articles under 7 CFR Part 340.6.
2. Regulatory approval from the EPA of the *B.t.k.* Cry1Ac insecticidal protein as expressed in IR Tomato Line 5345 under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).
3. Monsanto will also consult with the U.S. FDA following their May 29, 1992 policy statement "Foods Derived from New Plant Varieties" to demonstrate the compositional and nutritional equivalence of IR Tomato Line 5345 to the currently available tomato varieties.

The EPA has exempted the NPTII and Cry1Ac proteins and the genetic material necessary for the production of the proteins from the requirement of a tolerance in or on all agricultural commodities when used as a plant pesticide inert ingredient (EPA 1994, 1997). FDA has also amended the food additive regulations to provide for the safe use of NPTII as a processing aid in the development of new varieties of tomato, oilseed rape and cotton (Calgene, Inc. 1993; FDA 1994). No additional regulatory approvals are necessary for the use of NPTII and Cry1Ac proteins in the U.S.

Table 1. Field Trials of IR Tomato Line 5345

USDA #	Location
94-362-01N	Lee County, FL
94-362-01N	Collier County, FL
94-362-01N	Collier County, FL
94-362-01N	Lee County, FL
95-138-04N	Lee County, FL
95-138-04N	Collier County, FL
95-151-04N	Fresno County, CA
95-216-03N	Pinal Country, AZ
96-011-01N	Lee County, FL
96-011-01N	Collier County, FL
96-011-01N	Lee County, FL
96-011-01N	Collier County, FL
96-011-01N	Lee County, FL
96-011-01N	Collier County, FL
96-247-14N	Puerto Rico
96-247-14N	Puerto Rico
97-013-01N	Collier County, FL
97-013-01N	Lee County, FL
97-013-01N	DeSoto County, FL
97-013-01N	Gadsden County, FL
97-013-01N	Fresno County, CA
97-013-01N	Fresno County, CA
97-013-01N	Fresno County, CA
97-015-02N	San Joaquin County, CA
97-013-01N	Decatur County, GA
97-013-01N	Lee County, FL
97-013-01N	DeSoto County, FL
97-013-01N	Collier County, FL
97-182-04N	Lee County, FL

II. THE TOMATO FAMILY

Description of the Genetics and Breeding of Tomato and its Production in the U.S.
Steven D. Tanksley, Cornell University, NY.

A. Tomato as a Crop

Lycopersicon esculentum (cultivated tomato) originated in Latin America where it was domesticated by native people in pre-Columbian times. While the exact site of domestication is unknown, the bulk of the evidence points to Mexico (Jenkins, 1948; Rick, 1976). Studies of morphological and enzymatic variation show the greatest similarity between modern cultivated tomatoes and wild forms of this same species (*L. esculentum* var. *cerasiforme*) from Mexico.

By the time Spanish explorers arrived in the New World, tomato was already a well-developed cultigen and it was apparently from Mexico that Spanish explorers obtained tomato seeds that were subsequently transported back to Europe in the 1600's. Acceptance of the tomato as a vegetable crop in Europe was slow, due at least in part to the fact that tomato belongs to the Nightshade family (Solanaceae) which contains a number of poisonous plant species (*e.g.*, black nightshade). While tomato fruit do not contain the toxins found in many wild nightshades, the association with poisonous plants remained an obstacle to general acceptance until the early 20th century (Rick, 1978).

Tomatoes were introduced into what is now the United States, not from Latin America, but from Europe by colonists. The first references to this crop are found in writings in the 1700's and early 1800's by the herbalist William Salmon and by Thomas Jefferson (Rick, 1978). Production and consumption of tomatoes remained at a fairly constant but low level until the mid 1900's when demand for the fruit increased, not only as a fresh vegetable, but also as the primary ingredient of soups, sauces and catsup.

B. Taxonomy of the *Lycopersicon* genus

Tomato is a member of the genus *Lycopersicon*, which is native to tropical and subtropical Central America and western South America. The majority of the *Lycopersicon* species are concentrated in the Andean region of Peru, Chile and Ecuador and it is in this region that the genus likely originated. Under natural conditions, all of the *Lycopersicon* species persist as perennials in those regions. Lack of cold tolerance dictates that the tomato now be grown as an annual in the temperate regions where it is currently commercially produced.

The genus is split into two subgenera: *Eulycopersicon* and *Eriopersicon*. Species belonging to *Eriopersicon* have small fruit which remain green at maturity whereas *Eulycopersicon* have fruit that develop the familiar red and orange pigments (lycopene and b-carotene) at maturity. It is to *Eulycopersicon* that the cultivated tomato (*L. esculentum*) belongs. Other members of the *Eulycopersicon* include *L. pimpinellifolium* and *L. cheesmanii*. *L. pimpinellifolium* has very small fruit and is found in large concentrations in coastal regions of Peru and Ecuador and often occupies disturbed or abandoned lands. It also occurs as a weed in fields of the same region (Rick *et al.*, 1977). *L. cheesmanii* is endemic to the Galapagos Islands off Ecuador and has never been reported to occur any other place in the world (Rick and Forbes, 1975a).

The wild form of the cultivated tomato, *L. esculentum* var. *cerasiforme*, typically bears fruit (and flowers) larger than those of *L. pimpinellifolium* but is otherwise very similar in appearance to *L. pimpinellifolium*. It occupies a broader range than *L. pimpinellifolium* and in pre-Columbian times was common to the flora of western South America, Central America and Mexico. Since the Spanish explorations of Latin America, seeds of cerasiforme have been transported around the world and it now occurs as a weed in Africa and parts of Southeast Asia (Rick, 1976; Rick and Forbes, 1975b).

All of the red-fruited species (*L. esculentum, L. pimpinellifolium and L. cheesmanii*) are naturally self-pollinating, but are sexually compatible with one another. Hybrids among these species can be readily obtained only with manual crossing. Interspecific hybrids are highly fertile as are subsequent progeny (*e.g.*, F_2, F_3, etc.). *L.*

pimpinellifolium (and *L. cheesmanii* to a lesser extent) has been used extensively by breeders as a source of disease resistance genes and other genes of agronomic importance to tomato culture.

The green-fruited species (*L. chmielewskii, L. parviflorum, L. hirsutum, L. pennellii, L. peruvianum, L. chilense*) are more distantly related to the cultivated tomato. Most of these species are self-incompatible and occur as highly variable populations in valley and coastal regions of Peru, Chile and Ecuador. There are no known natural populations of any of these species elsewhere in the world. Hybrids can be obtained between the cultivated tomato and all of the green-fruited species; however in some instances (especially with *L. peruvianum* and *L. chilense*) embryo rescue techniques are required. Interspecific hybrids are vegetatively vigorous and display various levels of fertility. Sterility is a common occurrence in progeny derived from these interspecific hybrids and represents a barrier to natural gene flow between these species and the cultivated tomato. Nonetheless, the green fruited species have been a source of many disease resistance genes that have been transferred into the cultivated tomato via backcrossing by breeders (Rick, 1982).

Outside of the genus *Lycopersicon*, the closest relatives of cultivated tomato are species in the genus *Solanum*. While *Solanum* and *Lycopersicon* species share the same basic chromosome number (x=12), strong reproductive barriers prevent crossing (artificial or natural) except in a few rare instances. Crosses have been obtained between *L. esculentum* and *S. lycopersicoides* and *S. rickii* with the use of embryo rescue techniques, but the hybrids are generally highly sterile.

C. Genetics of tomato

Tomato is a diploid species and contains 12 pairs of chromosomes. Among crop species it has a relatively small amount of DNA (*ca.* 1000 megabases). The genetics of this species is well characterized. A linkage map based on morphological mutations was established by the middle of this century and it is currently one of the most extensively mapped species (plant or animal) with more than 200 morphological and 1000 molecular markers having been localized to chromosomes (Tanksley, 1993). Numerous cytogenetic stocks have also been developed for tomato, including a full set of primary trisomics, which has greatly facilitated the genetics and cytogenetics of this species.

D. Pollination of tomato

The cultivated tomato is naturally self pollinating. Under field conditions in the United States, self-pollination occurs at a rate of approximately 99% (Currence and Jenkins, 1942; Lesley, 1924). While many of the wild tomatoes have stigmas that are exerted beyond the anther cone and experience high levels of cross-pollination, modern tomato cultivars have been selected (probably inadvertently for high fertility) for stigmas recessed inside the anther cone and are therefore not available for receipt of outside pollen. The self-pollinating nature of tomatoes make them ideal for the pedigree method of breeding for improvement of yield and other quantitative horticultural characteristics. Two plants (usually different varieties) are hybridized to produce an F_1 which is allowed to self pollinate. Single desirable plants are selected at the F_2 generation and their progeny (F_3) are similarly selected. The process is repeated for several generations until homozygous lines are obtained. Tomato is an annual, day-neutral crop, requiring 4-6 months from seeding to fruit harvest. Flowers are perfect and, due to recessed stigmas, they automatically self-pollinate. Cross hybridization between tomato plants can be accomplished by removing the anthers from immature flowers and placing pollen from another plant on the exposed stigma surface. Ovules are receptive to fertilization even before pollen of the same flower has matured. A single tomato fruit will produce 20-150 seeds depending on the variety and environmental conditions. Seeds mature 40-60 days after pollination and a single plant can produce as many as 25,000 seeds.

Tomato pollen is binucleate and remains viable under room temperature for several weeks. Pollen stored under low temperature and humidity can remain viable for 6 months or more. While cultivated tomatoes are typically self-pollinating, occasional cross pollination can occur and, in the field, is usually attributable to activity of common pollinating insects, especially bees. The incidence of cross-pollination typically less than 1% in field tomatoes grown in the United States, but can be higher in areas of the world (i.e., Latin America) where tomatoes

originally evolved. The higher incidence of cross pollination is probably attributable to greater natural populations of pollinating insects.

E. Weediness of Tomato

Tomato is not considered a weed pest itself and breeding of cultivated tomatoes has never produced a weed pest (USDA, FR 57:202). Many other members of the nightshade family are found as weeds in tomato fields and *Lycopersicon esculentum* is sexually incompatible with all these weedy relatives (Rick, 1979).

F. Potential for outcrossing

1. Out-crossing with nontransgenic cultivars. Tomato does not cross-pollinate with other plants in the United States without the intervention of man. Cultivated tomato is self-fertile and also is almost exclusively self-pollinating, due, in part to the presence of an inserted stigma developed through over 50 years of breeding (Rick, 1976). Cultivated tomato is not wind pollinated and insect pollination is limited (Rick, 1976).

The cherry tomato, *L. esculentum* var. *cerasiforme*, was most likely the wild progenitor of the cultivated tomato (Rick, 1983). Although *L. esculentum* var. esculentum and var. *cerasiforme* can cross with either plant as male or female parent (Rick, 1979), the probability of IR Tomato Line 5345 naturally introgressing into var. *cerasiforme* in the United States is almost nil, since the rate of outcrossing in var. *esculentum* is low (Rick, 1949) and var. *cerasiforme* is not present in areas of the U.S. that are devoted to large-scale cultivation of tomatoes (USDA, FR57:202). There are no published reports that visible traits of cultivated tomato have introgressed into var. *cerasiforme* from cultivated tomatoes in areas where the wild cherry tomato commonly grows.

2. Hybridization with species in the same genus. *L. pimpinellifolium*, or the currant tomato, is the only species in the tomato genus for which there is good evidence for natural hybridization with the cultivated tomato (Rick, 1958). *L. pimpinellifolium* is a weedy, short-lived perennial plant native to the coastal regions of Ecuador and Peru. It produces small red fruit (< 1 cm diameter) and, although it is not grown commercially, it is occasionally harvested from the wild for human consumption.

3. Hybridization with species outside the genus. *Solanum* is the genus most closely related to the tomato genus (*Lycopersicon*). *Solanum* is a large genus comprised of hundreds of species including such agronomic species as potato and eggplant. However only two *Solanum* species (*S. lycopersicoides* and *S. rickii*) have been successfully crossed with the tomato and this was accomplished only in the laboratory. Hybrids between the tomato and *S. lycopersicoides* or *S. rickii* are almost always sterile, making further gene introgression very difficult. *S. lycopersicoides* and *S. rickii* are found only in restricted habitats of Peru and Chile and do not normally occupy agricultural lands where tomatoes are commercially grown. This fact, combined with the strong barriers to hybridization, make it extremely unlikely that gene transfer would ever occur between transgenic cultivated tomatoes and these wild species.

The *Solanum* species that occur naturally in the United States (*e.g., S. nigrum*, black nightshade or *S. elaeagnifolium*, silver nightshade) do not hybridize with the cultivated tomato and thus present no significant risk for gene exchange.

G. Characteristics of the Non-transformed Cultivar

Lycopersicon esculentum cv. UC82B is the tomato cultivar which was genetically modified to be resistant to feeding by Lepidopteran insects and is a commercial variety developed at the Department of Vegetable Crops, University of California, Davis CA. UC82B is a processing variety that has been grown extensively in California (Stevens *et al.*, 1976). UC82B is readily transformed using *Agrobacterium tumefaciens* T-DNA vectors (McCormick *et al.*, 1986).

III. DESCRIPTION OF TRANSFORMATION SYSTEM

A. TRANSFORMATION SYSTEM

IR Tomato Line 5345 was produced by *Agrobacterium tumefaciens*-mediated transformation of the commercial tomato variety UC82B. The plant expression vector was assembled and then transformed into *E. coli* and mated into the ABI *Agrobacterium* strain by the triparental conjugation system, using the helper plasmid pRK2013 (Ditta *et al.*, 1980). The binary ABI strain contains the disarmed (*i.e.*, lacking the T-DNA phytohormone genes) pTiC58 plasmid pMP90RK (Koncz and Schell, 1986), in a chloramphenicol resistant derivative of the *Agrobacterium tumefaciens* strain A208. The disarmed pMP90RK Ti plasmid does not carry the T-DNA phytohormone genes and is, therefore unable to cause crown gall disease. The pMP90RK Ti plasmid was engineered to provide the *trfA* gene functions required for autonomous replication of the plasmid vector after conjugation into the ABI strain. When the plant tissue is incubated with the ABI:plasmid vector conjugate, the vector is transferred to the plant cells via the *vir* functions encoded by the disarmed MP90RK Ti plasmid (Klee *et al.*, 1983; Stachel and Nester, 1986). The Ti plasmid does not transfer to the plant cells, but remains in the *Agrobacterium.* Further information on the plant transformation system based on *Agrobacterium tumefaciens* delivery can be found in a review by Klee and Rogers (1989). Procedures for *Agrobacterium* transformation and regeneration of tomato tissues were performed as described by McCormick *et al.* (1986).

Generally only the T-DNA is transferred and integrated into the plant genome (Zambryski, 1992). It is generally accepted that T-DNA transfer into plant cells by *Agrobacterium* is irreversible (Huttner *et al.*, 1992). The border sequence itself is not entirely transferred during the process of insertion of the T-DNA into the plant genome (Bakkeren *et al.*, 1989). This means that the inserted DNA is no longer a functional T-DNA; *i.e.*, once integrated, there is no known mechanism for transfer into the genome of another plant, even if acted on again by *vir* genes.

B. PLANT EXPRESSION VECTOR PV-LEBK04

The *cry1Ac* and *nptII* genes were introduced into plants of commercial tomato variety UC82B using an *Agrobacterium tumefaciens* binary single border transformation vector, PV-LEBK04 (Bevan, 1984; Wang *et al.*, 1984). This vector is identical to the plasmid vector PV-GHBK04 used to transform Bollgard cotton lines 531 and 757 which was renamed for use in tomato. The vector (Figure 1) contains well-characterized DNA segments required for selection and replication of the plasmid in bacteria, as well as a right border for initiating the region of DNA (T-DNA) transferred into plant genomic DNA. It is composed of several genetic components. The 0.70 Kb *ori-V* fragment from the RK2 plasmid (Stalker *et al.*, 1981) provides the origin of replication for maintenance in *Agrobacterium tumefaciens* and is fused to the 3.0 Kb *Sal*I to *PVU*I segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (*ori-322*) and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* (Bolivar *et al.*, 1977; Sutcliffe 1978). This was fused to a 0.09 Kb DNA fragment from the pTiT37 plasmid which contains the nopaline-type T-DNA right border (Depicker *et al.*, 1982; Zambryski *et al.*, 1982; Bevan *et al.*, 1983). The remaining portion of plasmid DNA consists of two chimeric genes (genes with signals for plant expression), that encode the Cry1Ac and NPTII proteins and a bacterial selectable marker protein gene (*aad*) under the control of a bacterial promoter. A summary of the specific DNA components in vector PV-LEBK04 is listed in Table 2.

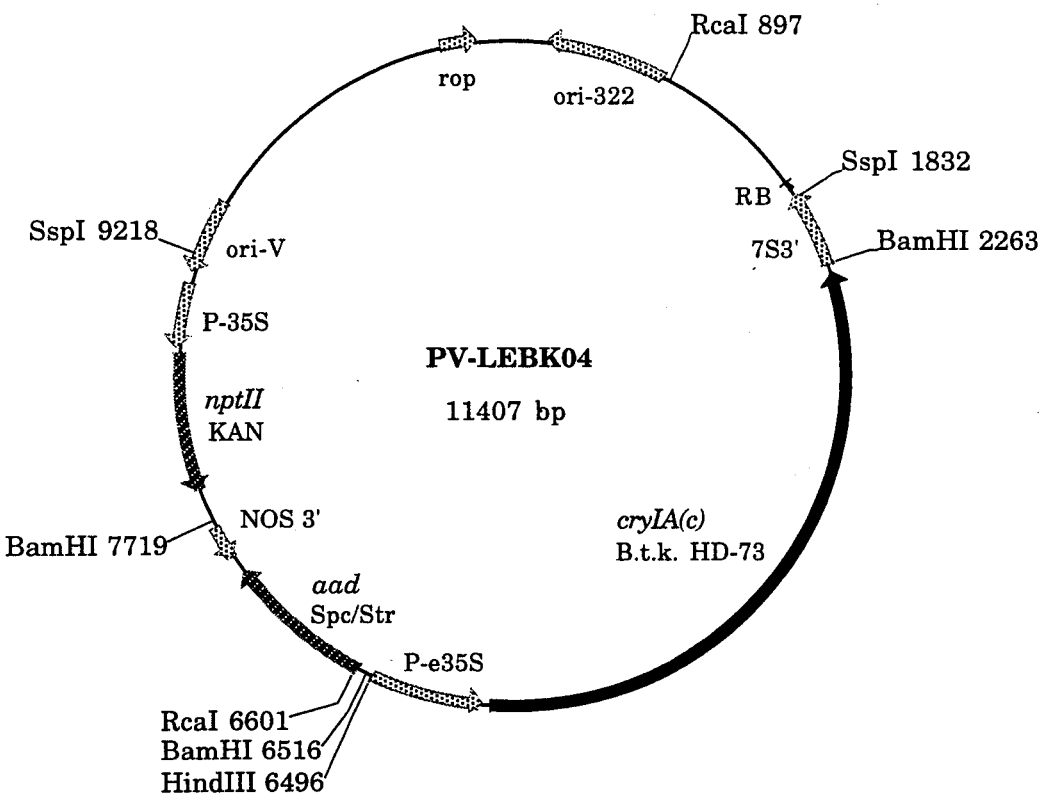


Figure 1: Plasmid map of PV-LEBK04 (pMON10518).

Plasmid map of the 11.4 Kb binary vector, PV-LEBK04, used to transform tomato line 5345. Restriction sites, and their locations in bp, utilized during Southern blot analysis are shown.

Table 2. Summary of DNA Components in PV-LEBK04

Genetic Element	Size (kb)	Function
right border (RB)	0.09	A DNA fragment from the pTiT37 plasmid containing the 24 bp nopaline-type T-DNA right border used to initiate the T-DNA transfer 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).
cry1Ac	3.5	The gene which confers insect resistance, encoding the Cry1Ac protein. The modified gene encodes an amino acid sequence that is 99.4% identical to the *Bacillus thuringiensis* subsp. *kurstaki cry1Ac* gene as described by Adang *et. al* (1985)
7S 3'	0.43	A 3' nontranslated 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 streptomycin adenylyltransferase that allows for bacterial selection on spectinomycin or streptomycin, in steps prior to plant transformation (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' nontranslated 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).
ori-V	0.62	Origin of replication for ABI *Agrobacterium* derived from the broad-host range plasmid RK2 (Stalker *et al.*, 1981).
ori-322/rop	1.8	A segment of pBR322 which provides the origin of replication for maintenance of the PV-LEBK04 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).

IV. DONOR GENES AND REGULATORY SEQUENCES

A. The *cry1Ac* Gene

The *cry1Ac* gene is under the control of the enhanced 35S promoter (Kay *et al.*, 1987; Odell *et al.*, 1985). The 3' end of the gene is from the 3' non-translated region of the soybean alpha subunit of the beta-conglycinin gene and provides the mRNA polyadenylation signals (Schuler *et al*, 1982). The *cry1Ac* gene encodes a full-length Cry1Ac protein of 1178 amino acids, which when subjected to trypsin yields an active N-terminal protein product of approximately 600 amino acids *in planta* and *in vitro*. The *cry1Ac* gene was constructed by combining the first 1398 amino acids of the *cry1Ab* gene (corresponding to amino acids 1 to 466) (Perlak *et al.*, 1990) with nucleotides number 1399 to 3534 of the *cry1Ac* gene (corresponding to amino acids 467 to 1178)(Adang *et al.*, 1985). With the exception of 6 amino acid differences, the Cry1Ab region is identical to the analogous region of the Cry1Ac protein encoded by the *cry1Ac* gene as described by Adang *et al.* (1985). The *cry1Ac* portion of the gene encodes a protein that is identical to the Cry1Ac protein found in nature (Adang *et al.*, 1985) with the exception of one amino acid at position 766 (contained in the C-terminal region that is clipped away from the active portion of the protein). The encoded protein produced in IR Tomato Line 5345 is greater than 99.4% identical to the naturally occurring Cry1Ac protein. The *cry1Ac* gene in IR Tomato Line 5345 is identical to that in Bollgard cotton lines 531 and 757. See Figure 2 for the amino acid sequence of the Cry1Ac protein expressed in IR Tomato Line 5345.

B. The *nptII* Gene

Following the *aad* gene in PV-LEBK04 is the gene for the enzyme neomycin phosphotransferase type II (NPTII). This enzyme confers resistance to the aminoglycoside antibiotics kanamycin and neomycin. The coding sequence for the *nptII* gene is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982) and is fused between the 35S promoter and the nopaline synthase 3' nontranslated sequences (NOS) for expression in plant cells (Rogers *et al.*, 1985). See Figure 3 for the amino acid sequence of the NPTII protein expressed in IR Tomato Line 5345.

C. The *aad* Gene

The *cry1Ac* gene is followed by the *aad* gene, isolated from transposon Tn7 (Fling *et al.*, 1985). The *aad* gene is under the control of its own bacterial promoter and is used as a selectable marker in laboratory steps prior to plant transformation. The *aad* gene encodes the enzyme aminoglycoside adenylyltransferase (AAD) which allows for the selection of bacteria containing the PV-LEBK04 plasmid on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter and its lack of detectable expression in the tomato plant was confirmed by western blot developed for the AAD protein (Appendix 6).

Figure 2. Amino acid sequence of the Cry1Ac protein expressed in IR Tomato Line 5345.

1	MDNNPNINEC	IPYNCLSNPE	VEVLGGERIE	TGYTPIDISL	SLTQFLLSEF
51	VPGAGFVLGL	VDIIWGIFGP	SQWDAFLVQI	EQLINQRIEE	FARNQAISRL
101	EGLSNLYQIY	AESFREWEAD	PTNPALREEM	RIQFNDMNSA	LTTAIPLFAV
151	QNYQVPLLSV	YVQAANLHLS	VLRDVSVFGQ	RWGFDAATIN	SRYNDLTRLI
201	GNYTDHAVRW	YNTGLERVWG	PDSRDWIRYN	QFRRELTLTV	LDIVSLFPNY
251	DSRTYPIRTV	SQLTREIYTN	PVLENFDGSF	RGSAQGIEGS	IRSPHLMDIL
301	NSITIYTDAH	RGEYYWSGHQ	IMASPVGFSG	PEFTFPLYGT	MGNAAPQQRI
351	VAQLGQGVYR	TLSSTLYRRP	FNIGINNQQL	SVLDGTEFAY	GTSSNLPSAV
401	YRKSGTVDSL	DEIPPQNNNV	PPRQGFSHRL	SHVSMFRSGF	SNSSVSIIRA

451	PMFSWIHRSA	EFNNIIASDS	ITQIPAVKGN	FLFNGSVISG	PGFTGGDLVR
501	LNSSGNNIQN	RGYIEVPIHF	PSTSTRYRVR	VRYASVTPIH	LNVNWGNSSI
551	FSNTVPATAT	SLDNLQSSDF	GYFESANAFT	SSLGNIVGVR	NFSGTAGVII
601	DRFEFIPVTA	TLEAEYNLER	AQKAVNALFT	STNQLGLKTN	VTDYHIDQVS
651	NLVTYLSDEF	CLDEKRELSE	KVKHAKRLSD	ERNLLQDSNF	KDINRQPERG
701	WGGSTGITIQ	GGDDVFKENY	VTLSGTFDEC	YPTYLYQKID	ESKLKAFTRY
751	QLRGYIEDSQ	DLEIYSIRYN	AKHETVNVPG	TGSLWPLSAQ	SPIGKCGEPN
801	RCAPHLEWNP	DLDCSCRDGE	KCAHHSHHFS	LDIDVGCTDL	NEDLGVWVIF
851	KIKTQDGHAR	LGNLEFLEEK	PLVGEALARV	KRAEKKWRDK	REKLEWETNI
901	VYKEAKESVD	ALFVNSQYDQ	LQADTNIAMI	HAADKRVHSI	REAYLPELSV
951	IPGVNAAIFE	ELEGRIFTAF	SLYDARNVIK	NGDFNNGLSC	WNVKGHVDVE
1001	EQNNQRSVLV	VPEWEAEVSQ	EVRVCPGRGY	ILRVTAYKEG	YGEGCVTIHE
1051	IENNTDELKF	SNCVEEEIYP	NNTVTCNDYT	VNQEEYGGAY	TSRNRGYNEA
1101	PSVPADYASV	YEEKSYTDGR	RENPCEFNRG	YRDYTPLPVG	YVTKELEYFP
1151	ETDKVWIEIG	ETEGTFIVDS	VELLLMEE		

Figure 3. Amino acid sequence of the NPTII protein expressed in IR Tomato Line 5345.

1	MIEQDGLHAG	SPAAWVERLF	GYDWAQQTIG	CSDAAVFRLS	AQGRPVLFVK
51	TDLSGALNEL	QDEAARLSWL	ATTGVPCAAV	LDVVTEAGRD	WLLLGEVPGQ
101	DLLSSHLAPA	EKVSIMADAM	RRLHTLDPAT	CPFDHQAKHR	IERARTRMEA
151	GLVDQDDLDE	EHQGLAPAEL	FARLKARMPD	GEDLVVTHGD	ACLPNIMVEN
201	GRFSGFIDCG	RLGVADRYQD	IALATRDIAE	ELGGEWADRF	LVLYGIAAPD
251	SQRIAFYRLL	DEFF			

V. GENETIC ANALYSIS AND AGRONOMIC PERFORMANCE

A. CHARACTERIZATION OF THE INSERTED GENETIC MATERIAL

As described in Part III-A, IR Tomato Line 5345 was generated by *Agrobacterium tumefaciens* mediated transformation with the plasmid PV-LEBK04. DNA analyses were performed to characterize the inserted T-DNA in terms of:

- insert number (number of integration events)
- copy number (number of T-DNA copies at a particular genetic locus)
- insert integrity (gene size, composition and linkage)

The characterization was performed by Southern blot analyses (Southern, 1975) on genomic DNA isolated from the young leaf tissue of the control and IR Tomato Line 5345 plants. Genomic DNA isolated from IR Tomato Line 5345 was digested separately with *Hind*III, *Ssp*I, *BamH*I and *Rca*I restriction enzymes, and probed with the whole plasmid (PV-LEBK04), the *cry1Ac*, *nptII*, *aad* and 7S 3' genetic elements.

1. Insert Number and Copy Number

Genomic DNA from IR Tomato Line 5345 was digested with *Hind*III and probed with the whole plasmid, PV-LEBK04. For a single copy and a single insertion event, the *Hind*III digest was predicted to yield two fragments each joined to the plant genomic DNA, referred to as border fragments. Three bands of approximate sizes 16.7, 10.8, and 5.9 Kb were observed (Figure 4A, lane 14). The 5.9 Kb fragment also hybridized to the *cry1Ac* and 7S3' probes (Figures 5 and 8, lane 14) identifying this as one of the border fragments. The 10.8 Kb fragment hybridized to the *aad* and *nptII* probes (Figure 6 and 7, lane 14), identifying this as the other border fragment. The 10.8 Kb fragment also hybridized to the *cry1Ac* and 7S3' probes (Figures 5 and 8, lane 14), showing that the T-DNA transfer included the entire plasmid and continued through the right border into the 3' region of the *cry1Ac* gene. The size of the faint band at approximately 16.7 Kb is equal to the sum of the sizes of the other two bands and is the result of incomplete digestion. The bands visible at >21 Kb (Figure 4A, lanes 12-14) are background bands resulting from non-specific hybridization and are visible for line UC82B, the plasmid control, and IR Tomato Line 5345. It is concluded that there is a single T-DNA insert in the genome of IR Tomato Line 5345. Results of *Hind*III digest of plant genomic DNA are summarized schematically in Figures 4C, 5B, 6B, 7B, and 8B.

2. Insert Composition and Structure

a. SspI Digest

There are two *Ssp*I sites within PV-LEBK04; one is near the right border and the second is approximately 7.4 Kb downstream of the first site (Figure 1). For a single insert of the entire plasmid into the plant genome, the *Ssp*I digest was predicted to yield three distinct DNA fragments. A 7.4 Kb fragment, a border fragment containing the bacterial origin of replication region, and a border fragment containing less than 100 bp of the plasmid DNA. Digestion of genomic DNA of line 5345 with *Ssp*I yielded three DNA fragments that hybridized to the entire plasmid PV-LEBK04 probe (Figure 4, lane 4). The approximate sizes of the fragments were 8.3, 4.0, and 1.3 Kb. The 8.3 Kb fragment also hybridized to the *cry1Ac*, *aad*, *nptII*, and 7S3' probes (Figure 5, 6, 7, and 8, respectively, lane 4) as expected for the predicted 7.4 Kb fragment. The larger size of the 8.3 Kb fragment indicates that the T-DNA transfer to the plant genome initiated after the *Ssp*I site at the right border, and the 8.3 Kb fragment is joined to the plant genomic DNA as a border fragment. The 4.0 Kb fragment hybridized very faintly to the 7S3' probe (Figure 8, lane 4) indicating that the *Ssp*I restriction site at bp 1832 of PV-LEBK04 is present in the read through section of the T-DNA and this fragment contains the bacterial origin of replication. The 1.3 Kb fragment hybridized to the *cry1Ac* and 7S3' probes (Figures 5 and 8, lane 4) indicating that it is a border fragment containing the 7S3' element and maximally 0.9 Kb of the 3' region of the *cry1Ac* gene. The bands visible at >21 Kb (Figure 4A, lanes 2-4) are background bands and are visible for line UC82B, the plasmid control, and IR Tomato Line 5345. Results of *Ssp*I digest of plant genomic DNA are summarized schematically in Figures 4C, 5B, 6B, 7B, and 8B.

b. BamHI Digest.

There are three *Bam*HI sites within plasmid PV-LEBK04 (Figure 1). For a single insert of the entire plasmid into the plant genome, the *Bam*HI digest was predicted to produce four distinct DNA fragments, consisting of two plasmid fragments and two border fragments. Digestion with *Bam*HI resulted in five bands (Figure 4A, lane 7). The 18.9 Kb fragment is a border fragment containing the 7S3' element and hybridized to the 7S3' probe (Figure 8, lane 7). The 4.3 Kb fragment corresponds to the *cry1Ac* gene and hybridized to the *cry1Ac* probe as expected (Figure 5, lane 7). The 1.2 Kb fragment contains the *aad* gene and hybridized to the *aad* probe (Figure 6, lane 7). The 6.0 Kb fragment corresponds to the fragment containing the *nptII* gene, the bacterial origin of replication, and the 7S3' element from the read through section of the T-DNA. This fragment hybridized to the *nptII* and 7S3' probes (Figures 7 and 8, lane 7). The 1.1 Kb fragment is a border fragment containing the 3' region of *cry1Ac* from the read through section, and hybridized to the *Cry1Ac* probe (Figure 5, lane 7). Results of *Bam*HI digest of plant genomic DNA are summarized schematically in Figures 4C, 5B, 6B, 7B, and 8B.

c. RcaI Digest

There are two *Rca*I sites within plasmid PV-LEBK04 (Figure 1). For a single insert of the entire plasmid into the plant genome, the *Rca*I digest was predicted to produce three distinct DNA fragments. Digestion with *Rca*I resulted in three bands as expected (Figure 4A, lane 11). The 7.1 Kb fragment is a border fragment containing the 7S3' element and the *cry1Ac* gene and hybridized to the *cry1Ac* and 7S3' probes (Figures 5 and 8, lane 11). The 5.7 Kb fragment corresponds to the fragment containing the *aad* and *nptII* genes and the bacterial origins of replication. This fragment hybridized to the *aad* and *nptII* probes (Figures 6 and 7, lane 11). The 2.4 Kb fragment is a border fragment and contains the 7S3' element and the 3' region of the *cry1Ac* gene from the read through section, and hybridized to the *cry1Ac* and 7S3' probes (Figures 5 and 8, lane 11). The bands visible at >21 Kb (Figure 4A, lanes 9-11) are background bands and are visible for line UC82B, the plasmid control, and line 5345. Results of *Rca*I digest of plant genomic DNA are summarized schematically in Figures 4C, 5B, 6B, 7B, and 8B.

3. Insert Stability

Insert stability was assessed by comparing the T-DNA of the original transformed line, IR Tomato Line 5345, to the T-DNA of the seventh generation, line IR-BC-W. Line IR-BC-W is the BC_7F_1 progeny of line 5345 backcrossed to non-transgenic parent line BHN-W. Line CONT-BC-W, the BC_7F_1 progeny of a negative segregant of line 5345, backcrossed to line BHN-W, does not contain the *cry1Ac* gene and was used as the negative control. Genomic DNA of each plant line was digested separately with either SspI or BamHI and probed with either the whole plasmid (Figures 8A and 8B) or with the *cry1Ac* gene (Figure 9). As shown in Figures 9A, 9B, and 10, the pattern of hybridization for line IR-BC-W is identical to that observed for the original line 5345. These findings verify the stable integration of the T-DNA in IR Tomato Line 5345 through seven generations.

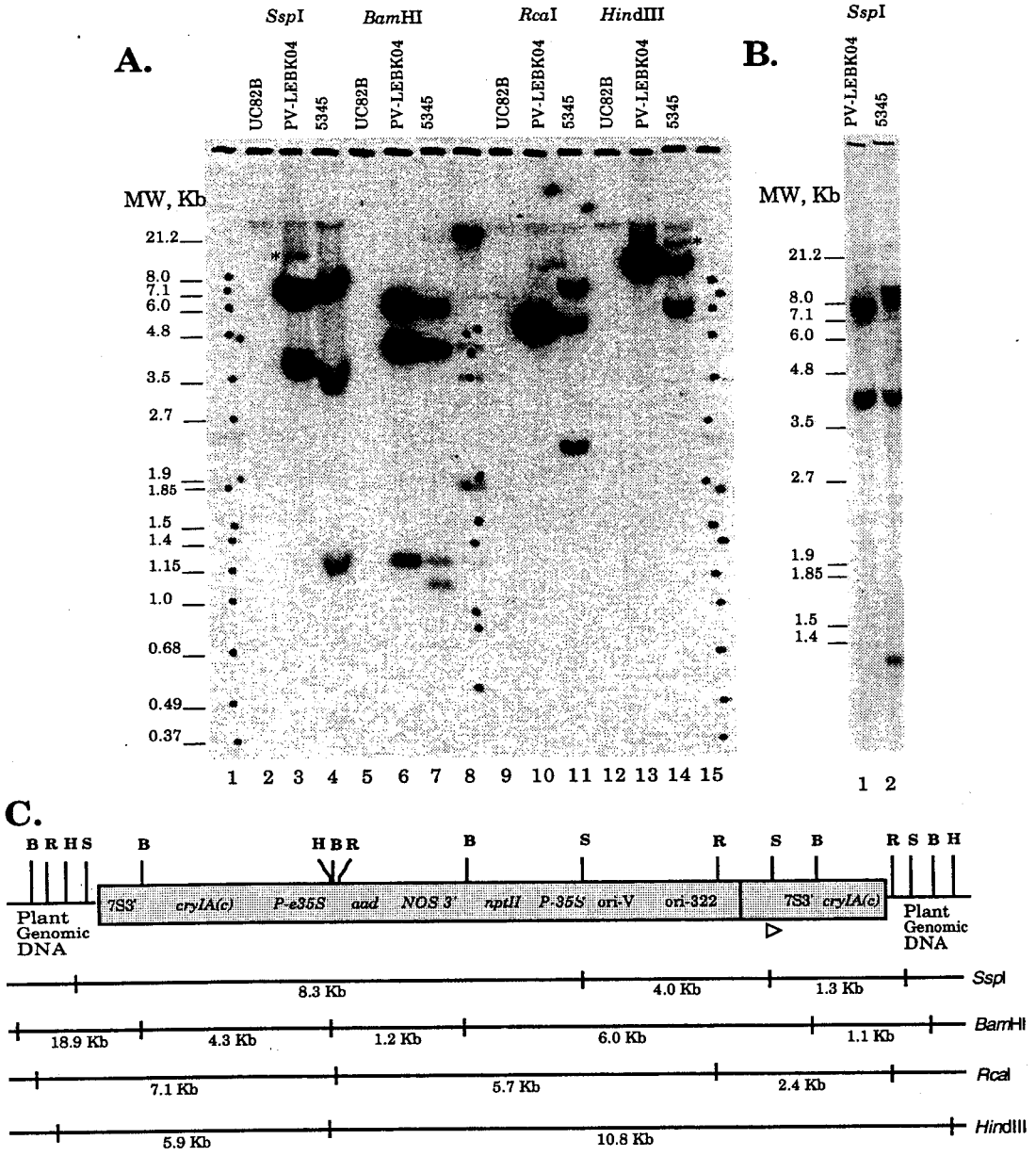


Figure 4: Southern blot analysis of genomic DNA from tomato line 5345 probed with the undigested plasmid, PV-LEBK04.

A: Southern blot of line 5345, parental control line UC82B, and plasmid vector PV-LEBK04. DNA was digested with *Ssp*I (S), *Bam*HI (B), *Rca*I (R), and *Hin*dIII (H), and probed with the undigested plasmid, PV-LEBK04. Lanes 1, 8 and 15 contain molecular weight markers. Asterisk (*) denotes a band due to incomplete digestion. **B:** Repeat of lanes 3 and 4 of the Southern blot in panel A, to confirm fragment sizes produced by the *Ssp*I digest. **C:** A schematic illustration of the Southern blot results indicating the orientation of the T-DNA in tomato line 5345 (not to scale). The grayed region within the box illustrates the location of the probe homology. The vertical lines denote the locations of the restriction sites within the T-DNA. The open triangle denotes the Right Border sequence of the read through section, for orientation purposes. All border fragment sizes are estimates.

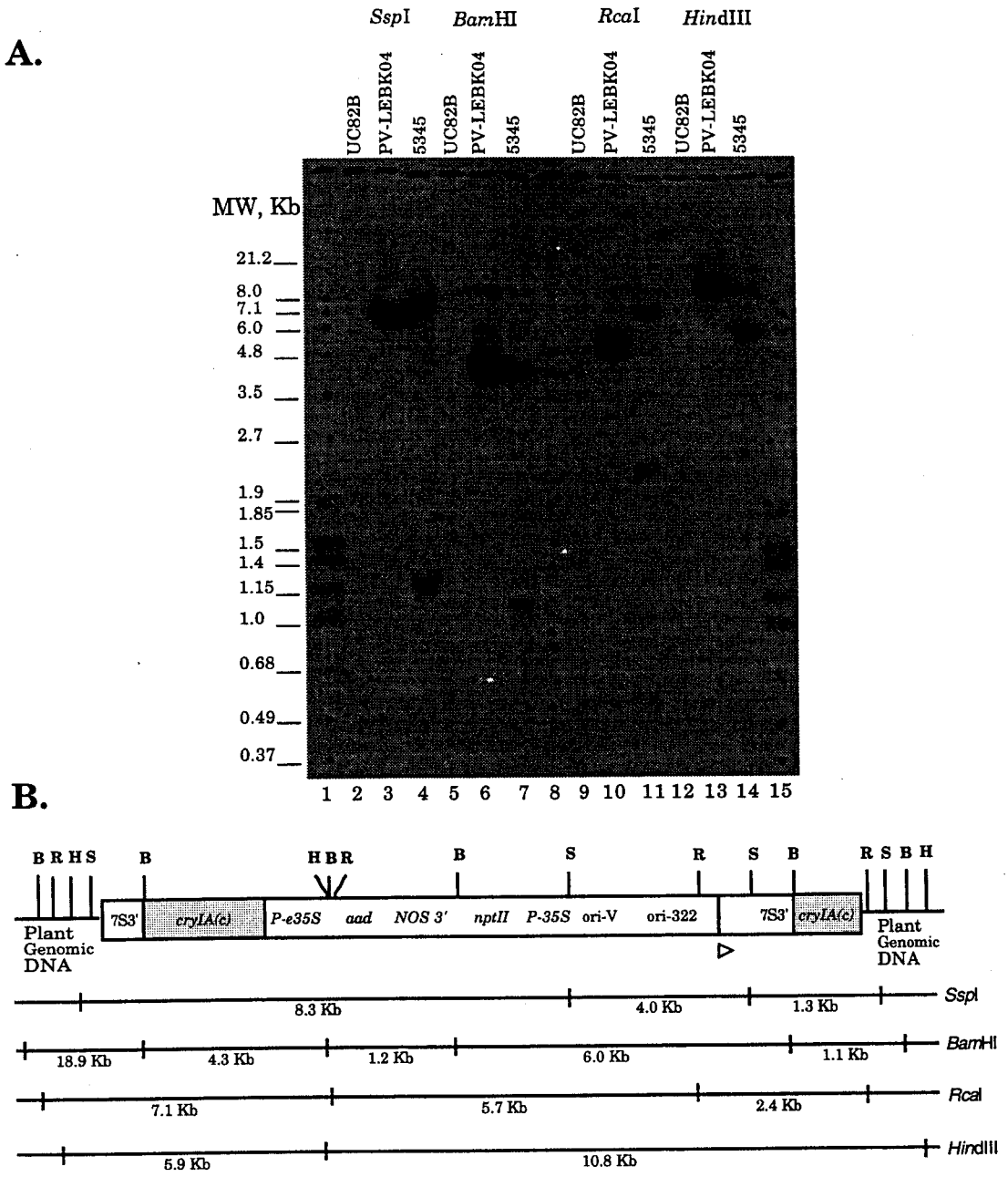


Figure 5: Southern blot analysis of genomic DNA from tomato line 5345 probed with the *cryIAc* gene.

A: Southern blot of line 5345, parental control line UC82B, and plasmid vector PV-LEBK04. DNA was digested with *Ssp*I (S), *Bam*HI (B), *Rca*I (R), and *Hin*dIII (H), and probed with the *cryIAc* gene. Lanes 1, 8 and 15 contain molecular weight markers.
B: A schematic illustration of the Southern blot results indicating the orientation of the T-DNA in tomato line 5345 (not to scale). The grayed region within the box illustrates the location of the probe homology. The vertical lines denote the locations of the restriction sites within the T-DNA. The open arrow denotes the Right Border sequence of the read through section, for orientation purposes. All border fragment sizes are estimates.

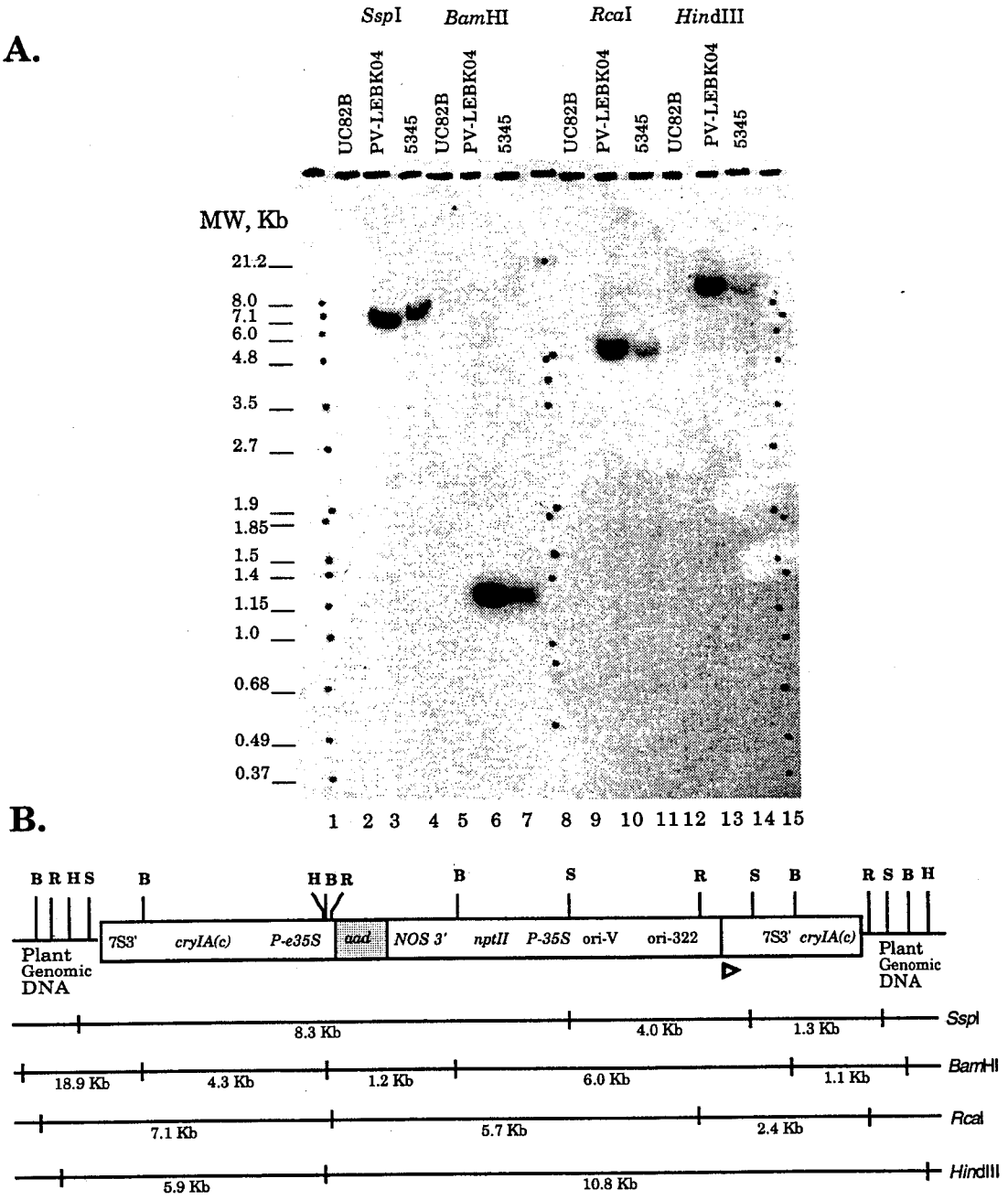


Figure 6: Southern blot analysis of genomic DNA from tomato line 5345 probed with the *aad* gene.

A: Southern blot of line 5345, parental control line UC82B, and plasmid vector PV-LEBK04. DNA was digested with *Ssp*I (S), *Bam*HI (B), *Rca*I (R), and *Hin*dIII (H), and probed with the *aad* gene. Lanes 1, 8 and 15 contain molecular weight markers.
B: A schematic illustration of the Southern blot results indicating the orientation of the T-DNA in tomato line 5345 (not to scale). The grayed region within the box illustrates the location of the probe homology. The vertical lines denote the locations of the restriction sites within the T-DNA. The open triangle denotes the Right Border sequence of the read through section, for orientation purposes. All border fragment sizes are estimates.

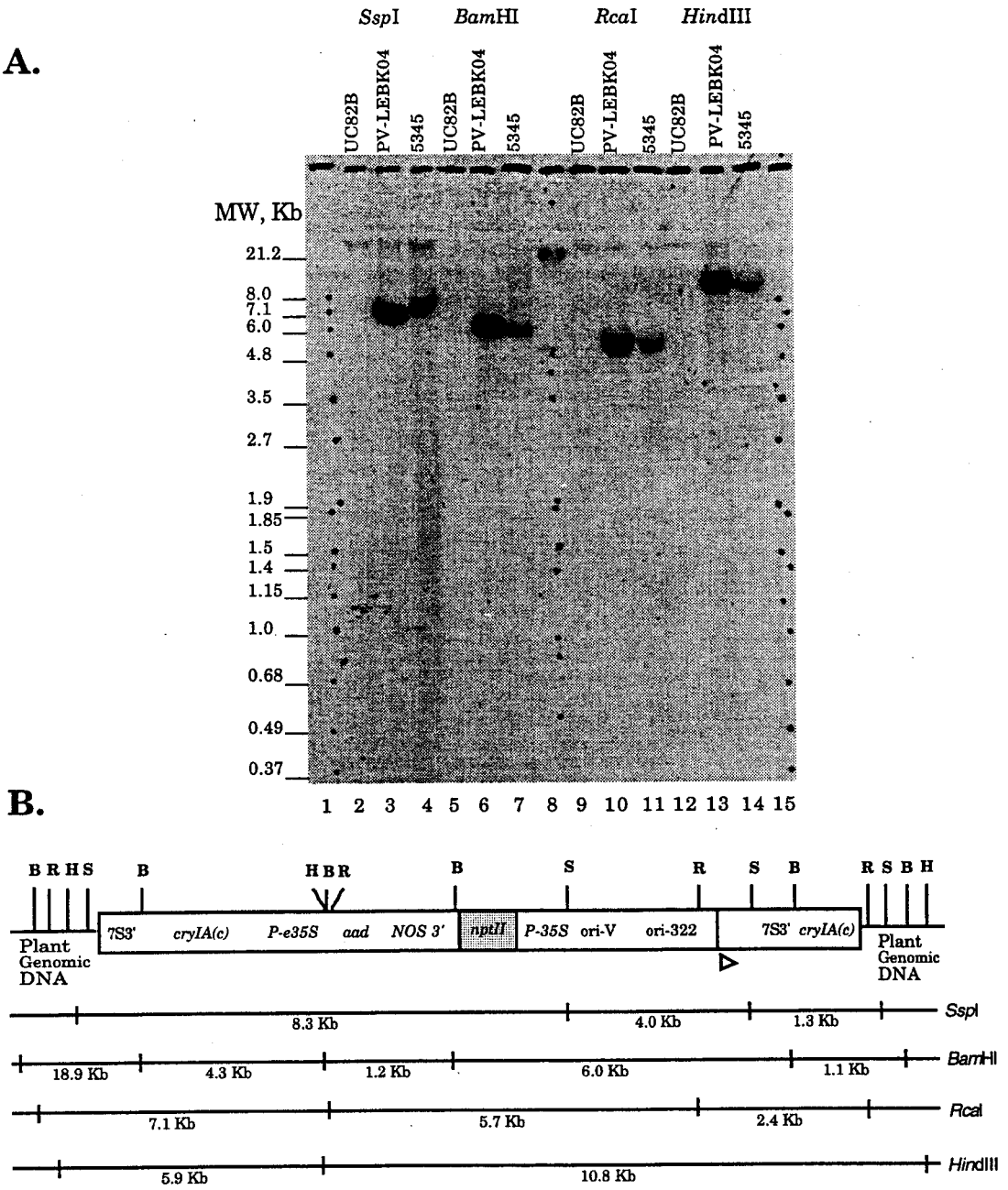


Figure 7: Southern blot analysis of genomic DNA from tomato line 5345 probed with the *nptII* gene.

A: Southern blot of line 5345, parental control line UC82B, and plasmid vector PV-LEBK04. DNA was digested with *Ssp*I (S), *Bam*HI (B), *Rca*I (R), and *Hin*dIII (H), and probed with the *nptII* gene. Lanes 1, 8 and 15 contain molecular weight markers.
B: A schematic illustration of the Southern blot results indicating the orientation of the T-DNA in tomato line 5345 (not to scale). The grayed region within the box illustrates the location of the probe homology. The vertical lines denote the locations of the restriction sites within the T-DNA. The open triangle denotes the Right Border sequence of the read through section, for orientation purposes. All border fragment sizes are estimates.

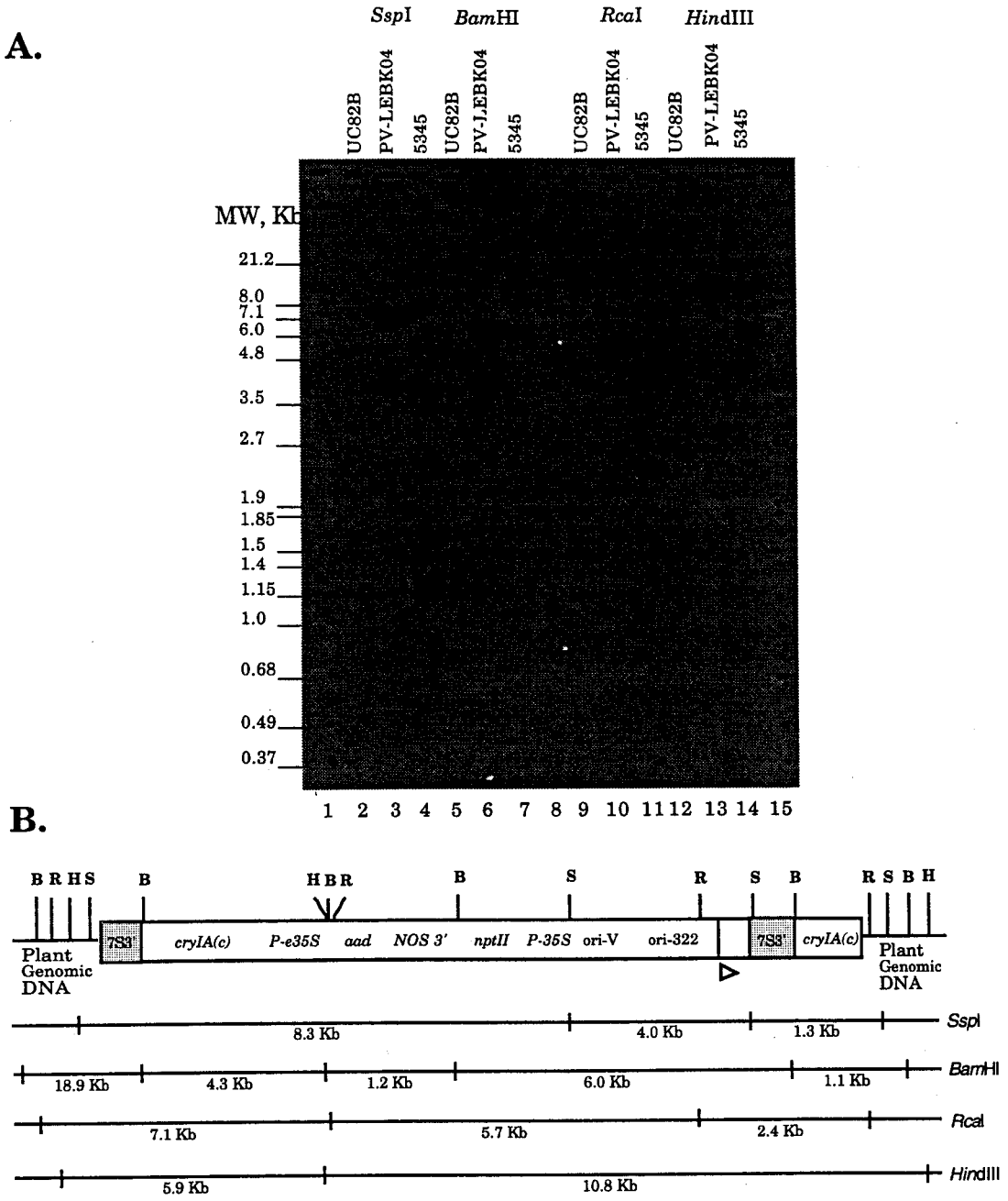
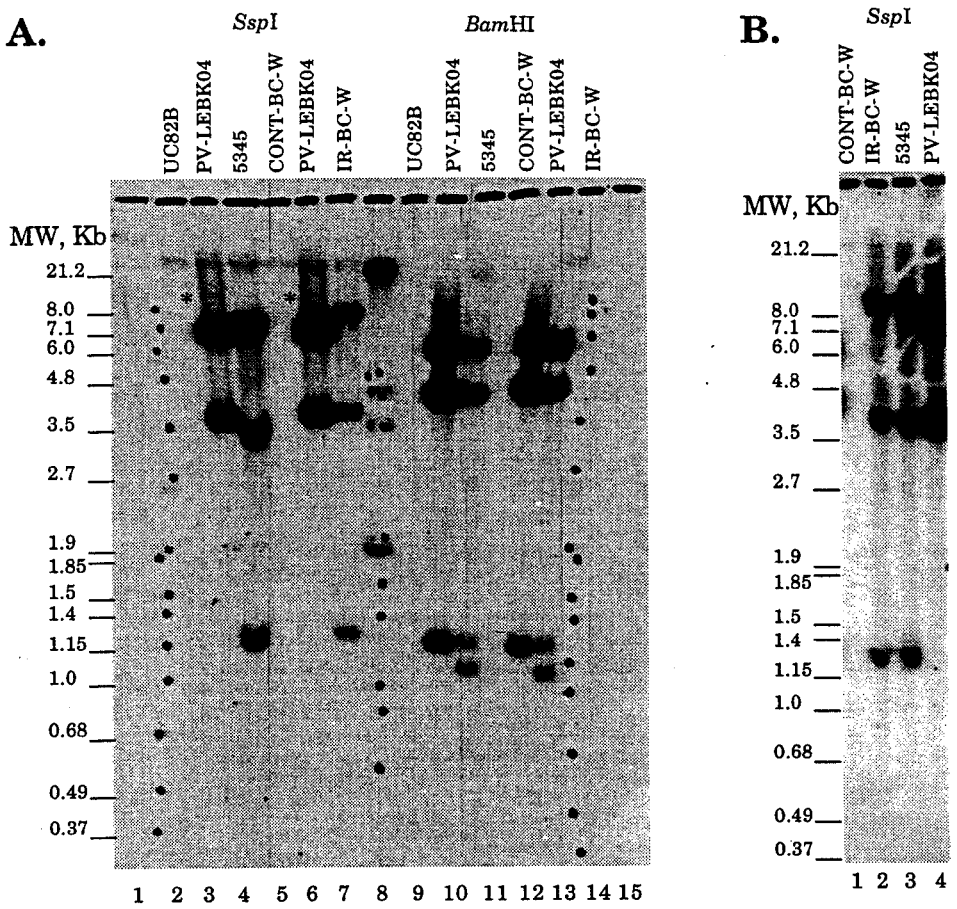


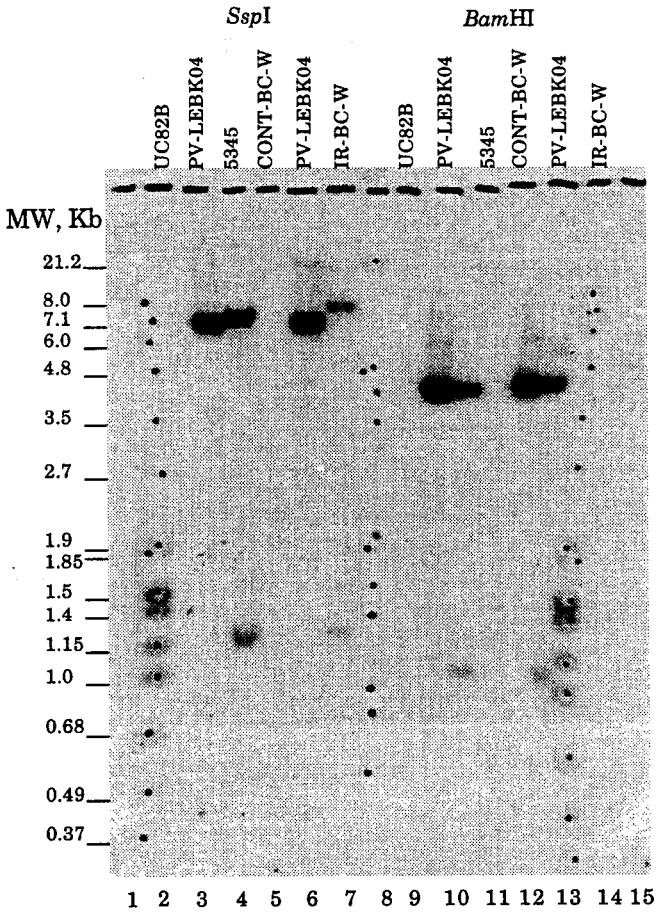
Figure 8: Southern blot analysis of genomic DNA from tomato line 5345 probed with the 7S3' element.

A: Southern blot of line 5345, parental control line UC82B, and plasmid vector PV-LEBK04. DNA was digested with *Ssp*I (S), *Bam*HI (B), *Rca*I (R), and *Hin*dIII (H), and probed with the 7S3' element. Lanes 1, 8 and 15 contain molecular weight markers.
B: A schematic illustration of the Southern blot results indicating the orientation of the T-DNA in tomato line 5345 (not to scale). The grayed region within the box illustrates the location of the probe homology. The vertical lines denote the locations of the restriction sites within the T-DNA. The open triangle denotes the Right Border sequence of the read through section, for orientation purposes. All border fragment sizes are estimates.



Figures 9: Southern blot analysis comparing tomato lines 5345 (R_1) and IR-BC-W (BC_7F_1) for insert stability. The blot was probed with the undigested plasmid, PV-LEBK04.

A: Southern blot of lines 5345 and IR-BC-W, control lines UC82B and CONT-BC-W, and plasmid vector PV-LEBK04. DNA was digested with *Ssp*I and *Bam*HI and probed with the undigested plasmid, PV-LEBK04. Lanes 1, 8 and 15 contain molecular weight markers. An edge effect of the blot caused the outer lanes to compress inward. Asterisk (*) denotes band due to incomplete digestion.
B: Repeat of lanes 5 through 7 of the Southern blot in panel A to confirm fragment sizes produced by the SspI digest.



Figures 10: Southern blot analysis comparing tomato lines 5345 (R_1) and IR-BC-W (BC_7F_1) for insert stability. The blot was probed with the *cryIAc* gene.

Southern blot of lines 5345 and IR-BC-W, control lines UC82B and CONT-BC-W, and plasmid vector PV-LEBK04. DNA was digested with SspI and BamHI and probed with the *cryIAc* gene. Lanes 1, 8 and 15 contain molecular weight markers. An edge effect of the blot caused the outer lanes to compress inward.

B. MENDELIAN INHERITANCE

Southern blot analyses showed a single DNA insert in the genome of IR Tomato line 5345 (Section V.A.1). These analyses also showed then the DNA insert is stably maintained during the life cycle of the plant, from the original transformed line 5345 to backcross progeny of this line.

Further evidence demonstrating a single active copy of the *cry1Ac* gene stably inserted in the genome of IR Tomato Line 5345 was obtained from segregation data for the gene inherited in progeny of IR Tomato Line 5345 backcrossed to nontransgenic tomato cultivars. Inheritance of the *cry1Ac* gene in progeny of the backcrosses was determined by detection of the Cry1Ac protein in plant leaf tissue, either by insect bioassay or by an enzyme linked immunosorbent assay (ELISA). Homozygous IR Tomato Line 5345 was crossed to a nontransgenic tomato cultivar and the heterozygous progeny further backcrossed to seven different nontransgenic commercial cultivars. Progenies of these backcrosses out to BC_7F_1 material yielded the expected segregation ratio of approximately 1:1 with respect to Cry1Ac protein expression (Table 3).

Virtually 90% of the cases showed the expected ratio of 1:1 (Table 3). These data establish that the DNA insert in IR Tomato Line 5345 remains stably integrated in the plant genome over successive backcross generations, and behaves as a single dominant gene inherited in progeny in a Mendelian fashion. The data are consistent with there being a single active copy of the *cry1Ac* and marker genes inserted into the genome of IR Tomato Line 5345.

Table 3. Cry1Ac Segregation data for Backcross progeny of IR Tomato Line 5345 with Different Nontransgenic Tomato Varieties.

Generation	Inbred Parental Line	Number Expressing	Number Negatives	$(X^2)^1$
BC4F1	A	9	7	0.3
BC5F1	A	7	9	0.3
BC6F1	A	6	10	1.0
BC7F1	A	9	9	0.0
BC4F1	B	8	8	0.0
BC5F1	B	6	10	1.0
BC6F1	B	9	7	0.3
BC7F1	B	11	6	1.5
BC4F1	C	4	12	4.0*
BC5F1	C	10	6	1.0
BC6F1	C	11	5	2.3
BC7F1	C	10	8	0.2
BC4F1	D	7	9	0.3
BC5F1	D	7	9	0.3
BC6F1	D	6	10	1.0
BC7F1	D	9	8	0.1
BC4F1	E	5	11	2.3
BC5F1	E	7	9	0.3
BC6F1	E	5	11	2.3
BC7F1	E	3	15	8.0*
BC4F1	F	3	13	6.3*
BC5F1	F	10	6	1.0
BC6F1	F	10	6	1.0
BC7F1	F	12	6	2.0
BC4F1	G	9	7	0.3
BC5F1	G	7	9	0.3
BC6F1	G	10	6	1.0
BC7F1	G	12	6	2.0
			TOTAL[2]	39.8

* Statistically significant chi-square values at a significance level of 95% ($X^2_{0.05,1d.f.}$=3.8)

1 Uncorrected goodness-of-fit test for hypothesis of 1:1 segregation.

2 Total Chi-square value not significant at significance level of 95% ($X^2_{0.05,1d.f.}$=42.6)

C. EXPRESSION OF THE INSERTED GENES

The levels of Cry1Ac, NPTII and AAD proteins expressed in IR Tomato Line 5345 were determined from plant samples collected from three different field sites in 1995. The field site locations were: Bonita Springs, FL; Collier County, FL and Huron, CA. These sites provided a variety of environmental conditions and insect pressure from agronomically-important pests. IR Tomato Line 5345 and control lines were successfully grown and harvested under conditions typical for each region.

Expression levels of the Cry1Ac and NPTII proteins in tomato plant tissues were measured by a validated enzyme linked immunosorbent assay (ELISA). Expression levels of the AAD protein were determined by Western blot using Enhanced Chemiluminescence detection. A description of the methods employed and the descriptive features of the ELISAs developed to measure the Cry1Ac, NPTII and AAD protein levels in the various tomato tissues are summarized in Appendix 1 and validation information is in Appendix 5. Expression levels of each protein were measured in newly expanded leaf tissue and red ripe fruit tissue. Additionally, Cry1Ac protein

expression was determined in whole plant tissues harvested at the end of the season and leaf and fruit tissues collected over time.

The levels of Cry1Ac and NPTII proteins expressed by IR Tomato Line 5345 comprise an extremely small percentage of the total fresh weight (fwt) of leaf and fruit tissue from each of the field sites (Tables 4 -8). As expected, the AAD protein was not detected in IR Tomato Line 5345 in leaf or fruit tissue (Appendix 6).

The Cry1Ac and NPTII proteins were detected in leaf tissue collected from two of the four replicate control plots at the Bonita Springs, FL field site and also in one replicate plot at the Collier Co., FL site. This indicates that some IR Tomato Line 5345 plants were present in control plot replicates at these field sites. These replicates were therefore excluded from the calculation of mean protein expression values for control lines at these sites. As expected, the Cry1Ac and NPTII proteins were not detected in control leaves collected from the other replicate plots at these sites, or from any of the control plots at the other field site.

1. **Cry1Ac Expression**

Expression levels of the Cry1Ac protein were measured in newly expanded leaf tissue, in red ripe fruit tissue, and in whole plant tissues harvested at the end of the season using validated ELISA. The Cry1Ac protein is a minor component of IR tomato fruit, accounting for 0.007% of total fruit protein. Expression was highest in the young leaf tissue (13.32 µg/g fwt, mean across field sites) and was extremely low in the red ripe tomato fruit (0.70 µg/g fwt, mean across field sites).

Leaves. Young leaf tissue from 6 plants in each plot was collected for protein expression analysis approximately 3 weeks after transplanting seedlings to the field. The mean expression (across three field sites) of Cry1Ac protein in newly expanded leaf tissue of IR Tomato Line 5345 was 13.32 µg/g fresh weight (Table 4). The expression of Cry1Ac in leaf tissue was consistent across all plots and field sites, with a range of 11.76-16.63 µg/g fresh weight.

Newly-expanded leaves harvested throughout the growing season at the Bonita Springs, FL, field site were also analyzed for expression of the Cry1Ac protein. The levels of the Cry1Ac protein increased slightly throughout the growing season (Table 5). Expression levels of the Cry1Ac protein ranged from 12.95 to 15.21 µg/g fresh weight (Table 5).

Fruit. Expression of Cry1Ac protein was measured in red ripe tomato fruit collected from three field sites, and also measured in fruit at different ripening stages (mature green, pink, and red ripe) collected from field site at Bonita Springs, FL. Approximately 10 fruits were selected from each of the four plots at each site for protein expression determination. Expression of the Cry1Ac protein varied by a factor of 2 across the three field sites. The mean expression (across three field sites) of Cry1Ac protein in IR Tomato Line 5345 red ripe tomato fruit was 0.70 µg/g fresh weight (Table 6). The range of Cry1Ac expression in IR Tomato Line 5345 red ripe fruit across all plots and field sites was 0.39-1.26 µg/g fresh weight. Since tomato fruit contains approximately 1% protein (Davies and Hobson, 1981), the Cry1Ac protein is a minor component of the IR Tomato Line 5345 fruit, accounting for 0.007% of total fruit protein.

The Cry1Ac expression levels were also measured in IR Tomato Line 5345 fruits at different fruit maturity stages: mature green fruit, pink fruit and red ripe fruit. Expression levels of the Cry1Ac protein in fruit declined as the fruit ripened. Expression levels of the protein in mature green, pink and red ripe fruit from the Bonita Springs, FL site were 1.47, 1.35, and 1.00 µg/g fresh weight respectively (Table 7).

Whole Plants. The Cry1Ac protein was also measured in a single whole, mature IR Tomato Line 5345 plant collected from each plot at the field sites in CA, and Bonita Springs, FL. The whole plants included the above-ground vegetation and most of the root, with all of the fruit removed prior to sampling. This was determined to estimate the total protein that would enter the environment after harvest if the plant material were soil incorporated. Average expression levels (across replicate plots at each field site) of Cry1Ac protein in whole plant tissues were extremely low at both sites (Table 8). Cry1Ac protein levels of 3.28 µg/g fresh weight were detected at the field site in CA, and 1.62 µg/g fresh weight at the Florida field site. The range of Cry1Ac protein expression in IR Tomato Line 5345 whole plant tissue across all plots and both field sites was 1.01-4.82 µg/g fresh weight.

2. **NPTII Expression**

A monoclonal antibody based NPTII ELISA was used for measurement of NPTII expression levels in leaf and fruit tissues of IR tomatoes. The precision, accuracy, and sensitivity of a polyclonal antibody based NPTII ELISA was established in a previous study. The two ELISAs were shown to be equivalent for measurement of the NPTII protein. This led to the conclusion that the monoclonal antibody based ELISA is a valid assay for measurement of NPTII protein levels in leaves and red ripe fruit of IR Tomato Line 5345. The mean expression of the NPTII protein in new, fully expanded leaves collected from IR tomatoes grown at three field locations was 4.69 µg/g fresh weight. The mean expression of the NPTII protein in red ripe fruit collected from IR tomatoes grown at three field locations was 0.36 µg/g fresh weight. The NPTII protein is a minor component of IR Tomato Line 5345 fruit, accounting for 0.004% of total fruit protein.

Leaves. The NPTII expression levels in leaves of IR Tomato Line 5345 are shown in Table 4. A single healthy, fully-expanded, terminal leaflet of the youngest fully-expanded leaf was harvested from six plants per plot and pooled by plot. Protein expression was determined by ELISA, and results are presented as a mean of expression values across four plots at each of the three field sites. The mean expression of NPTII in leaves of IR Tomato Line 5345 across three field sites was 4.69 µg/g fresh weight. The range of expression of NPTII in IR Tomato Line 5345 leaves across all plots and field sites was 1.67-8.09 µg/g fresh weight.

Fruit. The NPTII expression levels in red ripe fruit from IR Tomato Line 5345 are shown in Table 6. Approximately 40-60 tomato fruit at the mature green or breaker stage (<10% color) of ripening were harvested from each plot and pooled by plot. Fruits were ripened with either 200 ppm ethylene or in a controlled environment chamber with no exogenous ethylene. Protein expression results are presented as a mean of expression values across plots at each of the three field sites. The mean expression of NPTII in fruit of IR tomato across three field sites was 0.36 µg/g fresh weight. The range of expression of NPTII in IR Tomato Line 5345 red ripe fruit across all plots and field sites was 0.0091-0.85 µg/g fresh weight.

Expression of the NPTII protein in IR Tomato Line 5345 fruit collected from the Collier Co., FL field site was consistently low across all replicate plots, compared to NPTII expression values for fruit collected from the other two field sites. This difference in fruit NPTII expression levels between field sites may reflect differences in the maturity of the fruit collected from the field sites. Fruit collected from the Huron, CA and Bonita Springs, FL field sites were at a less mature stage of ripening and were subsequently treated with ethylene to accelerate ripening, whereas fruit from the Collier Co., FL field site were ripened without use of exogenous ethylene.

3. **AAD Expression**

Leaf and fruit tissues of field grown IR Tomato Line 5345 and control tomatoes were analyzed for the presence of the AAD protein by Western blot (Appendix 6), using Enhanced Chemiluminescence detection. The accuracy and limit of detection of the Western blot assay in control plant extracts was assessed prior to sample analysis. The AAD protein produced and purified from *Escherichia coli* was spiked into control samples prior to extraction. Recovery of the AAD protein was approximately 100% from both leaf and fruit tissue samples. Serial dilutions of the purified AAD protein spiked into control plant tissues showed that the limit of detection of the protein by Western blot was approximately 20 ng/g tissue fresh weight. The IR Tomato Line 5345 and control tomato leaf and fruit tissues were extracted in SDS-PAGE sample buffer, and analyzed for the AAD protein. The AAD protein was not detected in either IR Tomato Line 5345 or control plant tissues by Western blot analysis. The results confirm that the AAD protein is not detected in IR Tomato Line 5345 tomato plants. The *aad* gene, which is driven by a bacterial promoter, allows for selection of bacteria in media containing spectinomycin or streptomycin, in steps prior to plant transformation. The *aad* gene, lacking a plant promoter, is not expected to express in IR Tomato Line 5345 tomato plants.

Table 4. Cry1Ac and NPTII Protein Expression Levels in Young Leaf Tissue from IR Tomato Line 5345 at Multiple Field Sites in 1995 [a].

Field Site	DAS [d]	Cry1Ac		NPTII	
		Mean µg/g fwt [b]	Range [c]	Mean µg/g fwt [b]	Range [c]
Huron, CA	83	13.86	12.62-16.63	3.04	1.67-4.07
Collier Co., FL	66	13.14	11.76-15.44	4.28	3.93-4.63
Bonita Springs, FL	88	12.95	12.43-13.47	6.76	5.91-8.09
Overall Mean		13.32	11.76-16.63	4.69	1.67-8.09

a For each protein, extracts of a pooled 6-leaf sample from each of the four plots at each field site were analyzed by ELISA
b Means are the averages across four plots.
c Range denotes the lowest and highest individual assay results across plots.
d DAS = Days After Sowing

Table 5. Cry1Ac Protein Expression Levels in Young Leaf Tissue Collected Over Time from IR Tomato Line 5345 at Two Field Sites in 1995 [a].

Collection Date	DAS [d]	Cry1Ac	
		Mean µg/g fwt [b]	Range [c] µg/g fwt
11/14/95	88	12.95	12.43-13.47
12/18/95	122	14.81	11.43-17.19
1/24/96	159	15.21	13.47-16.75
Mean Across Sites		14.32	11.43-17.19

a Extracts of leaf samples from each harvest date were analyzed by ELISA
b Means are the averages for each line across four plots.
c Range denotes the lowest and highest individual assay results across plots.
d DAS = Days After Sowing

Table 6. Cry1Ac and NPTII Protein Expression Levels in Tomato Fruit from IR Tomato Line 5345 at Multiple Field Sites in 1995 [a].

Field Site	DAS [d]	Cry1Ac		NPTII	
		Mean µg/g fwt [b]	Range [c] µg/g fwt	Mean µg/g fwt [b]	Range [c] µg/g fwt
Huron,CA	144	0.47	0.39-0.58	0.54	0.50-0.61
Collier Co., FL	130	0.64	0.40-0.85	0.03	0.0091-0.042
Bonita Springs, FL	171	1.00	0.54-1.26	0.50	0.27-0.85
Overall Mean		0.70	0.39-1.26	0.36	0.0091-0.85

a Extracts of a pooled 10-fruit sample from each of the four plots at each field site were analyzed by ELISA
b Means are the averages across four plots.
c Range denotes the lowest and highest individual assay results across plots.
d DAS = Days After Sowing

Table 7. Cry1Ac Protein Expression Levels in Fruit Tissue from IR Tomato Line 5345 Collected at Different Ripening Stages at Two Field Sites in 1995 [a].

Ripening Stage	Cry1Ac	
	Mean µg/g fwt [b]	Range [c] µg/g fwt
Mature Green	1.47	1.25-1.72
Pink	1.35	1.20-1.66
Red Ripe	1.00	0.54-1.26

a Extracts of leaf samples from each harvest date were analyzed by ELISA
b Means are the averages for each line across four plots.
c Range denotes the lowest and highest individual assay results across plots.

Table 8. Cry1Ac Protein Expression Levels in Whole Plant Tissue from IR Tomato Line 5345 at Two Field Sites in 1995 [a].

Field Site	DAS [d]	Cry1Ac	
		Mean µg/g fwt [b]	Range [c] µg/g fwt
Huron, CA	153	3.28	2.16-4.82
Bonita Springs, FL	171	1.62	1.01-2.49
Overall Mean		2.45	1.01-4.82

a Extracts of leaf samples from each harvest date were analyzed by ELISA
b Means are the averages for each line across four plots.
c Range denotes the lowest and highest individual assay results across plots.
d DAS = Days After Sowing

D. DISEASE AND PEST RESISTANCE CHARACTERISTICS

IR Tomato Line 5345 transformed with the plasmid vector, PV-LEBK04, was tested in replicated trials in the United States in 1995, 1996 and 1997 at more than 10 different field locations, as well as Puerto Rico, under notifications acknowledged by the USDA (#94-362-01N, 95-138-04N, 95-151-04N, 95-216-03N, 96-011-01N, 96-247-14N, 97-013-01N). Detailed monitoring for growth and development characteristics, disease and insect susceptibility of these lines versus nontransgenic control plants was performed approximately once every two weeks during the growing season at the sites listed in Table 1. No differences in disease or insect infestation or severity other than Lepidopteran insect control were detected between IR Tomato Line 5345 and control plants. The USDA final reports for the trials conducted in 1995 and 1996 have been submitted to the Agency (Appendix 2), however final reports for 1997 field trials will not be submitted until the required year of observation following planting. The observations were obtained by tomato breeders and/or agronomists, as well as University of Florida researchers, who compared the general vigor and disease and insect susceptibility of control tomato plants and IR Tomato Line 5345 plants. These observations are typical of those taken by crop consultants, agronomists, entomologists and breeders in detecting the presence and magnitude of a disease or insect infestation and assessing varietal performance.

Plots of the IR Tomato Line 5345 and control plants were visually checked for the appearance of possible disease symptoms such as spotted leaves, leaf necrosis, stunted or distorted plants, and wilting of the plants, which are indicative of, but not limited to: *Xanthomonas campestris* (bacterial spot), *Corynespora cassiicola* (target spot), *Alternaria alternata* (early blight), *Phytophthora infestans* (late blight), *tomato mosaic virus, tomato mottle virus, tomato spotted wilt virus, and Fusarium oxysporum* (Fusarium wilt and Fusarium crown rot). Plants were also checked for insect populations, including tomato pinworm (*Keiferia lycoperisicella*), tomato fruitworm (*Helicoverpa zea*), fall armyworm (*Spodoptera fugiperda*), beet armyworm (*Spodoptera exigua*), and cabbage looper (*Trichoplusia ni*).

Based on the results of the field monitoring program, there were no significant differences between IR Tomato Line 5345 and control tomato plants, except for the intended difference of resistance to lepidopteran insect pests. No differences in growth, developmental characteristics, disease or insect infestation or severity were detected between IR Tomato Line 5345 and control tomato lines at any of the three field sites. This conclusion was confirmed by two horticulturists, expert in tomato production who inspected the IR Tomato Line 5345 and control plants at the Bonita Springs, FL field site. The letters from the expert tomato horticulturists are located in Appendix 3.

E. COMPOSITIONAL ANALYSES OF IR TOMATO LINE 5345

Monsanto Co. is in consultation with the FDA following their policy, "Foods Derived from New Plant Varieties" on the food safety of IR Tomato line 5345. Studies were carried out to compare the nutritional constituents of tomato fruit from IR Tomato line 5345 with tomato fruit obtained from control plants grown, processed and analyzed under the same conditions. The study demonstrated that tomato fruit produced by IR Tomato line 5345 was substantially equivalent to that produced by nontransformed tomato plants in the production of total solids, protein, fat, ash, carbohydrates, calories, vitamins A and C and folic acid, as well as the pH level of the fruit.

F. TOXICANTS

In addition to analyses for nutrients and fruit pH, tomatine, a naturally occurring endogenous plant toxicant, was measured in mature green and red ripe fruit of the IR Tomato Line 5345 and control tomato plants from each of the three U.S. field sites in 1995. Analytical methods are listed in Appendix 4. The levels of tomatine in fruit of the two lines were averaged across field sites by line and fruit ripening stage. The results are presented in Table 9. Tomatine levels in fruit of both lines declined as fruit ripened from mature green to red ripe. This was expected since it is known that tomatine is degraded during fruit ripening (Roddick, 1974; Davies and Hobson, 1981). The tomatine content of mature green fruit was slightly higher in IR Tomato Line 5345 than in the control line. Although this difference was statistically significant, the level of tomatine in mature green fruit of the IR Tomato Line 5345 was well within and at the low end of the range of tomatine levels reported in the literature for tomatoes (Table 9). This minor compositional difference most likely represents the inherent variability of tomatine levels between tomato plants, and is not attributed to the insertion of the gene for insect

resistance into the tomato genome. For example, Bushway *et al.* (1994) report that tomatine in mature green fruit can range from none detected to 64.9 µg/g fruit fresh weight among fruit of a single tomato variety. Tomatine levels in red ripe fruit, the most commonly consumed fruit type, was very low in both IR Tomato Line 5345 and control lines. There was no statistical difference in content between the lines.

Table 9. Tomatine content of mature green and red ripe fruit from IR Tomato Line 5345 and control tomatoes. Values reported are the means of analysis of fruit collected from three separate field sites

	Tomatine µg/g fresh fruit wt. (range)[b]		
Fruit ripening stage	**Control**	**IR Tomato Line 5345**	**Literature range[c]**
Mature green	37.9 (20.3-47.4)	51.0 (31.8-63.1)[a]	49.0-900.0
Red ripe	1.4 (N.D.-4.3)	1.4 (N.D.-4.3)	N.D.-360.0

[a] Significantly different from the control line at the 5% level (paired t-test).

[b] Range denotes the lowest and highest individual values across sites for each line.

[c] Literature ranges of tomatine in mature green fruit are from Bajaj *et al.* (1987); Davies and Hobson (1981). Literature ranges of tomatine in red ripe fruit are from Takagi *et al.* (1994); Roddick (1974).

N.D.-Not detected

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

A. Cry1Ac PROTEIN

Bacillus thuringiensis are crystalliferous spore-forming gram-positive bacterium that have been used commercially over the last 30 years to control insect pests. These microbes are found naturally in soil worldwide. Numerous different strains have been identified, characterized and used commercially. The protein produced in IR Tomato Line 5345 is more than 99% identical to the protein produced by the *B.t.k.* HD-73 bacterial strain. This strain controls insects pests by the production of crystalline insecticidal proteins known as sporulation phase and can account for approximately one-third of the weight of the bacterial cell. 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 (Wolfersberger *et al.* 1986; Hofmann *et al.*, 1988a and 1988b; Van Rie, *et al.*, 1989; Van Rie, *et al.*, 1990). These events disrupt the digestive processes and cause the death of the insect. Thus, the insecticidal effect is highly specific to Lepidopteran insect pests (MacIntosh *et al.*, 1990; Klausner, 1984; Aronson *et al.*, 1986; Dulmage, 1981; Whitely and Schnepf, 1986).

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, 1983; Siegel and Shadduck, 1989). Results of unpublished safety studies generated by registrants of *B. thuringiensis* commercial preparations have also been summarized in the EPA Registration Standard for *B.t.* Formulations (EPA, 1988). In addition to the lack of receptors for the *B.t.k.* protein, the absence of adverse effects in non-target animals is further supported by the poor solubility and stability of the *B.t.k.* proteins in the acid milieu of the stomach. The acid conditions in the stomach and the presence of bile acids denature the *B.t.k.* protein, facilitating their rapid degradation by pepsin and intestinal proteases.

The safety of *B.t.k.* HD-73, which is greater than 99% equivalent to Cry1Ac, to non-target insects has also been extensively studied (Flexner *et al.*, 1986; Krieg and Langenbruch, 1981; EPA 1988; Vinson, 1989; Melin and Cozzi, 1989). Potential toxicity was assessed in feeding studies to honey bee larvae and adults (*Apis mellifera* L.), parasitic Hymenoptera (*Nasonia vitripennis*), ladybugs (*Hippodamia convergens*) and green lacewing larvae

(*Chrysopa carnea*) (Appendix 7) and two Collembola species, *Folsomia candida* and *Xenylla grisea*. The results clearly demonstrate the overall safety of the Cry1Ac protein to beneficial insects (Table 10).

The Cry1Ac protein from *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*) has been determined by the EPA and other regulatory agencies worldwide through the review of extensive safety and health testing to pose no significant risks to human health or non-target organisms (EPA, 1988). Since the Cry1Ac protein produced by IR Tomato Line 5345 is more than 99% identical to that produced by *Bacillus thuringiensis* subsp. *kurstaki*, the data also support the safety of the protein as produced by IR Tomato Line 5345.

Table 10. Cry1Ac Toxicity in Beneficial Insect Species

Species	LC_{50}	NOEL
Apis mellifera	>20 ppm	20 ppm
Nasnia vitripennis	>20 ppm	20 ppm
Hippodamia convergens	>20ppm	20 ppm
Chrysopa carnea	>20 ppm	20 ppm
Folsomia candida	-	200 ppm
Xenylla grisea	-	200 ppm

B. NPTII PROTEIN

The NPTII protein which has no insecticidal effect, is ubiquitous in the environment and is found in microbes present on food and within the human digestive system (Flavell *et al.*, 1992; Calgene, Inc., 1993). This protein has also been used as a selectable marker for animal and human cell transformation and for human gene therapy experiments (Culver *et al.*, 1991; Brenner *et al.*, 1993). The safety of NPTII and other selectable markers were addressed in reviews by Fuchs *et al.* (1993a and 1993b), Flavell *et al.* (1992) and Nap *et al.* (1992). The EPA approved a tolerance exemption for the NPTII protein on September 28, 1994 (EPA, 1994) and an exemption from tolerance in all crops in 1997. The U.S. Food and Drug Administration (FDA) also approved the use of this protein as a processing aid food additive in several tomato, cotton and oilseed rape in 1994 (FDA, 1994). All data support the safety of the NPTII protein for use as a selectable marker in crops grown for human and animal consumption. This conclusion was also supported by a document published by the World Health Organization (WHO, 1993).

C. CURRENT AGRONOMIC PRACTICES AND THE IMPACT OF IR TOMATO LINE 5345 ON PEST MANAGEMENT

Tomato varieties can generally be divided into two categories: fresh market tomatoes and processing tomatoes. Fresh market tomatoes are harvested from the field or greenhouse, then packed and shipped to supermarkets where they are consumed as a fresh vegetable. Processing tomatoes are harvested from the field (usually by machines) and shipped directly to a cannery where they are sorted, peeled and directed to one or more canned tomato products (*e.g.* tomato juice, paste, catsup, sauce, salsa, diced or whole peeled tomatoes).

1. Processing tomatoes. In the past 30 years, California has become the predominant location for production of processing tomatoes in the United States. Warm sunny summer weather, fertile soils and low humidity contribute to high yields and good tomato quality. Level fields and typical lack of substantial summer rain also favor mechanical harvesting of tomatoes which in turn reduces labor costs. The leading counties in California for production of processing tomatoes are Fresno, Yolo and San Joaquin with a combined production area in excess of

more than 50,000 hectares. The total production of processing tomatoes in California typically exceeds 5 million tons and accounts for nearly 90% of the total U.S. processing tomato production. The remainder of processing tomato production occurs in isolated areas of the Midwest (*e.g.*, Ohio).

2. Fresh market tomatoes. Commercial fresh market tomatoes are grown over a larger geographic area than processing tomatoes with production occurring in more than 20 states. However, for most of these states, production is limited to what can be consumed locally. Only California and Florida have large acreages of fresh market tomatoes and both participate in broad distribution throughout the U.S. Together these two states account for nearly two-thirds of the U.S. fresh market tomato crop with Florida being the larger producer (Anonymous, 1993). Unless consumed locally, fresh market tomatoes are normally picked in the mature-green state and transported to local packing houses from which they are shipped to various locations throughout the U.S.

Although advances in IPM technologies have fostered improved insect management systems, insect control is still largely based on the use of chemical insecticides (Farrar and Kennedy, 1991), which include all classes of chemical insecticides. Economic damage to tomatoes by insects is considerable (Schwartz and Klassen, 1981). Continued dependence on chemical insecticides results in cyclic problems with insecticide-resistant pest populations and outbreaks of secondary pests. The need for alternative insect control measures is becoming more critical to profitable tomato production in the United States. Environmental concerns limit the availability of existing insecticide chemistry and increase the developmental costs of new chemistries.

IR Tomato Line 5345 expressing the *B.t.k.* Cry1Ac protein is a unique, innovative alternative to traditional chemical control measures. Although alternative insect control tactics are often cited as components of IPM, few alternative insect control methods are of sufficient efficacy to replace chemical control methods. Other methods, such as biological control, host plant resistance and cultural control, provide suppression of pest populations without disrupting natural control, but generally lack the high efficacy and curative action of conventional insecticides.

The use of IR Tomato Line 5345 will enable farmers to effectively control many lepidopteran pests of tomato, providing yield protection and a reduction in the use of chemical insecticides for these insect pests. IR Tomato Line 5345 will provide benefits to growers, the general public, and the environment, including:

1. A more reliable, economical and less labor intensive means to control lepidopteran insect pests.

2. Insect control without harming non-target species, including humans.

3. A means for growers to significantly reduce the amount of chemical insecticides now applied to the crop while maintaining comparable yields. Therefore, lepidopteran insect control can be achieved in a more environmentally compatible manner than is currently available.

4. A reduction in the manufacturing, shipment and storage of chemical insecticides used on tomato.

5. A reduction in the exposure to workers to the pesticide and pesticide spray solution.

6. A reduction in the number of empty pesticide containers and amount of pesticide spray solution that must be disposed of according to applicable environmental regulations.

7. An excellent fit with Integrated Pest Management Programs (IPM) and sustainable agricultural systems.

In conclusion, the consistent lepidopteran control offered by IR Tomato Line 5345 will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop for control of many lepidopteran insect pests which damage tomatoes. As a result, they will be able to utilize a host of IPM practices that cannot presently be implemented because of the lack of options other than use of chemical insecticides to control these pests. An increase in the biological and cultural control of non-target tomato pests and a more judicious use of

chemical insecticides will result in a positive impact on the environment, which will ultimately be advantageous to the grower and the public as well.

D. DEVELOPMENT OF PEST AND RESISTANCE MANAGEMENT STRATEGIES FOR IR TOMATO LINE 5345

To achieve the numerous benefits, previously discussed, it is important that IR Tomato Line 5345 be implemented and managed properly. The development of an IPM strategy includes the following components that are consistent with the seven elements of an adequate insect resistance management (IRM) plan identified by the EPA's Pesticide Resistance management Working Group (Matten & Lewis, 1995):

- Knowledge of pest biology and ecology
- Employment of integrated pest management practices that encourage ecosystem diversity and provide multiple tactics for insect control
- Monitoring and reporting of incidents of pesticide resistance development
- Optimal dose, full-season, constitutive *cry1Ac* gene deployment strategy, to control insects heterozygous for resistance alleles
- Refuges to support the development of *B.t.k.*-susceptible insects
- Development and deployment of products with alternative modes of action
- Communication and education plan

The successful implementation of these strategies will require a concerted effort on the part of the applicant, who assures that the benefits of IR Tomato Line 5345 will be fully realized and sustained.

E. CROSS POLLINATION OF CULTIVATED AND NATIVE SPECIES OF TOMATO

1. Outcrossing with wild species

Although there are wild relatives of tomato with which it can outcross, none of these are found in the United States, but rather are limited to Latin America (Rick, 1976).

2. Outcrossing to the cultivated tomato

Cultivated tomatoes are almost exclusively self-pollinating and outcrossing is rare due to the presence of an inserted stigma. There is no wind pollination and insect pollination is rare (Rick, 1976).

3. Transfer of genetic information to organisms with which it cannot interbreed

As stated in the Animal and Plant Health Inspection Service / USDA's Interpretative Ruling on Calgene, Inc. FLAVR SAVR™ tomato (1992), "There is no published evidence for the existence of any mechanism, other than sexual crossing by which genes can be transferred from a plant to other organisms." Evidence presented in the Calgene petition and supplementary information and summarized in the FR Notice suggests that, based on limited DNA homologies, transfer from plants to microorganisms may have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of the *cry1Ac* or *nptII* gene to a microbe would not pose any plant pest risk. There is no known mechanism for transfer to microbes and even if it did, there is no significant consequence from a plant pest point of view.

F. POTENTIAL FOR IR TOMATO LINE 5345 TO BECOME A WEED

The introduction of the *cry1Ac* and *nptII* genes into a tomato cultivar should not increase the "weediness" of the plant. A general consensus of the traits common to many weeds was developed by Baker (1974). They include:

1) germination requirement fulfilled in many environments
2) discontinuous germination and great longevity of seed
3) rapid growth through vegetative phase to flowering
4) continuous seed production for as long as growing conditions permit
5) self-compatibility but not completely autogamous and apomictic
6) when cross-pollinated, unspecialized visitors or wind pollinated
7) high seed output in favorable environment and some seed production in a wide range of environments

8) adaptation for short- and long-distance dispersal
9) if perennial, vegetative production or regeneration from fragments and brittleness (so not easily removed from the ground)
10) ability to compete interspecifically by special means (rosette formation and presence of allelochemicals). Not all weeds have all of these characteristics.

Tomato does not possesses the characteristics of plants that are notably successful weeds. It is an annual crop in the U.S. which is considered to be highly domesticated, and is not persistent in undisturbed environments without human intervention. *Lycopersicon esculentum* cv. UC82B, the cultivar which has been genetically modified, is not considered to be a weed, and introduction of the insect resistant trait into this cultivar has not imparted any new "weedy" characteristics.

No significant changes were noted with respect to the germination characteristics of seeds. Seeds of both lines germinated mostly within 2 weeks after sowing, with 78.8% of the transgenic germinated versus 81.8% of the non-transgenic controls. There was no significant difference in seedling vigor between the tomato lines, measured as seedling height, weight, and stem width at approximately one month after sowing seeds in the field. Time-to-flowering for plants of IR Tomato Line 5345 was 12 to 46 days, compared to 12 to 50 days for control plants. Average seed number per fruit for IR Tomato Line 5345 and the control was 127 and 135, respectively, and the difference between the lines was not statistically significant. The average number of fruit per plant for the IR Tomato Line 5345 and control plants were 59.5 and 62.0, respectively. There was no statistically significant difference in average fruit number per plant. The average seed weight of IR Tomato Line 5345 and the control was 0.269 and 0.260 g/100 seed, respectively, and the difference between the lines was not statistically significant at the 5% level (Student's t-test).

G. INCREASED NUMBERS OF BENEFICIAL INSECTS

Aside from the benefit of a decrease in the use of chemical insecticides, an additional benefit is anticipated, that being an increase in the numbers of beneficial insects present in the tomato fields. This effect has already been observed in Bollgard cotton lines 757 and 531, which also expresses the Cry1Ac protein expressed by IR Tomato line 5345. The worst enemies of most insects are parasitic and predatory insects. These arthropods feed on other insects, thus providing a "natural" level of control. Most chemical insecticides used in tomato are fairly general in the range of insects controlled, and therefore, most insects and other arthropods including the beneficial predators and parasites are controlled. Over the period of a growing season, their numbers can be depleted to the point that control of pests by the predators is essentially non-existent. Since the *B.t.k.* Cry1Ac insect control protein is very specific in its range of control, an increase in the numbers of beneficial insects has been observed in the field and are expected to supplement the control of the tomato insect pests. This expected increased presence of beneficials will likely reduce the need for insecticide applications targeted to control of tomato pests not susceptible to the *B.t.k.* insect control protein.

H. CONCLUSIONS

IR Tomato Line 5345 is not expected to have a negative impact on the environment and may in fact have a positive benefit. IR Tomato Line 5345 is not expected to become a weed or have any other adverse impact on the environment or production agriculture in the United States. The positive consequences of reduced pesticide use, increases in the numbers of beneficial insects and the overall positive impacts to tomato production fully justifies approval of this request for a determination of non-regulated status.

VII. ADVERSE CONSEQUENCES OF INTRODUCTION

The results of all field studies and laboratory tests establish that there are no unfavorable grounds associated with IR Tomato Line 5345 developed using the PV-LEBK04 plasmid vector, the vector identical to that used to develop Bollgard cotton lines 531 and 757™ cotton. Therefore, on the basis of the substantial potential benefits to the farmer, the environment and the significantly reduced risk to public health, Monsanto requests that IR Tomato Line 5345 and any progenies derived from crosses between this line and other commercial tomato cultivars no longer be regulated under 7 CFR part 340.6.

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Appendix 1. Summary of the Methods used to Conduct Protein Extraction, Analysis and Quantitation of the Cry1Ac, AAD and NPTII Proteins Expressed in IR Tomato Line 5345

Cry1Ac ELISA Procedure.

Step 1. Polystyrene microtiter plates were pre-coated overnight at 4°C with purified mouse monoclonal antibody diluted 1:6000 in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 0.15 M sodium chloride).

Step 2. Plant extracts and the standard CryIAc protein spiked into control plant extracts were trypsinized (3.5 µL of 5 mg/mL trypsin [Calbiochem] per 100 µL of sample or standard) for 3 h at room temperature. Trypsinization was terminated by addition of 2.5 µL of 50 mM phenylmethylsulfonyl fluoride (PMSF). The samples and standards were diluted in PBST plus ovalbumin (PBSTO) prior to addition to the plates.

Step 3. The coating antibody was washed from the plates (two short washes with PBST), and 50 µL of secondary rabbit polyclonal antibody (diluted 1:900 in PBSTO) added to each well. Samples and standards were added to the plates in a total volume of 200 µL, and incubated overnight at 4°C in a humid box.

Step 4. Secondary antibody, samples and standards were washed from the plates (two short washes with PBST), and 250 µL of donkey-anti-rabbit antibody conjugated to alkaline phosphatase (diluted 1:2500 in PBSTO) added to each well. The plates were incubated at room temperature for approximately 1.5 to 3 h. The antibody-alkaline phosphatase conjugate was washed from the plates (one short wash, two 5 min washes with PBST). p-Nitrophenyl phosphate (pNPP) substrate (Sigma, St. Louis, MO) was added to the plates (250 µL per well), and allowed to react for approximately 1 h at room temperature. Absorbance was read at 405 nm (reference wavelength 655 nm) using a Bio-Rad (Richmond, CA) Model 3550 microplate processor. Quantitation of CryIAc protein concentration in samples was accomplished by extrapolation from a logistic curve fit of the CryIAc standard curve.

Extraction of the NPTII Protein from Tomato Tissues.

Frozen tissue powders of IR and control tomato lines were extracted in phosphate buffered saline plus Tween-20 (PBST; 0.14 M sodium chloride, 8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride, and 0.05% Tween-20) at tissue-to-buffer volumes of 1:20 for leaf tissue, and 1:40 for red ripe fruit. A Brinkman overhead Polytron (Kinematica AG, Switzerland) was used for tissue extraction, as described in SOP# DRT-PRO-021. Extracts were centrifuged, and the supernatant assayed for NPTII protein by NPTII ELISA.

NPTII ELISA Procedure.

Step 1. Polystyrene microtiter plates were pre-coated overnight at 4°C with 1 µg of rabbit polyclonal antibody in 250 µL of coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6).

Step 2. Wells were blocked with 5% skim milk in PBST for 30 min.

Step 3. The mouse monoclonal secondary antibody was added to each well (225 µL) at a final dilution of 1:10,000 in sample buffer (50 mM sodium phosphate, pH 7.5; 150 mM sodium chloride; 0.1% bovine serum albumin; 1% polyvinylpyrrolidine; 0.05% Tween-20). Appropriately diluted samples and standards were added to the plates in a total volume of 25 µL, and incubated overnight at 4°C in a humid box.

Step 4. 250 µL of anti-mouse monoclonal antibody conjugated to horse-radish peroxidase (diluted 1:1500 in sample buffer) was added to each well. The plates were incubated at room temperature for approximately 1.5 to 3 h. The antibody-horse-radish peroxidase conjugate was washed from the plates and 250 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and allowed to react for approximately 7 to 12 minutes at room temperature. The reaction was stopped with 100 µL of 3 M phosphoric acid. Absorbance was read at 450 nm using a Bio-Rad (Richmond, CA) Model 3550 microplate processor. Quantitation of NPTII protein concentration in samples was accomplished by extrapolation from a logistic curve fit of the NPTII standard curve.

Washing Steps. Plate wells were washed with ELISA wash buffer (50 mM sodium phosphate, pH 7.4; 150 mM sodium chloride; 0.05% Tween-20) between each of the above steps. Each wash step was one short wash and two 5 min washes.

Appendix 2. USDA Field Reports

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Summer/Fall 1995
Insect Resistant Tomato Trials
USDA Permit #95-151-04N
MONSANTO #95-174XR
BHN-Research, Huron, CA

Andrew Reed

Tomato, *Lycopersicon esculentum*, has been genetically engineered to be resistant to selected insect pests (Lepidoptera). Resistance was accomplished by the stable insertion of a gene encoding the CryIAc protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 (CryIAc) into the tomato genome. Tomato varieties expressing the CryIAc protein, referred to as Insect Resistant (IR) tomato, are expected to significantly reduce chemical insecticide use in tomato production and, therefore, provide a major benefit to the tomato growers and to the environment. The purpose of the field trial was to evaluate the agronomic performance, measure protein expression of the genes introduced into the tomato genome, and fruit composition of an IR tomato. Transformation event 5345 is used as an example line in this study. The field trial was conducted at BHN-Research Farm, Huron, CA. Data collected from the field trial will be used for regulatory approval of IR tomatoes containing the *cryIAc* gene.

Experimental Layout:

The trial was conducted at BHN-Research Farm, [CBI DELETED] Huron CA (coded as CA). IR tomato line 5345 was backcrossed to a nontransgenic tomato line, BHN-W, in the greenhouses at BHN-Research, Bonita Springs, FL. Insect resistant (contain the *cryIAc* gene) and control (do not contain the *cryIAc* gene) tomato plants were produced from each backcross, and progeny of the seventh backcross (BC_7F_1) were grown at the CA field site. Seed were shipped from BHN-Research, FL to BHN-Research Farm, CA, under permit #95-151-04N. Seed were sown in the greenhouse at BHN-Research Farm, CA and held until plantlets attained adequate size for transplanting. Dates of sowing and transplanting are listed in Appendix 1.

The plot design at the CA field site was a randomized complete block. There were four replicates each of the IR and control lines. Each plot consisted of a single row of 24 plants each, planted approximately 18 inches apart in a row. Row spacing was approximately 60 inches. Normal California fresh market production practices were used for plant culture at each of the field sites. These practices included resetting weak transplants, plant staking, tying, irrigation, and use of registered pesticides within the labeled application rates for control of weeds, insects (including Lepidoptera), and diseases. Plot maps, cultural practices and weather data for field site CA are contained in Appendix 1.

Data Collection/Analysis

Approximately 50 kg of fruit were harvested from the field site. Fruit were shipped in accordance with USDA permit # 95-151-03N. Fruit were harvested at either mature green, breaker, pink, or red. Sampling and shipping dates are listed in Appendix 1.

Fruit were shipped to Monsanto Co., Chesterfield, MO. The fruit were analyzed for protein expression and for nutrient/toxicant composition.

Plant Growth and General Observations

Detailed monitoring for plant growth characteristics, disease and insect infestations of the IR tomato line versus the control line was performed at each of the two field sites. Tomato breeders and/or agronomists were responsible for collecting this data and reporting their findings. Plots were evaluated in the same fashion as a typical tomato breeder would examine tomato plots to decide on the acceptability of a new line for commercial release. Plots of the IR and control tomato lines were visually checked for the appearance of possible disease symptoms such as spotted leaves, leaf necrosis, stunted or distorted plants, and wilting of the plants. Plants were also checked for insect populations, including fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), cabbage loopers (*Trichoplusia ni*), tomato fruitworm (*Helicoverpa zea*), and tomato pinworm (*Keiferia lycopersicella*). Notes were also taken on the growth habit and morphology of IR and control tomato plants.

No differences in disease or insect infestation or severity were detected between the IR and control tomato lines at any of the two field sites. Survival of transplants and overall vigor of plants in the field was excellent. There were no differences in growth and development characteristics between the IR and control tomato lines. The fruit yield of the IR tomato line was statistically equivalent to yield of the control line at each of the two field sites.

The plots were also monitored for *Agrobacterium tumefaciens* infection symptoms. None was found at either of the two field sites.

Responses to Specific Issues:

1. Horizontal Gene Movement:

Expression levels of the CryIAc protein in fruit and leaf samples of IR and control tomato plants were measured by ELISA. The CryIAc protein was detected in samples of IR plants only. There was no evidence of movement of the *cryIAc* gene to other tomatoes through outcrossing.

2. Changes in Survival Characteristics:

There was no evidence of changes in the survival characteristics of the IR tomato plants compared to the control plants. Under normal cultural practices in California tomato production, fields are routinely disked immediately after harvest of fruit from the field, and disking is repeated every 4 to 5 weeks after to control weeds and volunteer tomatoes. As such, disking typical for the area was conducted at the CA site. A few volunteers were observed initially after fruit harvest, but there was no difference in incidence of volunteers between the IR and control tomatoes. These volunteers were destroyed by disking. No differences were observed in survival of volunteers between IR and control tomatoes. The plots were left fallow after termination of the study.

3. Stability and Pattern of Inheritance of the Insect Resistant Trait:

Breeding programs involving several nontransgenic tomato lines crossed with IR tomato line 5345 showed normal Mendelian inheritance of the *cryIAc* gene, as determined by expression of the CryIAc protein in progeny. Continued testing of selected lines shows that the insect resistant trait is stable over backcross generations.

4. Published Data

At this time (9/4/96) results of these studies have not been published.

APPENDIX 1

Test Site and Field Trial Details for the CA Field Site

BHN-Research, [CBI DELETED]
Huron,
CA 33923

Test Site and Field Trial Details for the CA site:

A. Test Site Location

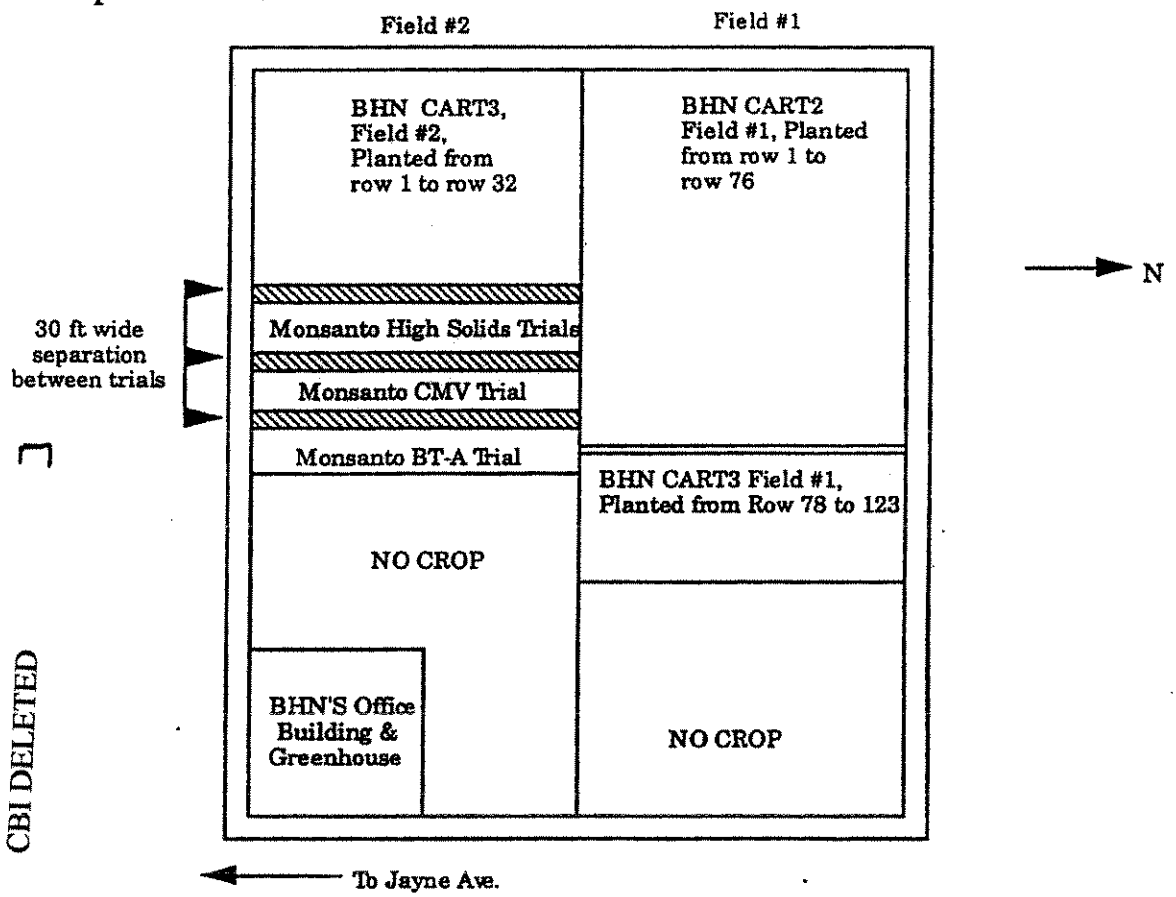
BHN Research Farm
[CBI DELETED]
Huron, CA [CBI DELETED]
[CBI DELETED]

B. Farm Manager and Principal Investigator

Farm Manager: [CBI DELETED]

Principal Investigator: [CBI DELETED]

C. Map of Field Site Location



General Location of the BT-A Trial at the BHN Farm; Huron, CA. Fall 1995. Map is not to scale.

Soil Type

Clay loam

E. Experimental Design

Seed sow date 6/16/95
Seedling transplant date .. 8/11/95

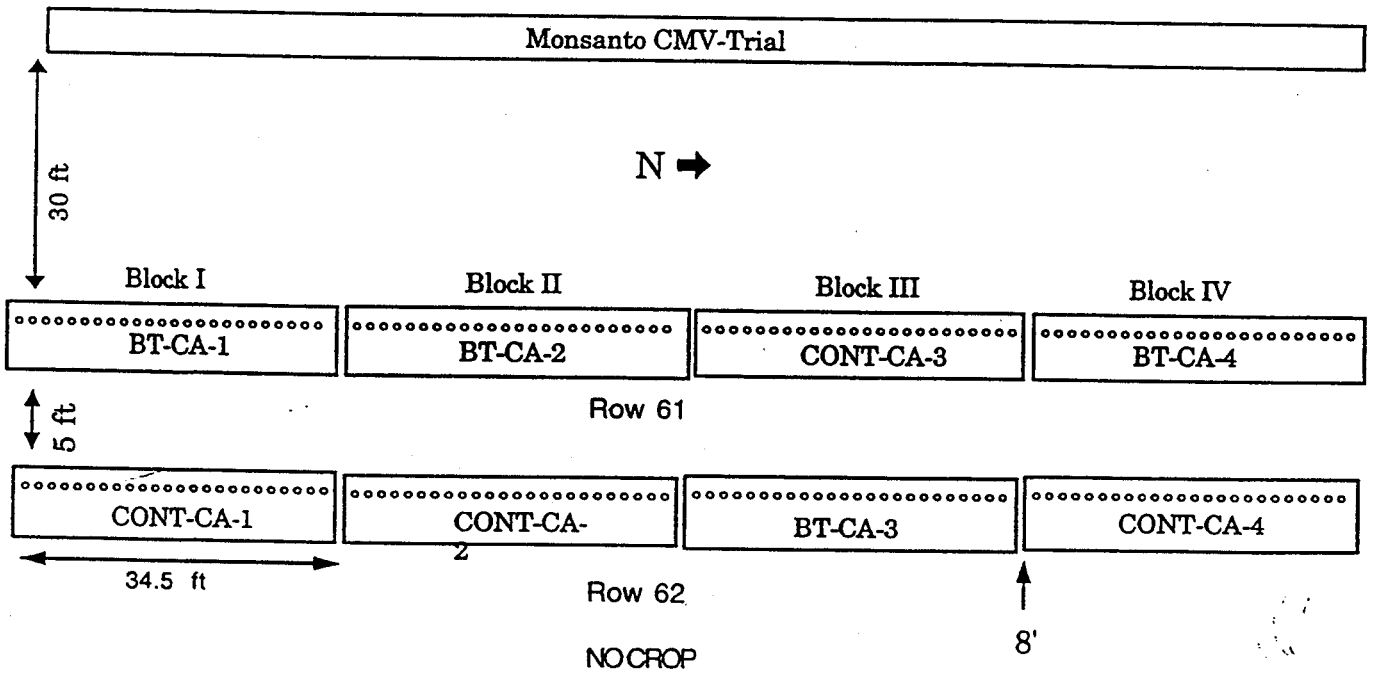
Field Design
- Randomized complete block
- 4 replicate plots per line
- Each plot consisted of a single row of 24 plants, planted approximately 18 inches apart in the row. Row spacing was approximately 60 inches unless otherwise indicated in the plot diagram. The field test was separated from other commercial or breeding tomatoes by a minimum border of 30 feet on all sides.

Field Plot Map (map is not to scale)

For each of the plots below:

- Plant spacing ≈ 18"
- Bed Width ≈ 40"
- Bed Height ≈ 6"
- No drive rows in plots
- space between plots ≈ 8'

- Plots are identified by rectangles
- Plants are identified as the dots inside the rectangle



F. Cultural Practices -CA

-Drip Irrigation applications to field plants:
8/11/95, 8/132/95, 8/16/95, 8/18/95, 8/20/95, 8/22/95, 8/24/95, 8/26/95, 8/28/95, 8/29/95, 8/31/95, 9/2/95, 9/4/95, 9/6/95, 9/8/95, 9/9/95, 9/10/95, 9/11/95, 9/13/95, 9/15/95, 9/17/95, 9/21/95, 9/22/95, 9/24/95, 9/27/95, 9/29/95, 10/1/95, 10/4/95, 10/6/95, 10/8/95, 10/11/95, 10/13/95, 10/15/95, 10/18/95, 10/20/95, 10/22/95, 10/25/95, 10/27/95, 10/29/95, 11/1/95, 11/3/95, 11/5/95, 11/8/95

-Fertilizer applications to field plants:
8/13/95, 8/16/95, 8/18/95, 8/20/95, 8/22/95, 8/24/95, 8/26/95, 8/28/95, 8/29/95, 8/31/95, 9/2/95, 9/4/95, 9/6/95, 9/8/95, 9/9/95, 9/10/95, 9/13/95, 9/15/95, 9/17/95, 9/22/95, 9/24/95, 9/27/95, 9/29/95, 101/95, 10/4/95, 10/6/95, 10/8/95, 10/11/95, 10/13/95, 10/15/95, 10/18/95, 10/20/95, 10/22/95, 10/25/95, 10/27/95, 10/29/95, 11/1/95, 11/3/95, 11/5/95, 11/8/95

-Fungicide Applications to field plants:
Rally 40W 8/18/95, 9/2/95, 9/13/95
Ridomil 2E 8/11/95

-Insecticide applications to field plants:

Agrimek	8/16/95, 8/18/95, 8/30/95, 9/8/95, 9/11/95, 9/13/95
Asana XL	8/18/95, 8/25/95, 9/2/95, 9/13/95, 9/15/95, 9/24/95
Diazion	9/59/5, 9/12/95, 9/13/95, 9/15/95, 9/24/95
Isotox	9/14/95
Lannate	8/18/95, 8/25/95, 9/2/95, 9/13/95, 9/15/95, 9/24/95
Malathion 50+	8/28/95, 8/31/95, 9/1/95
Provado	8/18/95, 8/25/95, 9/2/95

-Staking and Tying of plants:
9/8/95, 9/9/95, 9/26/95

-Test Material Destruction:
11/29/95

-Zinc applications to field plants:
8/29/95, 9/4/95, 9/13/95, 10/5/95

G. Weather Data -CA

Month	Average Air Temperature(°F) Maximum Normal	Maximum 1995	Minimum Normal	Minimum 1995	Precipitation (inches) Normal	Precipitation (inches) 1995
May	85	79	52	52	.33	0.35
June	91	86	57	56	0.07	0.16
July	94	92	61	61	0.04	0.08
August	93	94	61	60	0.07	0.08
September	89	89	57	57	0.22	0.00
October	82	87	51	51	0.61	0.00
November	67	75	41	47	0.38	0.00

H. Sampling and Shipping Log for IR and Control Tomato Fruit and Leaf Samples for Enzyme Expression and Nutrient/Toxicant Analyses.

Sample Description	Shipped to	Date Sampled	Date Received
Leaf	Monsanto	9/7/95	9/8/95
Fruit	Monsanto	11/7/95	11/8/95
Whole Plant	Monsanto	11/16/95	11/17/95

I. Tomato Fruit Collection Log for Yield Determination of IR and Control

Harvest date: 11/10/95

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Winter 1995 / Spring 1996
Insect Resistant Tomato Trials
USDA Permit #95-138-04N
MONSANTO #95-168XR
BHN-Research, Bonita Springs, FL

Andrew Reed

Tomato, *Lycopersicon esculentum*, has been genetically engineered to be resistant to selected insect pests (Lepidoptera). Resistance was accomplished by the stable insertion of a gene encoding the CryIAc protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 (CryIAc) into the tomato genome. Tomato varieties expressing the CryIAc protein, referred to as Insect Resistant (IR) tomato, are expected to significantly reduce chemical insecticide use in tomato production and, therefore, provide a major benefit to the tomato growers and to the environment. The purpose of the field trials was to evaluate the agronomic performance, measure protein expression of the genes introduced into the tomato genome, and fruit composition of an IR tomato. Transformation event 5345 is used as an example line in this study. The field trials were conducted at two sites in Florida in collaboration with BHN-Research, Bonita Springs, FL. Data collected from the field trials will be used for regulatory approval of IR tomatoes containing the *cryIAc* gene.

Experimental Layout:

The trials were conducted at two field sites:

BHN-Research, [CBI DELETED] Bonita Springs, FL (coded as BHN)

NTGargiulo Farm #6, [CBI DELETED] Collier Co., FL (coded as NTG).

IR tomato line 5345 was backcrossed to a nontransgenic tomato line, BHN-W, in the greenhouses at the BHN-Research site. Insect resistant (contain the *cryIAc* gene) and control (do not contain the *cryIAc* gene) tomato plants were produced from each backcross, and progeny of the seventh backcross (BC_7F_1) were grown at the two field sites. Seed were stored at the BHN-Research facility. Seed were sown in the greenhouse at BHN-Research and held until plantlets attained adequate size for transplanting. Dates of sowing and transplanting for field site BHN are listed in Appendix 1, and for field site NTG are listed in Appendix 2.

The plot design at each of the two field sites was completely random. The IR tomato line and the control line were evaluated at each of the test sites. There were four replicates of each line grown at the two field sites. At field site BHN, each plot consisted of 3 rows of 12 plants each, planted approximately 20 inches apart in a row. Row spacing was approximately 60 inches. At field site NTG, each plot consisted of a single row of 24 plants, planted approximately 20 inches apart in a row. Row spacing was approximately 60 inches. Normal Florida fresh market production practices were used for plant culture at each of the field sites. These practices included resetting weak transplants, plant staking, tying, irrigation, and use of registered pesticides within the labeled application rates for control of weeds, insects (including

Lepidoptera), and diseases. Plot maps, cultural practices and weather data are contained in Appendices 1 and 2 for field sites BHN and NTG, respectively.

Data Collection/Analysis

In total, approximately 100 kg of fruit were harvested from each of the two field sites. Fruit were shipped in accordance with USDA permit # 95-138-03N. Fruit were harvested at either mature green, breaker, pink, or red. Sampling and shipping dates are listed in Appendix 1 for fruit from field site BHN, and in Appendix 2 for fruit from field site NTG. Fruit were shipped to either Monsanto Co., Chesterfield, MO, or to NutraSweet, Mt. Prospect, IL. The fruit were analyzed for protein expression and for nutrient/toxicant composition.

Plant Growth and General Observations

Detailed monitoring for plant growth characteristics, disease and insect infestations of the IR tomato line versus the control line was performed at each of the two field sites. Tomato breeders and/or agronomists were responsible for collecting this data and reporting their findings. Plots were evaluated in the same fashion as a typical tomato breeder would examine tomato plots to decide on the acceptability of a new line for commercial release. Plots of the IR and control tomato lines were visually checked for the appearance of possible disease symptoms such as spotted leaves, leaf necrosis, stunted or distorted plants, and wilting of the plants. Plants were also checked for insect populations, including fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), cabbage loopers (*Trichoplusia ni*), tomato fruitworm (*Helicoverpa zea*), and tomato pinworm (*Keiferia lycopersicella*). Notes were also taken on the growth habit and morphology of IR and control tomato plants.

No differences in disease or insect infestation or severity were detected between the IR and control tomato lines at any of the two field sites. Survival of transplants and overall vigor of plants in the field was excellent. There were no differences in growth and development characteristics between the IR and control tomato lines. This conclusion was confirmed by two horticulturalists, expert in tomato production who inspected the IR and control tomato plants at the BHN field site. The letters from the expert tomato horticulturalists are contained in Appendix 3. The fruit yield of the IR tomato line was statistically equivalent to yield of the control line at each of the two field sites.

The plots were also monitored for *Agrobacterium tumefaciens* infection symptoms. None was found at either of the two field sites.

At field site BHN only, plants were monitored approximately once every two days for the onset of flowering. Monitoring was continued until all plants in each plot had flowered. Time-to-flowering for the IR and control tomato lines was calculated as an average of time-to-flowering for each plant within each replicate plot. Initiation of flowering ranged from 12 to 46 days after transplant to the field for the IR tomato line, and 12 to 50 days for the control line. The average time-to-flowering was 32.0 and 33.3 days for the IR and control lines, respectively. The difference between the two lines in average time-to-flowering was not statistically significant.

Responses to Specific Issues:

1. Horizontal Gene Movement:

Expression levels of the CryIAc protein in fruit and leaf samples of IR and control tomato plants were measured by ELISA. The CryIAc protein was detected in samples of IR plants only. There was no evidence of movement of the cryIAc gene to other tomatoes through outcrossing at either of the field sites.

2. Changes in Survival Characteristics:

There was no evidence of changes in the survival characteristics of the IR tomato plants compared to the control plants. Under normal cultural practices in Florida tomato production, fields are routinely disked immediately after harvest of fruit from the field, and disking is repeated every 4 to 5 weeks after to control weeds and volunteer tomatoes. As such, plants were destroyed by burning at the end of each trial and disking typical for the area was conducted at the BHN and NTG field sites. A few volunteers were observed initially after fruit harvest, but there was no difference in incidence of volunteers between the IR and control tomatoes. These volunteers were destroyed by disking. No differences were observed in survival of volunteers between IR and control tomatoes. The plots were left fallow after termination of the study at each test site.

3. Stability and Pattern of Inheritance of the Insect Resistant Trait:

Breeding programs involving several nontransgenic tomato lines crossed with IR tomato line 5345 showed normal Mendelian inheritance of the *cryIAc* gene, as determined by expression of the CryIAc protein in progeny. Continued testing of selected lines shows that the insect resistant trait is stable over backcross generations.

4. Published Data

At this time (9/4/96) results of these studies have not been published.

APPENDIX 1

Test Site and Field Trial Details for the BHN Field Site

BHN-Research, [CBI DELETED]
Bonita Springs,
FL 33923

Test Site and Field Trial Details for the BHN site:

A. Test Site Location

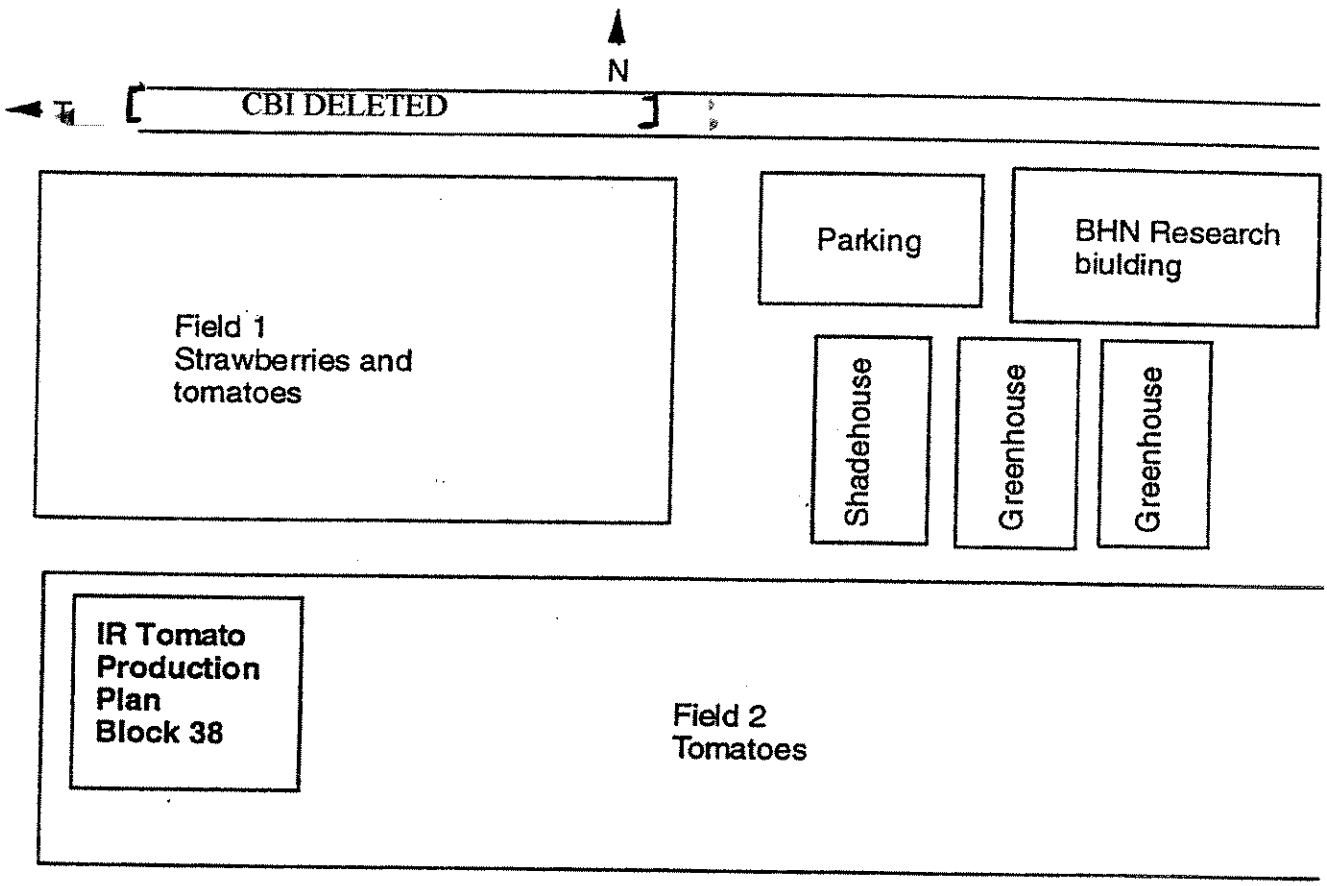
BHN Research
[CBI DELETED]
Bonita Springs, FL [CBI DELETED]

B. Farm Manager and Principal Investigator

Farm Manager: [CBI DELETED]

Principal Investigator: [CBI DELETED]

C. Map of Field Site Location (map is not to scale)



D. Soil Type

Arenic Haploquad - Immokalee fine sand

E. Experimental Design

Seed sow date 8/18/95
Seedling transplant date .. 10/27/95

Field Design

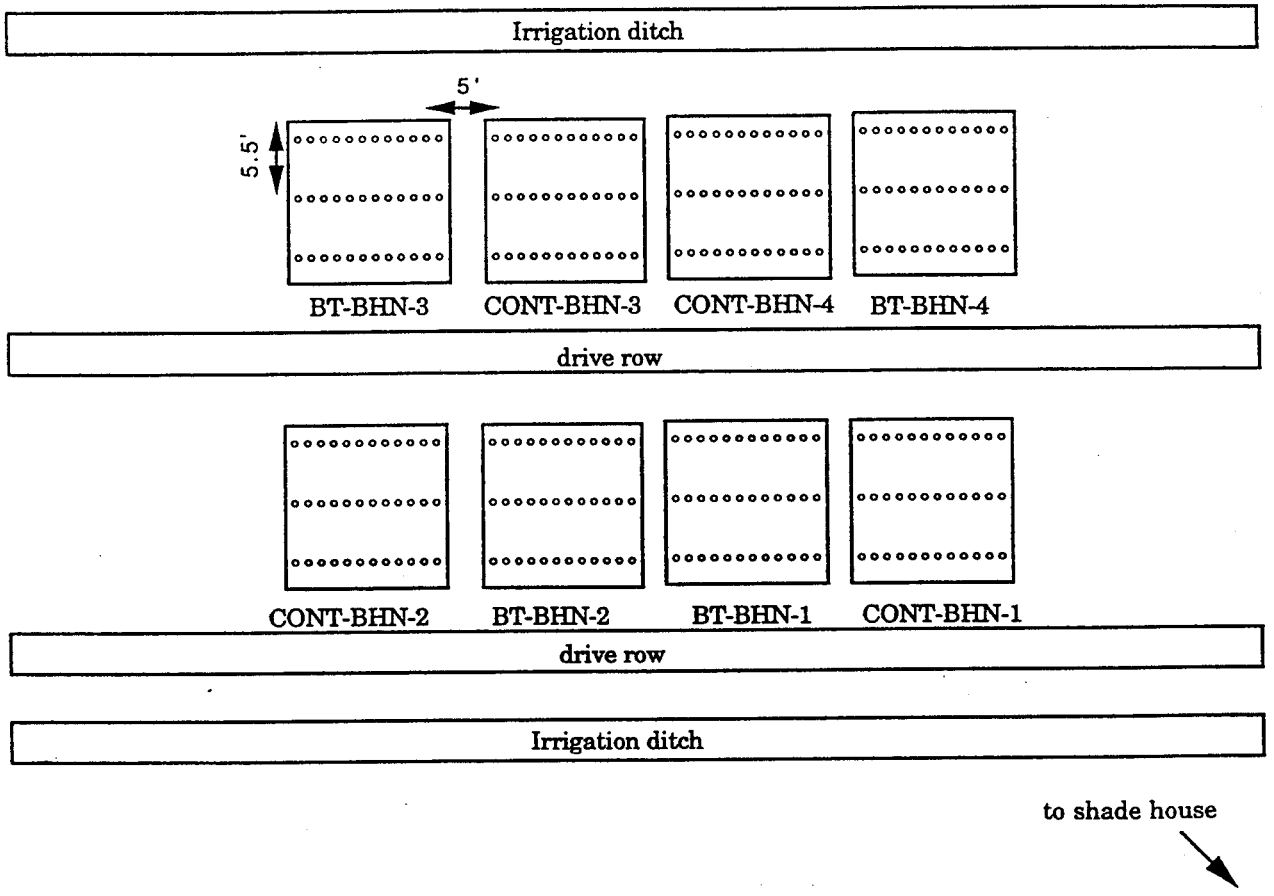
- Completely Randomized design
- 4 replicate plots per line• Each plot consisted of 3 rows of 12 plants each, planted approximately 20 inchesapart in a row. Row spacing was approximately 60 inches unless otherwise indicated in the plot diagram. The field test was separated from other commercial or breeding tomatoes by a minimum border of 30 feet on all sides.

Field Plot Map (map is not to scale)

For each of the plots below:

- Plant spacing ≈ 20"
- Bed Width ≈ 40"
- Bed Height ≈ 8"
- Drive rows on both sides of plot
- space between plots ≈ 5.5'

- Plots are identified by rectangles
- Plants are identified as the dots inside the rectangle



F. Cultural Practices -BHN

-Seep/slope irrigation applications to field plants:
Water level maintained at 15-16" from the top of the bed

Adjuvant applications to field plants:
Combine 10/27/95, 10/30/95, 11/1/95, 11/3/95, 11/6/95, 11/10/95, 11/13/95, 11/20/95, 11/22/95, 11/28/95, 12/5/95, 12/12/95, 12/20/95, 12/26/95, 1/1/96, 1/9/96, 1/16/96, 1/23/96, 1/26/96, 1/29/96, 2/2/96, 2/3/96, 2/5/96

-Fertilizer applications to field plants:

Calcium nitrate	11/1/95
THIS	11/1/95
Solubor	11/1/95

-Fungicide Applications to field plants:

Agrimycin 17	10/27/95, 10/27/95, 10/30/95, 11/1/95, 11/3/95
Benlate 50 WP	11/3/95
Kocide 101	10/27/95, 10/30/95, 11/1/95, 11/3/95, 11/6/95, 11/10/95, 11/13/95, 11/20/95, 11/22/95, 11/28/95, 12/5/95, 12/20/95, 12/26/95, 1/1/96, 1/3/96, 1/9/96, 1/16/96, 1/23/96, 1/26/96, 1/29/96, 2/2/96, 2/3/96, 2/5/96
Manzate 200 DF	10/27/95, 10/30/95, 11/1/95, 11/3/95, 11/6/95, 11/10/95, 11/13/95, 11/20/95, 11/28/95, 12/5/95, 12/12/95, 12/20/95, 12/26/95, 1/1/96, 1/3/96, 1/9/96, 1/16/96,1/22/95, 1/23/96, 1/26/96, 1/29/96, 2/2/96, 2/3/96, 2/5/96
Terranil	11/8/95, 11/17/95, 11/24/95, 21/1/95, 12/8/95, 12/15/95, 12/22/95, 12/29/95, 1/5/96, 1/14/96, 1/20/96, 1/31/96

-Insecticide applications to field plants:

Admire	10/27/95
Agrimek	11/1/95, 11/17/95, 11/24/95, 12/5/95, 12/12/95, 12/15/95, 12/20/95, 12/22/96, 12/26/95, 12/29/95, 1/3/96, 1/5/96,1/9/96, 1/14/96, 1/16/96, 1/20/96, 1/23/96, 1/26/96, 1/29/96, 2/2/96, 2/5/96,
Asana XL	11/1/95, 11/22/95, 12/15/95, 1/3/96
Butacide 8F	11/1/95
Lannate LV	10/30/95, 11/6/95, 12/5/95, 1/31/96
Thiodan 3EC	10/27/95

-Staking and Tying of plants: 11/16/95, 11/21/95, 12/6/95, 12/22/95

-Test Material Destruction: 2/25/96

G. Weather Data BHN

Month	Average Air Temperature(°F) Maximum Normal	Maximum '95/96	Minimum Normal	Minimum '95/96	Precipitation (inches) Normal	Precipitation (inches) '95/96
August	92	91	74	76	8.3	20.3
September	91	91	73	75	7.1	10.9
October	87	88	70	73	4.4	16.0
November	83	82	65	61	1.9	0.6
December	78	73	57	54	1.2	0.7
January	77	75	56	53	2.6	4.4
February	78	75	57	50	2.1	0.01

H. Sampling and Shipping Log for IR and Control Tomato Fruit and Leaf Samples for Enzyme Expression and Nutrient/Toxicant Analyses.

Sample Description	Shipped to	Date Sampled	Date Received
Leaf	Monsanto	11/14/95	11/15/95
	Monsanto	12/1895	12/19/95
	Monsanto	1/24/96	1/25/96
Mature Green Fruit	Monsanto	2/5/96	2/7/96
Red Fruit*	Nutrasweet	2/5/96	2/16/96
Pink and Red Fruit*	Monsanto	2/5/96	2/16/96
Whole Plant	Monsanto	2/5/96	2/7/96

*Picked as mature green fruit and ripened to red prior to shipping

I. Tomato Fruit Collection Log for Yield Determination of IR and Control Lines

Harvest date: 2/5/96

APPENDIX 2

Test Site and Field Trial Details for the NTG Field Site

NT Gargiulo, Farm # 6

[CBI DELETED]

Naples,

FL [CBI DELETED]

Test Site and Field Trial Details for the NTG site:

A. Test Site Location

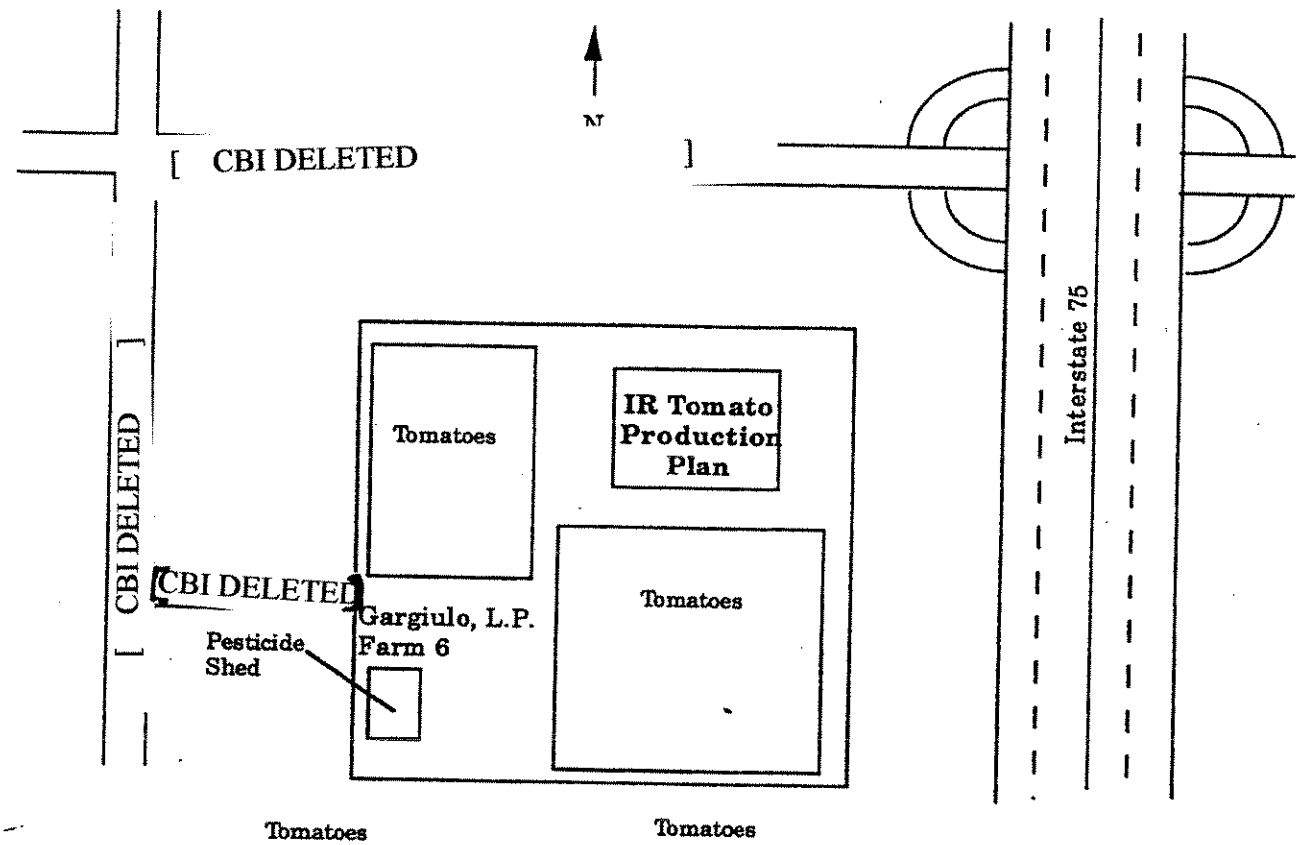
BHN Research
[CBI DELETED]
Naples, FL [CBI DELETED]

B. Farm Manager and Principal Investigator

Farm Manager:

Principal Investigator: [CBI DELETED]

C. Map of Field Site Location (map is not drawn to scale)



D. Soil Type

Arenic Haploquad - Immokalee fine sand

E. Experimental Design

Seed sow date 8/18/95
Seedling transplant date .. 9/22/95

Field Design

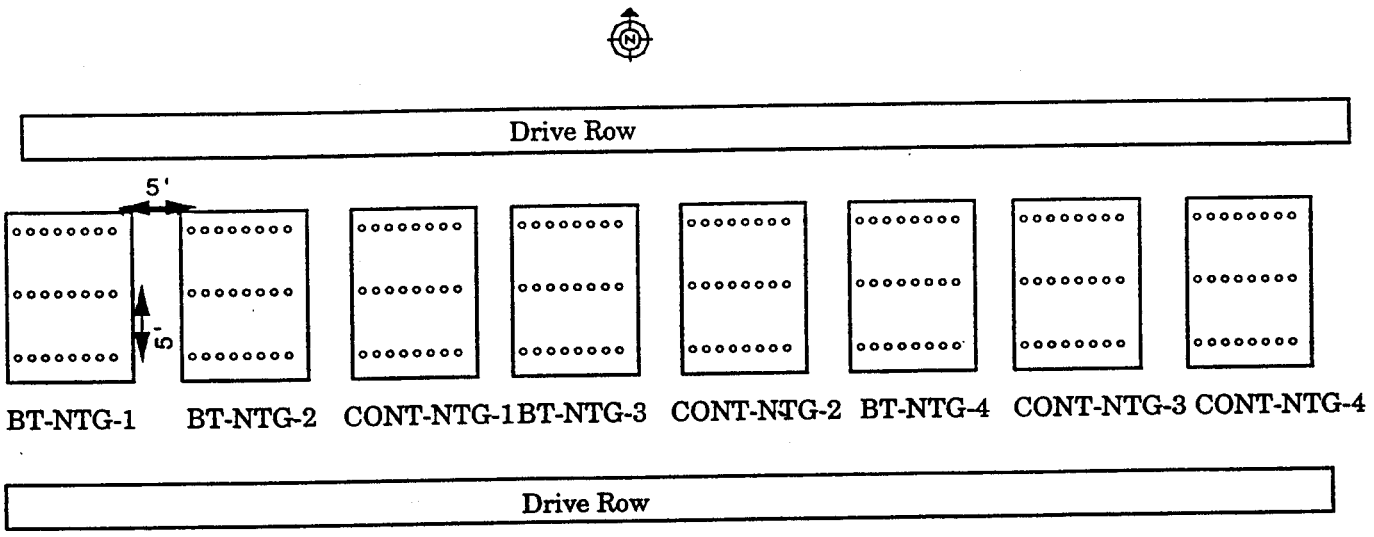
- Completely Randomized design
- 4 replicate plots per line
- Each plot consisted of a single row of 24 plants, planted approximately 20 inches apart in the row. Row spacing was approximately 60 inches unless otherwise indicated in the plot diagram. The field test was separated from other commercial or breeding tomatoes by a minimum border of 30 feet on all sides.

Field Plot Map (map is not drawn to scale)

For each of the plots below:

- Plant spacing ≈ 20"
- Bed Width ≈ 40"
- Bed Height ≈ 8"
- Drive rows on both sides of plot
- space between plots ≈ 5'

- Plots are identified by rectangles
- Plants are identified as the dots inside the rectangle



F. Cultural Practices -NTG

-Seep/slope irrigation applications to field plants:

System applied water every 36 out of 48 hours unless it rained to maintain a water level of 15-16" from the top of the bed.

-Fertilizer applications to field plants:

3-16-4	9/25/95
15-1-26	9/25/95
Calcium nitrate	9/25/95, 10/9/95, 11/21/95, 11/28/95, 12/5/95, 12/11/95, 12/26/95, 12/29/95, 1/2/96, 9/29/95, 10/9/95, 10/15/95, 11/21/95, 11/28/95, 12/4/95, 12/5/95, 12/11/95, 12/26/95, 12/29/95, 1/2/96,
THIS	9/25/95, 9/29/95, 10/9/95, 11/21/95, 11/28/95, 12/11/95, 12/5/95, 12/21/95, 12/26/95, 12/29/95, 1/2/96, 1/15/96, 1/17/96
Solubor	10/9/95, 11/21/95, 11/28/95, 12/5/95

-Fungicide Applications to field plants:

Ensign	10/8/95, 11/25/95, 12/2/95, 12/11/95, 12/14/95, 12/21/95, 12/21/95, 1/15/96, 1/17/96
Kocide 101	9/22/95, 9/23/95, 9/24/95, 9/25/95, 9/26/95, 9/27/95, 9/28/95, 9/29/95, 9/30/95, 10/1/95, 10/3/95, 10/4/95, 10/7/95, 10/8/95, 10/9/95, 10/10/95, 10/13/95, 10/24/95, 10/25/95, 10/26/95, 10/28/95, 10/30/95, 11/25/95, 12/2/95, 12/5/95, 12/7/95, 12/9/95, 12/11/95, 12/26/95, 1/2/96, 1/3/96, 1/17/96
Manzate	11/9/95, 1/3/96
Penncozeb 80 WP	9/22/95, 9/23/95, 9/24/95, 9/25/95, 9/26/95, 9/27/95, 9/29/95, 9/30/95, 10/1/95, 10/3/95, 10/4/95, 10/7/95, 10/10/95, 10/26/95, 10/28/95, 11/9/95, 11/21/95, 11/24/95, 11/25/95, 11/28/95, 11/30/95, 12/2/95, 12/5/95, 12/7/95, 12/9/95, 12/11/95, 1/2/96, 1/3/96, 1/17/96

-Herbicide Applications to field plants:

Enquick	11/27/95
Gramoxone	11/27/95
Poast	11/27/95

-Insecticide applications to field plants:

Agrimek	12/14/95, 1/17/96
JMS Stylet oil	11/27/95
Calgon Dish soap	1/3/96, 1/15/96, 1/17/96
Lannate	9/22/95, 9/25/95, 9/26/95, 10/8/95, 1/17/96
Lorsban	9/29/95, 10/7/95
Sunspray oil	12/14/95, 1/17/96
Warrior	10/3/95, 10/9/95

-Staking and Tying of plants:
10/31/95, 11/8/95, 11/22/95, 12/11/95

-Test Material Destruction:
1/18/96

G. Weather Data NTG

	Average Air Temperature(°F) Maximum		Minimum		Precipitation (inches)	
Month	Normal	'95/96	Normal	'95/96	Normal	'95/96
July	92	91	73	75	10.0	9.6
August	92	91	74	76	8.3	20.3
September	91	91	73	75	7.1	10.9
October	87	88	70	73	4.4	16.0
November	83	82	65	61	1.9	0.6
December	78	73	57	54	1.2	0.7
January	77	75	56	53	2.6	4.4

H. Sampling and Shipping Log for IR and Control Tomato Fruit and Leaf Samples for Enzyme Expression and Nutrient/Toxicant Analyses.

Sample Description	Shipped to	Date Sampled	Date Received
Leaf	Monsanto	10/23/95	10/24/95
Fruit (Composition)	Monsanto	12/26/95	12/27/95
Fruit (Expression)	Monsanto	1/8/96	1/10/96

I. Tomato Fruit Collection Log for Yield Determination of IR and Control lines

Harvest dates: 12/28/95, 1/8/96

APPENDIX 3

Expert Opinion Letters on the Growth and Morphology of Field-Grown Insect Resistant and Control Tomato Plants.

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Southwest Florida Research and Education Center
Institute of Food and Agricultural Sciences

PO Drawer 5127
Immokalee FL 33934
(813) 657-5221
Fax (813) 657-5224

January 26, 1996

Dr. Yossi Shapiro
BHN Research
16750 Bonita Springs Road
Bonita Springs, FL 33923

Dear Dr. Shapiro:

After viewing the transgenic Bt tomato line(s) designated BtBHN and BtNTG on January 10, 1996, it is my considered opinion that the characteristics expressed by said plants are consistent with those of other fresh market tomato cultivars used in commercial production. All physical parameters viewed including stem development, leaf and flower architecture, and fruit external and internal qualities appeared, to the naked eye, to be identical to known cultivars. If I can be of further service in this matter please do not hesitate to contact me.

Sincerely,

[CBI DELETED

]

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UNIVERSITY OF FLORIDA Cooperative Extension Service

Institute of Food and Agricultural Sciences

14700 Immokalee Road
Naples, FL 33964
Tele: (941)353-4244
Imm: (941)657-3306
FAX: (941)353-7127
Suncom: 974-5098

January 26, 1996

Dr. Yossi Shapiro
BHN Research
16750 Bonita Beach Rd.
Bonita Springs, Fl 33923

Dear Dr. Shapiro:

This is to confirm that on January 10, 1996, I evaluated two research trials on transgenic tomato breeding lines. One trial, located behind the BHN facility, compared Bt\BHN-2, the transgenic breeding line, with the control, Cont\BHN-2. The other trial located in a production field compared Bt\NTG, the transgenic, to Cont\NTG, the control.

In evaluating the trials I examined the morphology of the flowers, the appearance of the leaves and fruit, and the general habit of plant growth. The phenology of the plants in the two treatments of both trials, were identical. If you would like more information or have any further questions please feel free to contact me.

Sincerely,

[CBI DELETED

]

SS:tjb

0720F0138

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Fall 1995
Insect Resistant Tomato Trials
USDA Permit #95-216-03N
MONSANTO #95-202XR
[CBI DELETED], Maricopa, AZ

Andrew Reed

Tomato, *Lycopersicon esculentum*, has been genetically engineered to be resistant to selected insect pests (Lepidoptera). Resistance was accomplished by the stable insertion of a gene encoding the CryIAc protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 (CryIAc) into the tomato genome. Tomato varieties expressing the CryIAc protein, referred to as Insect Resistant (IR) tomato, are expected to significantly reduce chemical insecticide use in tomato production and, therefore, provide a major benefit to the tomato growers and to the environment. The purpose of the field trial was to evaluate the agronomic performance, measure protein expression of the genes introduced into the tomato genome, and fruit composition of an IR tomato. Transformation event 5345 is used as an example line in this study. The field trial was conducted at [CBI DELETED] Maricopa, AZ.

Experimental Layout:

Control and IR tomato plants were grown at field test site [CBI DELETED] Maricopa, AZ. However, the growth and development of both control and IR plants at this field site was not typical for the tomato variety used in these studies, and this field test was terminated before plant maturity. Based on reports from the Arizona Field Site Manager, [CBI DELETED] plant height varied from 1-3 ft above soil level at maturity, which is significantly smaller than normal growth (3-4 ft) of equivalent plants grown at the Florida field sites. The size of mature fruit harvested from either control or IR tomato plants at the Arizona field site was approximately one third the size of mature fruit harvested from the Florida field sites. Reasons for poor plant growth at the Arizona field site included heavy whitefly infestation at the time of seedling transplant, virus infestation during the growing season, off-season growth conditions (cool night temperatures), and that the tomato line is adapted for Florida growth conditions.

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CALGENE

Field Trial Report - Naples Trial #1 Fall 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - Farm 1A [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 9/6/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	7/29/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 1A [CBI DELETED] (Collier County)	9/6/96	11/25 - 12/24/96	1/15 - 1/24/97	2/97 - 8/97

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were wrapped in nylon netting for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN or Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 11/25, 12/4 and 12/24 for assessment of yield and size. Fruit were harvested into cardboard tomato packing boxes. Some fruit were transported to BHN Research in Bonita Springs for evaluation of ripening, firmness and flavor, after which they were buried at BHN, but most fruit were left at Farm 1A for burial in that field.

Trial Destruction

Upon completion of harvest, plants were destroyed by burning with propane flame-thrower (1/15/97), and disking (1/24/97). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between 2/97 and 8/97, the start of the next season.

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CALGENE

Field Trial Report - Naples Trial #2 Fall 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - Farm 3 [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 9/26/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	8/19/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 3 [CBI DELETED] (Lee County)	9/26/96	12/31/96 - 1/17/97	1/27 - 2/5/97	2/97 - 9/97

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were covered and labeled for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN or Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 12/31/96 and 1/17/97 for assessment of yield and size. Fruit were harvested into cardboard tomato packing boxes. Some fruit were transported to BHN Research in Bonita Springs for evaluation of ripening, firmness and flavor, after which they were buried at BHN, but most fruit were left at Farm 3 for burial in that field.

Trial Destruction

Within days after completion of the last harvest, plants were damaged by a hard freeze (1/20/97). To complete the destruction, plants were destroyed by burning with propane flame-thrower (1/24), and disking (1/31). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between 2/97 and 6/97, and will not be used for tomatoes in the 1997-1998 season.

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CALGENE

Field Trial Report - Naples Trial #3 Fall 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N 97-013-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 10/20/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	9/9/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 7 [CBI DELETED] (Collier County)	10/20/96	1/20 - 2/4/97	2/18 - 2/26/97	3/97 - 9/97

Trial Entries

The trial contained BHN Bt hybrids for which deregulation is currently being sought:

Variety	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN or Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 1/20 and 2/4/97 for assessment of yield and size. Fruit were harvested into cardboard tomato packing boxes. Some fruit were transported to BHN Research in Bonita Springs for evaluation of ripening, firmness and flavor, after which they were buried at BHN, but most fruit were left at Farm 7 for burial in that field.

Trial Destruction

Upon completion of the last harvest, plants were destroyed by burning with propane flame-thrower (2/22), and disking (2/26). After this treatment, no survivors nor

volunteers were found. The site was disked numerous times between 3/97 and 9/97, the start of the next season.

CALGENE

Field Trial Report - Naples Trial #4 Winter 1996 - 1997

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - Farm 7 [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N 97-013-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 11/20/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	10/7/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 7 [CBI DELETED] (Collier County)	11/20/96	No Harvest	3/12 - 3/21/97	4/97 - 10/97

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

Variety	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were wrapped in nylon netting for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN or Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

The intention was to assess fruit yield and size, however fruit from the trial was not harvested because plants had been damaged severely by a hard freeze on 1/20/97, rendering the trial uninformative. Plants and fruit were destroyed in the field as indicated below.

Trial Destruction

Following the decision not to harvest the trial, plants were destroyed by burning with propane flame-thrower (3/17/97), and disking (3/21/97). After this treatment, no survivors nor volunteers were found. The site was to be disked numerous times between April 1997 and October 1997, the start of the next season.

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CALGENE

Field Trial Report - Naples Trial #5 Spring 1997

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - Farm 4 [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N 97-013-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 12/28/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	11/11/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 4 [CBI DELETED] (Collier County)	12/28/96	4/3 - 4/23/97	4/24 - 5/5/97	5/97 - 12/97

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

Variety	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were wrapped in nylon netting for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN or Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 4/3, 4/14, and 4/23/97 for assessment of yield and size. Fruit were harvested into cardboard tomato packing boxes. Some fruit were transported to BHN Research in Bonita Springs for evaluation of ripening, firmness and flavor, after which they were buried at BHN, but most fruit were left at Farm 4 for burial in that field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower (4/29/97), and disking (5/5/97). After this treatment, no survivors nor volunteers were found. The site was to be disked numerous times between May 1997 and December 1997, the start of the next season.

CALGENE

Field Trial Report - Gargiulo GLP Trial Fall 1995

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - Farm 6 [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 94-362-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 9/22/95.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	8/18/95

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 6 [CBI DELETED] (Collier County)	9/22/95	12/28/95-1/8/96	1/18/96-2/28/96	3/96 - 8/96

Trial Entries

The trial contained BHN Bt inbreds for which deregulation is currently being sought:

	Construct	Event
BHN Bt inbreds	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 12/28/95 and 1/8/96 for yield assessment and assay. After weighing all the fruit and shipping a portion of it to Monsanto Co. in St. Louis, MO, the balance of the fruit was returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Plants were destroyed by burning with propane flame-thrower (1/18/96) and disking (2/28/96). After this treatment, no survivors nor volunteers were found. The site was

disked numerous times between March 1996 and August 1996, the start of the next season.

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CALGENE

Field Trial Report - Puerto Rico Trial #1 Fall 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo PR, Inc. [CBI DELETED] Santa Isabel, Puerto Rico	*Bt* [*cryIA(c)*]	Movement: 96-260-05N Field Release:96-247-14N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 10/24/96.

Chronology

Site of seedling production	Seeded in greenhouse	Shipped to Puerto Rico
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	9/9/96	10/21/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo PR, Inc. [CBI DELETED] Santa Isabel, Puerto Rico	10/24/96	1/0 2/13/97	2/20 - 3/18/97	4/97 - 10/97

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were wrapped in nylon netting for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored at least once every week to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants but did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 1/8, 1/21, 1/31 and 2/13/1997 for assessment of yield and size. Fruit were harvested into cardboard tomato packing boxes. Some fruit were transported to BHN Research in Bonita Springs, FL, for evaluation of ripening, firmness and flavor, after which they were buried at BHN, but most fruit were left in Puerto Rico for burial in the field.

Trial Destruction

Upon completion of the harvest, plants were sprayed with destroyed by burning with propane flame-thrower (2/25), and disking (3/18). After this treatment, no survivors nor volunteers were found. The site was to be disked numerous times between April 1997 and October 1997, the start of the next season.

CALGENE

Field Trial Report - Puerto Rico Trial #2 Fall 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo PR, Inc. [CBI DELETED] Santa Isabel, Puerto Rico	*Bt* [*cryIA(c)*]	Movement: 96-260-05N Field Release:96-247-14N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 12/11/96.

Chronology

Site of seedling production	Seeded in greenhouse	Shipped to Puerto Rico
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	10/21/96	12/2/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo PR, Inc. [CBI DELETED] Santa Isabel, Puerto Rico	12/11/96	2/25 - 3/31/97	4/10 - 6/6/97	6/97 - 11/97

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were wrapped in nylon netting for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored at least once every week to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 2/25, 3/6, 3/17, and 3/31/1997 for assessment of yield, size, ripening, firmness and flavor. Fruit were harvested into cardboard tomato packing boxes. After taking data, fruit were returned to the field for burial in the field. The vast majority of fruit were not harvested, and were destroyed along with the plants by disking and burial in the field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower (4/15), and disking (6/6). After this treatment, no survivors nor volunteers were found. The site will be disked numerous times between June and November 1997, the start of the next season.

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CALGENE

Field Trial Report - Port Charlotte Trial Spring, 1997

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - Farm 10 [CBI DELETED] (DeSoto County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N 97-013-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 1/27/97.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - Superior Plant Co. [CBI DELETED] (Collier County)	12/16/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 10 [CBI DELETED] (DeSoto County)	1/27/97	4/18 - 5/13/97	5/20 - 5/23/97	6/97 - 1/98

Trial Entries

The trial contained BHN Bt hybrids, for which deregulation is currently being sought:

Variety	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were packed for shipping in boxes separate from other transplants, and boxes were wrapped in nylon netting for containment.
5) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
6) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by Gargiulo personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN and Gargiulo personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN or Gargiulo personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 4/18, 5/2, and 5/13/97 for assessment of yield and size. Fruit were harvested into cardboard tomato packing boxes. Some fruit were transported to BHN Research in Bonita Springs for evaluation of ripening, firmness and flavor, after which they were buried at BHN, but most fruit were left at Farm 10 for burial in that field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower (5/20/97) and disking (5/23/97). After this treatment, no survivors nor volunteers were found. The site was to be disked numerous times between June 1997 and January 1998, the start of the next season.

CALGENE

Field Trial Report - BHN Research Fall Trial 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted between 9/3 and 9/25/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	7/15 - 8/16/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	9/3 - 9/25/96	12/12/96 - 1/24/97	1/27 - 1/31/97	2/97 - 8/97

Trial Entries

The trial contained BHN Bt hybrids, BHN Bt inbreds and BHN Bt breeding lines for which deregulation is currently being sought:

	Construct	Event
All BHN Bt materials	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Selected fruit from the trial was harvested between 12/12/96 and 1/24/97 for seed extraction. After grinding, remaining pulp and seed were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower and disking (1/31). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between 2/97 and 8/97, the start of the next season.

CALGENE

Field Trial Report - BHN Research Spring Trial 1995

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - Farm 8 [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 94-362-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trials were planted 3/1/95.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	1/19/95

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 8 [CBI DELETED] (Lee County)	3/1/95	6/12/95	6/12/95	6/95 - 1/96

Trial Entries

The trial contained BHN Bt inbreds for which deregulation is currently being sought:

	Construct	Event
BHN Bt inbreds	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Most plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. To assess efficacy of the *Bt* geneagainst tomato pinworm, in the efficacy plot, no sprays were applied to control that insect, and the natural population of tomato pinworms was augmented by a greenhouse-reared population. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trials were monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No

crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Selected fruit from the trials was harvested on 6/12/95 for assessment of yield and evaluation of insect damage, as well as for seed extraction. After taking data and grinding selected fruit for seed, remaining tomatoes, pulp and seed were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower and disked (6/12/95). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between June 1995 and January 1996, the start of the next season.

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CALGENE

Field Trial Report - BHN Research Trial Spring 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - Farm 3 [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trials were planted 3/6/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	1/26/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 3 [CBI DELETED] (Lee County)	3/6/96	6/18 - 7/3/96	7/5 - 7/7/96	7/96 - 12/96

Trial Entries

The trial contained BHN Bt hybrids, BHN Bt inbreds and BHN Bt breeding lines for which deregulation is currently being sought:

	Construct	Event
All BHN Bt materials	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the

Bt construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit was harvested between 6/18 and 7/3/96 for yield assessment and seed extraction. After taking data and grinding fruit for seed, remaining tomatoes, pulp and seed were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower (7/5/96) and disking (7/7/96). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between August and December 1996, the start of the next season.

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CALGENE

Field Trial Report - BHN Research Spring Trial 1997

Contains No Confidential Business Information

Field Trial Under USDA Notification

Location	Gene	Notification Number
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N 97-013-01N

Introduction

The objective of the trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 2/3 - 3/21/97.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	1/14 - 1/31/97

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	2/3 - 3/21/97	5/23 - 6/9/97	6/17 - 6/20/97	7/97 - 1/98

Trial Entries

The trial contained BHN Bt hybrids, BHN Bt inbreds and BHN Bt breeding lines for which deregulation is currently being sought:

	Construct	Event
All BHN Bt materials	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every one to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the

Bt construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Selected fruit from the trial was harvested between 5/23 and 6/9/97 for assessment of yield and seed extraction. After taking data and grinding selected fruit for seed, remaining tomatoes, pulp and seed were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower (6/17/97), and disking (6/20/97). After this treatment, no survivors nor volunteers were found. The site was to be disked numerous times between July 1997 and January 1998, the start of the next season.

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CALGENE

Field Trial Report - BHN Research GLP Trial Fall 1995

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	*Bt* [*cryIA(c)*]	Field Release: 94-362-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trials were planted 10/27/95.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	8/28/95

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	10/27/95	2/5/96	2/25/96	3/96 - 8/96

Trial Entries

The trials contained BHN Bt inbreds for which deregulation is currently being sought:

	Construct	Event
BHN Bt inbreds	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease and insect control according to standard cultural practices. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the *Bt* construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trials was harvested on 2/5/96 for yield assessment and assay. After weighing all the fruit and shipping a portion of it to Monsanto Co. in St. Louis, MO, the balance of the fruit was returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

On February 5, 1996, plants were destroyed by a hard freeze. Subsequently, they were, burned with propane flame-thrower and disked (2/25/96). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between March and August 1996, the start of the next season.

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CALGENE

Field Trial Report - Efficacy Trial Spring 1996

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - Farm 4 [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 96-011-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 3/28/96.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	2/14/96

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 4 [CBI DELETED] (Collier County)	3/28/96	6/18 -7/1/96	7/5 - 7/7/96	7/96 - 12/96

Trial Entries

The trial contained BHN Bt inbreds for which deregulation is currently being sought:

	Construct	Event
BHN Bt inbreds	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease control according to standard cultural practices. To assess efficacy of the *Bt* gene against tomato pinworm, no sprays controlling that insect were applied, and the natural population of tomato pinworms was augmented by a greenhouse-reared population. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the

Bt construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 6/18/96 and on 7/1/96 for yield and evaluation of insect damage. After taking data, fruit were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Plants were destroyed by burning with propane flame-thrower (7/5/96) and disking (7/7/96). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between July 1996 and January 1997.

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CALGENE

Field Trial Report - Efficacy Trial Spring 1995

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - Farm 2 [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 94-362-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 3/1/95.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	1/19/95

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - Farm 2 [CBI DELETED] (Collier County)	3/1/95	6/12/95	6/12/95	6/95 - 1/96

Trial Entries

The trial contained BHN Bt inbreds for which deregulation is currently being sought:

	Construct	Event
BHN Bt inbreds	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease control according to standard cultural practices. To assess efficacy of the *Bt* gene against tomato pinworm, no sprays were applied to control that insect, and the natural population of tomato pinworms was augmented by a greenhouse-reared population. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the

Bt construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Selected fruit from the trial was harvested on 6/12/95 for assessment of yield and evaluation of insect damage. After taking data, remaining tomatoes were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Upon completion of the harvest, plants were destroyed by burning with propane flame-thrower and disking (6/12/95). After this treatment, no survivors nor volunteers were found. The site was disked numerous times between June 1995 and January 1996, the start of the next season.

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CALGENE

Field Trial Report - Efficacy Trial Spring 1997

Contains No Confidential Business Information

Field Trial Under USDA Notification

Locations	Gene	Notification Number
Gargiulo, Inc. - BHN Research [CBI DELETED] (Collier County)	*Bt* [*cryIA(c)*]	Field Release: 97-013-01N

Introduction

The objective of this trial was to evaluate the *Bt* gene in fresh market tomatoes. The construct number evaluated was pMON 10518. The field trial was planted 3/12/97.

Chronology

Site of seedling production	Seeded in greenhouse
Gargiulo, Inc. - BHN Research [CBI DELETED] (Lee County)	2/18/97

Site of Field Release	Planting	Harvest	Destruction	Monitoring
Gargiulo, Inc. - BHN Research [CBI DELETED] (Collier County)	3/12/97	6/5/97	6/17-6/20/97	7/97 - 1/98

Trial Entries

The trial contained BHN Bt hybrids for which deregulation is currently being sought:

	Construct	Event
BHN Bt hybrids	pMON 10518	PV-LEBK04

The trial also contained non-transgenic controls.

Maintenance of Transgenic Plant Materials

To prevent the dissemination of propagules, the following precautions were taken:

1) Seed for this transgenic trial was kept separate from other seed before, during and after sowing.
2) Transplants for this transgenic trial were grown in seedling flats which were marked and kept separate from other flats.
3) Transplants for this transgenic trial were grown in limited-access planthouses.
4) Transplants for this transgenic trial were separated from all other tomatoes planted in the field by a 30-foot buffer.
5) Plants and fruit from this transgenic trial were destroyed after the trial.

Field Operations

Transplants were planted by hand and planting was supervised by BHN personnel. Leftover transplants were kept until resetting was completed, and then were destroyed. Plants were irrigated, fertilized, staked, tied, pruned and sprayed for disease control according to standard cultural practices. To assess efficacy of the *Bt* gene against tomato pinworm, no sprays controlling that insect were applied, and the natural population of tomato pinworms was augmented by a greenhouse-reared population. Harvesting of the fruit and data collection were conducted by BHN personnel.

Containment and Safety

No evidence of movement of the *Bt* gene from the transgenic tomatoes into other organisms was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored once every week to two weeks to check for potential problems with insects, diseases, stresses, and abnormal phenotypes. On these occasions, the field was walked by BHN personnel and observations were made, comparing plant morphology, flowering, fruit set, fruit ripening, and any abnormalities of the transgenic plants to their respective non-transgenic controls. Transgenic plants did not exhibit abnormal appearance or characteristics. No crown gall disease or cauliflower mosaic virus (CaMV) was observed. Plants transformed with the

Bt construct appeared normal with respect to plant morphology and fruit development when compared to the non-transgenic controls.

Harvest and Gene Function

Fruit from the trial was harvested on 6/5/97 for assessment of yield and evaluation of insect damage. After taking data, fruit were returned to the field for burial. The vast majority of fruit were not harvested, and were destroyed along with the plants, by disking and burial in the field.

Trial Destruction

Plants were destroyed by burning with propane flame-thrower (6/17/97) and disking (6/20/97). After this treatment, no survivors nor volunteers were found. The site was to be disked numerous times between July 1997 and January 1998.

Appendix 3. Expert Opinion Letters on the Growth and Morphology of Field-Grown IR Tomato Line 5345 and Control Tomato Plants

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UNIVERSITY OF FLORIDA

Southwest Florida Research and Education Center
Institute of Food and Agricultural Sciences

PO Drawer 5127
Immokalee FL 33934
(813) 657-5221
Fax (813) 657-5224

January 26, 1996

Dr. Yossi Shapiro
BHN Research
16750 Bonita Springs Road
Bonita Springs, FL 33923

Dear Dr. Shapiro:

After viewing the transgenic Bt tomato line(s) designated BtBHN and BtNTG on January 10, 1996, it is my considered opinion that the characteristics expressed by said plants are consistent with those of other fresh market tomato cultivars used in commercial production. All physical parameters viewed including stem development, leaf and flower architecture, and fruit external and internal qualities appeared, to the naked eye, to be identical to known cultivars. If I can be of further service in this matter please do not hesitate to contact me.

Sincerely,

[CBI DELETED]

97-287-01p

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UNIVERSITY OF FLORIDA Cooperative Extension Service

Institute of Food and Agricultural Sciences

14700 Immokalee Road
Naples, FL 33964
Tele: (941)353-4244
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January 26, 1996

Dr. Yossi Shapiro
BHN Research
16750 Bonita Beach Rd.
Bonita Springs, Fl 33923

Dear Dr. Shapiro:

This is to confirm that on January 10, 1996, I evaluated two research trials on transgenic tomato breeding lines. One trial, located behind the BHN facility, compared Bt\BHN-2, the transgenic breeding line, with the control, Cont\BHN-2. The other trial located in a production field compared Bt\NTG, the transgenic, to Cont\NTG, the control.

In evaluating the trials I examined the morphology of the flowers, the appearance of the leaves and fruit, and the general habit of plant growth. The phenology of the plants in the two treatments of both trials, were identical. If you would like more information or have any further questions please feel free to contact me.

Sincerely,

[CBI DELETED

]

SS:tjb

128 OF 0138

Appendix 4. Description of Methods used for Tomatine Analyses

Levels of an endogenous plant toxicant tomatine were measured in mature green and red ripe tomato fruit of IR Tomato Line 5345 and control lines collected from each field site. Analyses were according to Standard Operating Procedures (SOP) at Kelco-NutraSweet Inc. Tomatine levels were measured by an HPLC method described by Bushway *et al.* (1994). Fruit samples were extracted in tetrahydrofuran-water-acetonitrile-acetic acid (50:30:20:1), and C-18 Sep Paks and alumina were used for sample purification. Tomatine was quantitated by HPLC using a C-6 column and detection at 205 nm.

References

Bushway, R. J.; Perkins, L. B.; Paradis, L. R.; Vanderpan, S. High-performance liquid chromatographic determination of the glycoalkaloid, tomatine, in green and red tomatoes. *J. Agric. Food. Chem.* 1994, 42, 2824-2829.

Appendix 5. ELISA Validation Methodology and Results for Cry1Ac and NPTII Protein Analysis

Cry1Ac ELISA Validation: Precision, Accuracy, Sensitivity and Analyte Stability. Assay validation parameters were evaluated for measurement of Cry1Ac expression levels in tomato leaf, whole plant, and fruit tissues. Plant tissues used for assay validation were collected from the field sites described in this study.

Precision: Inter-assay precision was estimated from the analysis of aliquots of tissue extracts prepared from a pool of IR tomato tissue. These data were obtained from a minimum of 10 separate assays conducted over approximately a six month period.

Accuracy: i) Spike and Recovery. Tissues of control tomato plants were extracted in buffer spiked with known amounts of Cry1Ac protein, and recovery of Cry1Ac estimated by ELISA. The Cry1Ac protein was spiked at two different concentrations, that approximated to high and low values commonly observed in IR plant tissues. The percent recovery of Cry1Ac was estimated as the average for the two spike concentrations.

ii) Extraction Efficiency. The efficiency of Cry1Ac extraction from tomato tissues was estimated by the sequential extraction method. Extraction efficiency was calculated as the percent of Cry1Ac in the first extraction compared to the total amount of Cry1Ac extracted from the tissue in sequential extractions.

iii) Dilution Equivalence. A Cry1Ac dose-response curve was generated from serial dilutions of IR tomato extracts, using purified *E. coli*-produced Cry1Ac protein standard. The shape and slope of the dose-response curves for the Cry1Ac produced in IR tomatoes were compared to those for the *E. coli*-produced Cry1Ac to demonstrate dilution equivalence. Dilution equivalence shows that plant and *E. coli*-produced Cry1Ac proteins are immunologically and conformationally equivalent, and justifies use of the *E. coli*-produced protein for quantitation of Cry1Ac expression levels in IR tomato tissues.

iv) Confirming assay. Lyophilized leaf and fruit tissues of IR tomato were assayed for Cry1Ac protein concentrations by ELISA and a sensitive *Heliothis virescens* growth inhibition bioassay, to assess accuracy of the Cry1Ac ELISA for quantitation of Cry1Ac in IR tomato tissues.

Sensitivity: The sensitivity (lowest detectable dose) of the ELISA was defined as the amount of Cry1Ac that could be measured by an absorbance reading of 3 standard deviations above the background absorbance. The background absorbance and associated standard deviation were estimated from the ELISA absorbance values for more than 10 independent analyses of control samples.

Stability of CryIAc in Tomato Tissues and in Tissue Extracts. Because tomato tissues and tissue extracts were stored prior to analysis for Cry1Ac concentration, stability of Cry1Ac in tomato tissues and in tissue extracts stored at -80°C was determined. Tissues of IR tomato were extracted and assayed for Cry1Ac concentration over time intervals for approximately 4-6 months. Similarly, tissue extracts prepared at zero time and stored at -80°C were assayed at intervals over the same time period.

RESULTS

Cry1Ac ELISA Validation.

ELISA validation parameters (precision, accuracy, sensitivity, and storage stability) were established for measurement of Cry1Ac levels in plant tissues of IR tomato. The assay validation parameters are summarized in Table 1. The inter-assay precision of the Cry1Ac ELISA, measured as percent coefficient of variation (% CV), was 13.3% for leaf tissue and 10.2% for red ripe fruit tissue. Assay precision for the other tissue types ranged from a % CV of 11.8% (whole plant) to 27.8% (mature green fruit). Mean percent recovery (accuracy) of Cry1Ac spiked into plant tissues prior to extraction was 73.6% for leaf, 81.9% for red ripe fruit, and 88.2% for whole plant. There was no significant loss of Cry1Ac protein during extraction and assay. A single extraction of IR tomato tissues released 76% of the total Cry1Ac protein present in leaf tissue. Extraction efficiency of the Cry1Ac protein from the other IR tomato tissues was 94, 93, 81, and 26% for mature green, pink, red ripe fruit, and whole plant tissues respectively. The low value for extraction efficiency of Cry1Ac from whole plant tissue was most probably due to difficulties associated with protein extraction from mature, senescent, tissues. The sensitivity of the assay or lowest detectable level averaged 0.033 ng Cry1Ac per microtiter well. Under typical sample loading amounts per microtiter well, this is equivalent to a detection limit of approximately 0.1 ppm Cry1Ac in tomato tissues.

Table 1. Summary of Cry1Ac ELISA validation parameters for IR tomato line 5345 tissues

Measure	Tissue Type				
	Leaf	Fruit			Whole Plant
		Mature Green	Pink	Red Ripe	
Precision:					
Assay Variability (%CV)[1]	13.3	27.8	23.6	10.2	11.8
Accuracy					
Spike and Recovery (%)	73.6	N.A.[2]	N.A.	81.9	88.2
Extraction Efficiency (%)	76	94	93	81	26
Storage Stability:[3]					
Tissue and extracts (months)[4]	>6	>6	>5	>6	>6
Sensitivity:					
Lowest detectable dose[5]	0.033 ng/well				

1 CV= Coefficient of Variation

2 N.A. Not Analyzed

3 Stability of the Cry1Ac protein in IR Tomato tissues or tissue extracts stored at -80°C

4 Storage stability was determined over a 5 or 6 month period

5 Determined using control whole plant ELISA results

Table 2. Comparison of ELISA and insect bioassay[1] methods for measurement of CryIAc protein levels in leaf and fruit tissues of IR tomato.

Tissue	Line	Assay µg/g Tissue Dry Weight	
		ELISA[2]	Insect Bioassay
Leaf	control	N.D.[3]	N.D.
	IR	108.7	237
Red Fruit	control	N.D.	N.D.
	IR	7.5	N.D.

1 A *Heliothis virescens* growth inhibition assay

2 ELISA results have been corrected for recovery of Cry1Ac spike and for extraction efficiency

3 N.D. = Not Detected

NPTII ELISA Validation: Precision, Accuracy, Sensitivity and Analyte Stability.

Assay validation parameters were evaluated for measurement of NPTII expression levels in tomato leaf, whole plant, and fruit tissues from tissues of a delayed ripening tomato. The NPTII ELISA used for the delayed ripening tomato tissue was shown to be equivalent to that used for IR tomato tissues.

a. *Extraction Efficiency* Extractions were performed in triplicate for each tissue type. Percent extraction efficiency was calculated for each tissue by determining the percent of NPTII extracted in the first extract compared to the total amount of NPTII in all three extracts.

b. *Spike and Recovery* All extractions were conducted in triplicate using extraction solution of known concentration of NPTII and concentration of extracts determined by ELISA. Average percent recovery was determined by taking the mean percent recovery of all loading volumes and spike levels for each tissue type.

c. *Confirming Method* The confirming assay chosen for the NPTII ELISA was a western blot. Levels of NPTII were determined by visually comparing the intensity of the NPTII band in the samples with known concentrations of NPTII standard spiked into control tissue extracts. Concentrations of NPTII determined by western were compared with levels of NPTII calculated by ELISA.

d. *Criteria of Detection* The sensitivity of the NPTII ELISA was defined as the optical density (O.D.) corresponding to three standard deviations above the mean O.D. at zero analyte concentration.

e. *Limit of Detection* This was determined by taking the criteria of detection and converting this into an equivalent ng NPTII/well value.

f. *Stability of NPTII in Tomato Tissues and in Tissue Extracts* Because tomato tissues and tissue extracts were stored prior to analysis for NPTII concentration, stability of NPTII in tomato tissues and in tissue extracts stored at -80°C was determined. Tissues of DR tomato were extracted and assayed for NPTII concentration monthly for seven months. Similarly, tissue extracts prepared at zero time and stored at -80°C were assayed at intervals over the same time period.

g. Dilution Equivalence A comparison of the NPTII immuno-response dilution equivalence between *E. coli* produced NPTII and DR tomato produced NPTII was conducted by graphing ELISA assay absorbance versus the log concentrations.

RESULTS

Extraction efficiencies were well within the acceptable limits (>80%) (Table 3). Analyte spike and recoveries were also within the acceptable range of 80-120% (Table 3). Limits of detection ranged from 0.050-0.085 ng NPTII/well, which was below the amount of the lowest standard on the NPTII ELISA standard curve (0.1 ng/well). There was no statistical evidence of decay of NPTII in tomato tissues or extracts over a seven month period and therefore the storage conditions were sufficient to prevent degradation of samples.

Table 3. NPTII ELISA Validation Summary

Measure	Tissue Type: Fruit Red Ripe	Fruit Orange	Mature Green	Leaf
Precision:				
QC Sample Variability (%CV)	30.7	23.5	18.4	23.2
Assay Variability (%CV)[1]	30.1	25.2	24.5	34.2
Assay Working limits:				
Limit of Detection (ng/well)	0.075	0.065	0.050	0.085
Criteria of Detection (O.D.)	0.076	0.047	0.027	0.062
Accuracy:				
Extraction Efficiency (%)	98	98	93	91
Spike and Recovery (%)	109	86	106	96

[1] CV= Coefficient of Variation

Appendix 6. Western Blot Analysis for the Presence of the AAD Protein

MATERIALS and METHODS

Insect Resistant and Control Tomato Lines.
Insect Resistant (IR) tomato line 5345 was produced by *Agrobacterium tumefaciens*-mediated transfer of the *cry1Ac* gene encoding a protein from *Bacillus thuringiensis* var. *kurstaki* HD-73 (Cry1Ac) into the genome of commercial tomato variety UC82B. Line 5345 was backcrossed to a nontransgenic tomato cultivar, BHN-W. Insect resistant and control tomato plants were produced from each backcross. Progeny of the seventh backcross (BC_7F_1) of line 5345 were grown under field conditions at BHN-Research, Huron, CA (coded as CA in this study). There were four replicate plots of the IR and control tomato lines.

Test and Control Substances.
The test and control substances for analysis of detectable levels of the AAD protein were leaf and fruit tissues harvested from IR and control tomato plants, respectively, grown at the CA field site.

Reference Substance.
The reference substance used was AAD protein expressed and purified from *Escherichia coli* (*E. coli*) cells (lot number 4454557).

The Test System.
The test system is the Western blot, used to detect the AAD protein in tissues of the IR tomato line.

Collection, Transfer, and Treatment of Test and Control Substances.

Leaf: Approximately 3 weeks after transplanting seedlings to the field, one healthy, fully expanded, terminal leaflet of the youngest fully expanded leaf was harvested from six non-systematically selected plants in each plot. The leaves were pooled by plot, and immediately frozen on dry ice. Samples were packaged separately by plot, and shipped frozen by overnight carrier to Monsanto Co., St. Louis, MO. Leaf samples were crushed to frozen tissue powders, and approximately 150 mg leaf tissue per plot was composited across plots by line. The leaf samples were stored at approximately -80°C prior to analysis for the AAD protein.

Fruit: Tomato fruit at the mature green or breaker (less than 10% color) stages of ripening were harvested from each plot of the IR and control lines. The fruit were packaged separately by plot and shipped by overnight carrier at ambient temperature to Monsanto Co., St. Louis, MO. Fruit were washed in a 100 ppm chlorine solution, rinsed thoroughly in water, and dried at ambient temperature. The fruit were gassed with approximately 200 ppm ethylene to accelerate ripening to red ripe.

Approximately 10 red ripe fruit were non-systematically selected from each plot. The fruit were sectioned into quarters, and one section per fruit immediately frozen in liquid nitrogen. The frozen fruit sections were homogenized in liquid nitrogen to fine frozen tissue powders using a steel Waring blender, according to SOP# DRT-PRO-024. Approximately 5 g of frozen tissue per plot was composited across plots by line, and the frozen tissue powders were stored at -80°C prior to analysis.

Tissue Extraction.
Frozen leaf and fruit tissue powders of IR and control tomato lines were extracted in Laemmli buffer with 5% ß-mercaptoethanol at a tissue-to-buffer volume ratio of 1:20. A Brinkman overhead Polytron (Kinematica AG, Switzerland) was used for tissue extraction, as described in SOP# DRT-PRO-021. Extracts were centrifuged, and the supernatant stored frozen at -80°C prior to analysis.

Western Blot Analysis.
Tomato leaf and fruit extracts, and AAD protein standard were diluted into SDS-PAGE sample buffer and subjected to electrophoresis on 10-20% polyacrylamide gels (Integrated Separation Systems, Natick, MA)

according to SOP# BtC-PRO-026 to separate proteins by molecular weight. The proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham International, England) and hybridized with polyclonal antibodies raised in rabbit to purified AAD protein (F379, Bleed 3/7/91). Antibody bound to the blot was detected using a secondary donkey anti-rabbit antibody conjugated to horseradish peroxidase, enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham International, England), and exposure to X-ray film. Western blot analysis was conducted according to SOP # GEN-PRO-080.

Western Blot Validation Parameters: Accuracy, Limit of Detection, and Analyte Stability.
Assay validation parameters were evaluated for detection of AAD protein in IR tomato leaf and fruit tissues. Plant tissues used for assay validation were harvested from field grown plants.
Accuracy: i) Spike and Recovery. Tissues of control tomato plants were extracted in buffer spiked with known amounts of AAD protein, and recovery of AAD estimated by Western blot. The AAD protein was spiked at two different concentrations (1 and 5 pg per extract buffer volume). The percent recovery of AAD was estimated as the average for the two spike concentrations. Results were quantitated visually by comparison with band intensities of AAD standards on the same film.
Limit of Detection: Serial dilutions of the purified AAD protein spiked into control plant tissues were loaded onto the polyacrylamide gels at 0, 2, 5, 10, 20, 50, 100, 200 pg/gel lane. The limit of detection of the assay was the lowest AAD concentration that could be visually detected by Western blotting and ECL.
Stability of AAD in Tomato Tissues Extracts. The purified AAD protein was spiked into extracts of control tomato leaf and fruit tissues and stored frozen at -80°C. The samples were assayed for AAD concentration by Western blotting and ECL over time intervals for approximately 2 months.

RESULTS

Western Blot Validation Parameters.
The accuracy and limit of detection of the Western blot assay, and storage stability of the AAD protein in control plant extracts were assessed prior to sample analysis. The AAD protein produced and purified from *E. coli* was spiked into control samples prior to extraction. Recovery of the AAD protein was approximately 100% from both leaf and fruit tissue samples. Serial dilutions of the purified AAD protein spiked into control plant tissues showed that the visual limit of detection of the protein by Western blot and ECL was approximately 20 pg/well. Based on the quantity of plant tissue sample typically loaded for Western blot detection of the AAD protein, this is equivalent to a limit of detection of approximately 20 ng AAD/g tissue fresh weight. The AAD protein was stable in spiked plant extracts stored at -80°C for more than two months. Similar studies have shown that the AAD protein is stable as spiked into extracts of cotton leaf and seed extracts for at least 3 and 6 months, respectively, when stored at -80°C.

AAD Detection.
The IR and control tomato leaf and fruit tissues were extracted in SDS-PAGE sample buffer, and analyzed for the AAD protein. The AAD protein was not detected in either IR or control leaf (Figure 1) or fruit (Figure 2) tissues by Western blot analysis. As expected, the results confirm that the AAD protein is not expressed in IR tomato plants, within the limits of detection of the assay. Some immunoreactive bands were observed in lanes of IR leaf and fruit extracts, and were also observed in the control tissue lanes (Figures 1 and 2). These are background bands, and are a result of non-specific antibody binding to other proteins present in the tissue matrix. These non-specific immunoreactive bands did not interfere with the ability to detect the AAD-specific immunoreactive band.

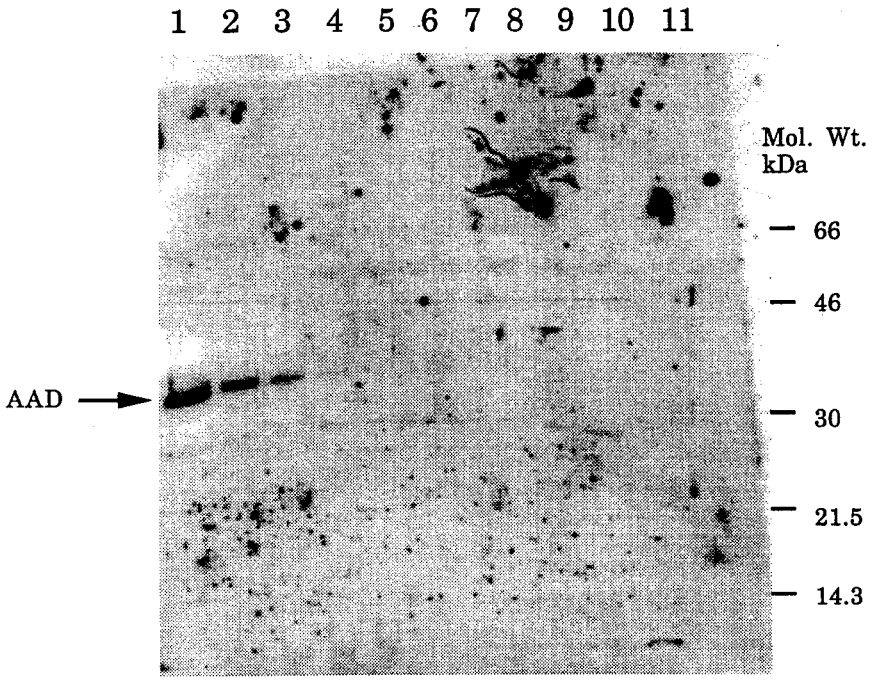
CONCLUSIONS

Insect resistant tomato varieties were developed by *Agrobacterium tumefaciens*-mediated transfer of the *cry1Ac* gene into the genome of a commercial tomato variety. Transgenic plants were generated by transformation with binary plasmid vector PV-LEBK04. The vector contains three genes that were transferred from *Agrobacterium tumefaciens* into the tomato genome: *cry1Ac*, *nptII* and *aad*. The *cry1Ac* and *nptII* genes are driven by plant-specific promoters and the corresponding Cry1Ac and NPTII proteins are expressed in IR tomato plant tissues. The *aad* gene is present in the vector to provide for selection in the laboratory. The *aad* gene, which is driven by a bacterial promoter, allows

for selection of bacteria in media containing spectinomycin or streptomycin, in steps prior to plant transformation. The *aad* gene, lacking a plant promoter, is not expected to express in IR tomato plants.

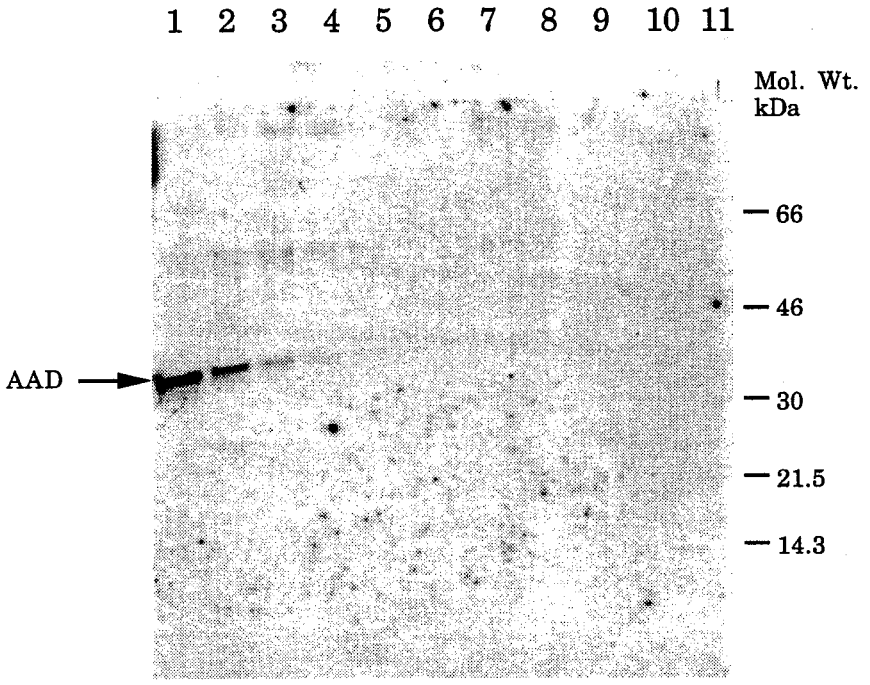
The results confirm that the AAD protein is not expressed in IR tomato plants, within the limits of detection of the assay.

Figure 1. Insect resistant tomato leaf does not express the AAD protein, as shown by Western blot analysis.



Lanes 1-8 contain AAD protein purified from *E. coli* and spiked into control leaf extract at 200, 100, 50, 20, 10, 5, 2, and 0 pg/well, respectively. Lanes 9 and 10 contain IR leaf extract loaded 20 µL/well. Lane 11 is empty. The positions of molecular weight markers are shown to the right of the blot.

Figure 2. Insect resistant tomato fruit does not express the AAD protein, as shown by Western blot analysis.



Lanes 1-8 contain AAD protein purified from *E. coli* and spiked into control red fruit extract at 200, 100, 50, 20, 10, 5, 2, and 0 pg/well, respectively. Lanes 9 and 10 contain IR red fruit extract loaded 20 µL/well. Lane 11 is empty. The positions of molecular weight markers are shown to the right of the blot.

Appendix 7. List of Non-Target Insect Toxicity Studies Submitted to EPA

MRID 43145204	Sims, S.R. "Sensitivity of Insect Species to the Purified CryIA(c) Insecticidal Protein from *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.* HD-73)" (1994), Study Number 92-01-36-17
MRID 4315205	Sims, S.R. "Stability of the CryIA(c) Insecticidal Protein of *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.* HD-73) in Sucrose and Honey Solutions Under Non-refrigerated Temperature Conditions" (1994), Study Number 92-01-36-15.
MRID 43145206	Maggi, V.L. "Evaluation of the Dietary Effect(s) of Purified *B.t.k.* Endotoxin Proteins on Honey Bee Larvae" (1993), Study Number 92-01-36-10.
MRID 43145207	Maggi, V.L. "Evaluation of the Dietary Effect(s) of Purified *B.t.k.* Endotoxin Proteins on Honey Bee Adults" (1993), Study Number 92-01-36-10.
MRID 43145208	Palmer, S.J. and Beavers, J.B. "*B.t.k.* HD-73 Protein: A Dietary Toxicity Study with Parasitic Hymenoptera (*Nasonia vitripennis*)" (1993), Study Number WL-93-234.
MRID 43145209	Palmer, S.J. and Beavers, J.B. "*B.t.k.* Hd-73 Protein: A Dietary Toxicity Study with Ladybird Beetles (*Hippodamia convergens*)" (1993), Study Number WL-93-232.
MRID 43145210	Palmer, S.J. and Beavers, J.B. "*B.t.k.* HD-73 Protein: A Dietary Toxicity Study with Green Lacewing Larvae (*Chrysopa carnea*)" (1993), Study Number WL-93-233.