95-053-01p

Petition for Determination of Nonregulated Status:

Tomatoes with a Delayed Ripening Gene

The undersigned submits this petition of 7 CFR 340.6 to request that the Director, BBEP, make a determination that the article should not be regulated under 7 CFR part 340.

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Contains No Confidential Business Information

Summary

The Agricultural Group of Monsanto Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding tomatoes with a delayed ripening gene. This petition requests a determination from APHIS that the delayed ripening (DR) tomato line 8338 and any progenies derived from crosses between line 8338 and traditional tomato varieties no longer be considered a regulated article under regulations in 7 CFR part 340.

Monsanto has developed tomato lines that are delayed in fruit ripening. These tomato lines have been modified to express the enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd), which catalyzes metabolism of ACC to ammonia and α-ketobutyrate. Because ACC is an essential precursor for ethylene biosynthesis and levels of ethylene initiate and control the rate of tomato fruit ripening, removal of ACC in these lines reduces ethylene production and delays ripening. Introduction of the delayed ripening trait into fresh tomatoes will allow harvest of vine-ripened tomatoes with extended market life, and supply good flavor quality fruit to the consumer nationwide.

The tomato line for which this determination is requested, DR tomato line 8338, contains a gene which encodes the enzyme ACCd from *Pseudomonas chloroaphis* strain 6G5. The ACCd protein is found in many different microorganisms that include several *Pseudomonas sp.* and *Enterobacter sp.*, the filamentous fungi *Paecilomyces variotti* and *Penicillium verrucosum*, and the yeasts *Hansenula saturnus* and *Hansenula polymorpha*. Delayed ripening tomato plants were produced by stable insertion of the *accd* gene into the genome of tomato cultivar UC82B. Based on Southern blot analysis, it was found that DR tomato line 8338 contains a single insert of DNA, and that this insert contains single copies of the *accd* and neomycin phosphotransferase (*nptII*) genes. The DR tomato line has reduced ethylene synthesis and delayed fruit ripening compared to the control line, but the DR and control lines are similar in all other aspects of plant growth and development.

Tomato plants containing the delayed ripening gene will enable growers to produce good taste quality fresh market tomatoes that have the market life attributes for a national distribution system. Current agronomic practices used for fresh market tomato production will not be changed for production of DR tomatoes. The delay in fruit ripening of DR line 8338 and other lines expressing ACCd is only observed after removal of the fruit from the plant. Fruit of DR tomato lines will be harvested at the breaker stage (first appearance of external fruit color), at most one to two days later than current harvest practice. Therefore, there will not be any significant increase in application of crop protection chemicals during production of DR tomatoes.

DR tomato line 8338 has been field tested since 1992 at seven locations under field release permits granted by APHIS (USDA # 92-049-01, 92-176-01, 93-054-01N, 93-063-04, 93-203-01, 94-014-01N, 94-234-01N). Data collected from these trials, literature references, and expert opinion letters presented in the following petition demonstrate that DR tomato line 8338: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than the non-modified parental varieties; 3) is unlikely to increase the weediness potential for any other cultivated plant or native wild species; 4) does not cause damage of processed agricultural commodities; and 5) is unlikely to harm other organisms that are beneficial to agriculture. Therefore, the Agricultural Group of Monsanto Company requests a determination from APHIS that the DR tomato line 8338 and any progenies derived from crosses between line 8338 and traditional tomato varieties no longer be considered a regulated article under regulations in 7 CFR part 340. Recently, the USDA has granted a determination of nonregulated status for a different tomato line that displays a similar delay in ripening due to decreased ethylene production (APHIS- USDA, 1994).

Reference:

Animal and Plant Health Inspection Service - United States Department of Agriculture (APHIS-USDA). 1994. Availability of Determination of Nonregulated Status for Genetically Engineered Tomato Line. Federal Register 60, 15:4588-4589.

ABBREVIATIONS AND SCIENTIFIC TERMS

AAD - aminoglycoside adenylyltransferase
ACC - 1-aminocyclopropane-1-carboxylic acid
ACCd - ACC deaminase
APH(3')-II - aminoglycoside-3'-phosphotransferase II
APHIS - Animal and Plant Health Inspection Service
bp - base pairs
DR- delayed ripening
ELISA - enzyme-linked immunosorbent assay
FDA - Food and Drug Administration
GLP - Good Laboratory Practices
kb - kilobase
kD - kilodalton
mg - milligram
NPTII - neomycin phosphotransferase II
NTSS - natural tomato soluble solids
µg - microgram
U.S. - United States
USDA - United States Department of Agriculture

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I. Rationale for Development of DR Tomatoes

A. Rationale

Monsanto has developed tomato lines that are delayed in fruit ripening. These tomato lines have been modified to express the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which catalyzes metabolism of ACC to ammonia and α-ketobutyrate (Honma and Shimomura, 1978). Because ACC is an essential precursor for ethylene biosynthesis (Adams and Yang, 1979; Yang, 1981), and levels of ethylene initiate and control the rate of tomato fruit ripening (Taiz and Zeiger, 1991), removal of ACC in these lines reduces ethylene production and delays ripening (Klee *et al.*, 1991; Klee, 1993).

1. Benefits of DR Tomatoes

Two major types of tomato products are grown in the United States: fresh tomatoes and processing tomatoes. Tomato varieties used for each of these applications are significantly different as are the cultural practices for producing each of these tomato products. However, both can benefit from the extension of ripening provided by the delayed ripening trait.

a. Fresh Tomatoes - The average annual *per capita* consumption of fresh tomatoes is 17 pounds and is increasing approximately 2 percent per year (Florida Tomato Committee Annual Report, 1991-1992). Sales of tomato, at the retail and food service level, have surpassed those of potato and lettuce. Annual sales of fresh tomatoes are valued at greater than $3.5 billion. However, while tomatoes have a large share of the U.S. produce market, tomatoes are universally considered by the consumer as having poor quality (Stevens, 1986).

The poor quality tomato product can primarily be attributed to a production system based on harvesting fruit at the mature green stage of development. Mature green fruit are firmer and have the handling and market life attributes necessary for a national distribution system. However, mature green fruit are indistinguishable externally from immature green fruit and immature fruit do not develop full flavor qualities when ripened by exposure to exogenous ethylene (Grierson and Kader, 1986; personal communication, Dr. D. Gull, Professor Emeritus, University of Florida, Appendix VII). During a typical commercial harvest, immature fruit can constitute 50% (ranging from 30-80%) of a total harvest (S. Chomchalow, 1991, Master's thesis, University of Florida, Appendix VII). To avoid contamination with inferior immature green fruit, many growers will harvest fruit showing color; (color formation indicates the fruit has progressed beyond the immature stage). These fruit, which are referred to as vine ripened, typically have a very short market life. To prolong the life of a vine ripened or mature green fruit, the retailer and/or consumer may refrigerate the tomato, which has been shown to destroy tomato flavor (Kader *et al.*, 1978; Buttery *et al.*, 1987).

Introduction of the delayed ripening trait into fresh tomatoes will allow the following benefits to be realized:

- Growers will be able to harvest fruit at the breaker stage (first break of color) eliminating the inferior immature green fruit from the harvest.
- Packers, shippers and retailers will be able to transport and store tomatoes at higher temperatures thereby saving energy and preserving flavor qualities.
- Packers, shippers and retailers will reduce fruit loss due to soft and over-ripe fruit thereby increasing the yield of marketable fruit.
- Packers and shippers will be able to expand the geographical distribution of the tomato product.

All of which provide the consumer with a better tasting tomato.

b. Processing Tomatoes - Processing tomatoes are allowed to remain on the vine until fully ripened. This allows the accumulation of flavor, texture and color components needed for high quality processed products. Fruit which over-ripen produce an inferior product. Once harvested, fruit are immediately shipped and processed into the desired end product. The processing facilities are generally designed to handle large volumes of tomatoes over a short period of time. In this system, growers need more flexibility with their harvest date to fit processing schedules and weather conditions that affect the harvest and crop quality. Since the delayed ripening trait delays over-ripening, the trait may provide greater vine holding capacity and allow the grower greater flexibility with processing and weather constraints.

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II. The Tomato Family

Description of the Genetics and Breeding of Tomato and its Production in the U.S.

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A. History of tomato

Lycoperiscon 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 *Lycoperiscon* species persist as perennials. 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 β-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 Fobes, 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 Fobes, 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. 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.

In recent years, tomato has been the focus of much molecular research and genetic engineering. It is an ideal candidate for this activity, not only because of its value as a vegetable crop, but because of excellent genetics, relatively-small genome and the fact that it is readily transformed with foreign DNA using *Agrobacterium*-based vectors. It was the first plant species in which the exact chromosomal positions were determined for DNA introduced via *Agrobacterium* (Chyi *et al.*, 1986). Results from those and subsequent studies have led to the conclusion that integration of foreign DNA is random, at least at the gross chromosomal level.

More than 50 known genes have been isolated from tomato. The list includes the genes encoding the small and large subunits of the carbon-fixing enzyme, ribulose bis-phosphate carboxylase (Pichersky *et al.*, 1987), the chlorophyll a/b binding polypeptides (Pichersky *et al.*, 1987), disease resistance (Martin *et al.*, 1993), ethylene biosynthesis (Picton *et al.*, 1993), fruit ripening (Penarrubia *et al.*, 1993), and self-incompatibility (Murfett and McClure, 1993).

D. Breeding of tomato

Tomatoes have been deliberately bred and selected by humans for more than 200 years and the Italians were the first to begin this endeavor. Most of the early selections emphasized variation in fruit size, shape and color and probably relied largely on chance spontaneous mutations since only limited natural variation existed in the European tomato germplasm. Cultivar development began in the United States in the late 1800's, but intensive breeding of tomato did not begin until the 1920's and was carried out at Land Grant universities and USDA facilities. Much of the recent breeding work on tomatoes (especially the past 25 years) has taken place in private breeding companies although government institutions continue to play a supportive role in germplasm development and local variety development.

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.

Prior to 1960, almost all tomato cultivars were true breeding, homozygous lines. In recent years, F_1 hybrids have gained in popularity. Currently, most commercial tomato varieties, both fresh market and processing, are hybrids. Most of the breeding efforts to develop F_1 hybrids has taken place in private companies and details of breeding methods are not generally available. However, it is common practice to test hybrid combinations using existing inbred lines, including previously released inbred varieties, or to derive new inbred lines from self-pollination and inbreeding of existing hybrid varieties.

Most of the qualitative improvement of tomatoes has been in the area of disease resistance. More than 50 single gene disease resistances have been identified in tomato, many having been introduced from the wild Lycopersicon relatives. Wild species have also been used as a source of the j-2 gene (jointless pedicels) which is important in mechanical harvest of field grown processing tomatoes (Rick, 1982).

E. Life cycle of tomato

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 seldom exceeds 5% 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.

Tomato seeds experience no natural dormancy and are readily germinated immediately after removal from ripe fruit. Seed viability is highly dependent on conditions of storage. In warm, humid climates, viability can drop substantially in a year or two. However, stored under dry, cool conditions, tomato seeds retain viability for 10 years or more.

In addition to sexual propagation, tomatoes can also be propagated by vegetative cuttings. Root formation occurs naturally on vegetative cuttings or can be promoted by exogenous hormone applications (e.g., auxin). Rooting of cuttings normally occurs in 1-2 weeks.

Commercially, nearly all tomatoes are propagated by seed. In the case of fresh market tomatoes, seeds are usually germinated in greenhouses and seedlings are then transplanted to the field. For processing tomatoes, direct seeding to the field is common. However, as hybrids become more popular and the price of seed increases, growers are also beginning to use transplants for processing tomatoes.

F. Tomato production -- practices /geography

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.

G. Potential for outcrossing

1. Out-crossing with non-transgenic cultivars. Cross pollination rates in modern tomato cultivars is very low -- typically less than 1% (Currence and Jenkins, 1942, Lesley, 1924). The risk of gene escape by outcrossing is further reduced since tomatoes are grown in relatively isolated conditions as pure lines (versus mixed populations). Under commercial growing conditions in the United States and most of the rest of the world, it is unlikely that transgenic tomatoes would cross naturally with other, non-transgenic cultivars. The only possible exceptions to this situation would be in Mexico, Central America and northwestern South America (Peru, Chile, Colombia and Ecuador) where primitive cultivars and the wild forms of tomato (*L. esculentum* var. *cerasiforme*) occur and can be found in or near commercial fields of tomatoes. In these areas, outcrossing rates in tomato can also be higher, possibly due to a greater abundance of pollinating insects (Rick, 1950).

2. Hybridization with species in the same genus. *L. pimpinellifolium* 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. Botanically it is very closely related to the cultivated tomato, and hybrids and hybrid progeny are readily obtained.

L. pimpinellifolium is restricted in its range to certain regions of Latin America (predominantly Peru and Ecuador) and therefore does not present a risk for gene exchange with transgenic cultivated tomatoes throughout most of the world. However, in the regions where *L. pimpinellifolium* does occur naturally, it is often found as a weed in commercial fields (including tomato

fields) and the possibility for gene exchange cannot be excluded. *L. pimpinellifolium* has not been reported in the United States, and, therefore, the risk of outcrossing from the transgenic tomatoes is negligible under commercial growing conditions.

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.

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III. Description of the Transformation System and Plasmid Utilized

The plasmid PV-LERP07 (pMON10117; Figure III.1), used to transform the parental tomato line UC82B to generate line 8338, contains two genes driven by plant promoters: the *accd* gene from *Pseudomonas chloroaphis,* strain 6G5 (Klee *et al.*, 1991) that codes for the 1-aminocyclopropane-1-carboxylic acid deaminase protein (ACCd), and the *nptII* gene encoding the neomycin phosphotransferase II protein, the kanamycin resistance marker gene (Beck *et al.*, 1982; Fraley *et al.*, 1983). These genes were introduced into tomato line UC82B using *Agrobacterium tumefaciens* as described below.

A. *Agrobacterium* Transformation System

The delayed ripening and marker genes were introduced into tomato using an *Agrobacterium tumefaciens* transformation system (Klee and Rogers, 1989). The intermediate vector, PV-LERP07, was assembled in *E. coli* K-12 cells and mated into an *Agrobacterium* strain using a triparental mating system (Ditta *et al.*, 1980). The *Agrobacterium* strain contains a disarmed plasmid which does not carry the T-DNA phytohormone genes. Therefore, the *Agrobacterium* is unable to cause crown gall disease and does not present a meaningful threat as a plant pest (Huttner *et al.*, 1992). Upon cultivation of plant tissue with the *Agrobacterium*, the T-DNA containing the delayed ripening and marker genes is excised and transferred to the plant cells by the *vir* functions encoded by the disarmed plasmid (Klee *et al.*, 1983; Stachel and Nester, 1986). The disarmed ABI *Agrobacterium* strain containing the PV-LERP07 vector was used to transform the tomato variety UC82B. T-DNA was transferred into individual tomato cells which were selected by their growth in the presence of kanamycin. Procedures for *Agrobacterium* transformation and regeneration of tomato tissue were performed as described by McCormick *et al.*, (1986).

The scientific literature supports the view that usually only the T-DNA is transferred and integrated into the plant genome (Fraley *et al.*, 1986). The sequence that is integrated includes only genes that are contained between the short, well-characterized border sequences of the T-DNA which are themselves essential for transfer and incorporation into the plant genome (Wang *et al.*, 1984; Gasser and Fraley, 1989) but are not precisely maintained during the process of insertion of the T-DNA into the plant genome (Zambryski *et al.*, 1982). Thus, the inserted DNA is no longer a functional T-DNA; i.e., once integrated, it cannot be remobilized into the genome of another plant even if acted on again by *vir* genes that effect transfer. All evidence available since the delineation of T-DNA in 1978, plus the accumulated epidemiology of crown gall disease, indicate that T-DNA transfer into plant cells by *Agrobacterium* is irreversible.

Molecular analysis of the inserted DNA in line 8338 demonstrates that only the delayed ripening and marker genes are present.

B. Recipient tomato variety, UC82B

Lycopersicon esculentum cv. UC82B is the tomato cultivar which was genetically modified to have a decreased rate of ripening 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). Our commercialization strategy for DR tomato is to use traditional backcrossing methods of breeding to transfer the delayed ripening locus from this cultivar to a wide range of varieties of processing and fresh market tomatoes.

C. Description of the Plasmid Utilized for Transformation, PV-LERP07

The lead delayed ripening tomato line 8338, was produced with the transformation vector PV-LERP07 (plasmid pMON10117; Figure III.1) which contains two genes that may be expressed in plants, ACC deaminase (*accd*) and the neomycin phosphotransferase (*nptII)* selectable marker gene (Fraley *et al.*, 1983). The proteins produced by these genes are described in more detail in following sections. PV-LERP07 is a double border vector containing the DNA sequences for the right and left borders of the T-DNA necessary for the *Agrobacterium* Ti plasmid transformation system. The genes and DNA components used to construct them are briefly described below and in more detail in following sections.

The cauliflower mosaic virus (CaMV) 35S promoter region (Sanders *et al.*, 1987; Gardner *et al.*, 1981) drives the expression of the *nptII* gene (Beck *et al.*, 1982). The nucleotide sequence of the vector *nptII* gene (Keck, 1993) is identical to the *nptII* gene sequence reported by Beck *et al.*, 1982, and differs from that reported by Calgene (1993) for *aph(3')-II* by a single nucleotide at position 180. This third position change does not alter the encoded amino acid and the NPTII protein is identical to the APH(3')-II protein that is an approved food additive (Food and Drug Administration, 1994). The marker gene is completed by the nopaline synthase (NOS) 3' region that directs polyadenylation of the mRNA (Fraley *et al.*, 1983; Depicker *et al.*, 1982). The gene encoding ACCd (isolated from *Pseudomonas chloroaphis*, strain 6G5; Klee *et al.*, 1991) is driven by a caulimovirus 35S promoter isolated from a cloned, modified figwort mosaic virus adapted for growth on *Datura stramonium* (Shepherd *et al.*, 1987; Richins *et al.*, 1987) and a 5' nontranslated leader from a *Petunia hybrida* HSP70 gene (Winter *et al.*, 1988). The *accd* gene is followed by a non-translated region of the pea rbc-E9 gene (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985) which directs polyadenylation of the mRNA. The caulimovirus 35S promoter drives expression constitutively resulting in production of mRNA in

most cells of the plant (Benfey *et al.*, 1989)

The vector also contains the bacterial selectable marker gene, aminoglycosideadenylyltransferase (*aad*), which confers spectinomycin resistance. The *aad* gene is driven by its own bacterial promoter (Fling *et al.*, 1985) and therefore is not expected to express in the plant. More importantly, direct analysis has shown that the *aad* gene is not present in the DNA of line 8338.

The vector PV-LERP07 is shown in Figure III.1. The location and extent of each genetic element that comprises plasmid PV-LERP07 (pMON10117) are listed below. The origin for numbering the nucleotides of the plasmid is located just inside the T-DNA Right Border at the 5' end of the P-FMV fragment.

Nucleotides 1 to 574: P-FMV. The 35S promoter from a modified figwort mosaic virus (Shepherd *et al.*, 1987; Richins *et al.*, 1987).
Nucleotides 575 to 681: PetHSP70-leader. The transcribed, nontranslated leader sequence from the petunia HSP70 gene (Winter *et al.*, 1988).
Nucleotides 682 to 1757: ACC deaminase. The ACC deaminase gene isolated from *Pseudomonas chloroaphis, strain* 6G5 (Klee *et al.*, 1991).
Nucleotides 1758 to 2416: E9 3'. The 3' end of the pea rbcS E9 gene which provides the polyadenylation sites for the ACC deaminase gene (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985).
Nucleotides 2417 to 2786: P-35S. The 35S promoter from cauliflower mosaic virus (Sanders *et al.*, 1987).
Nucleotides 2787 to 3631: KAN. The neomycin phosphotransferase type II gene confers resistance to kanamycin in plant cells (Beck *et al.*, 1982; Fraley *et al.*, 1983).
Nucleotides 3632 to 3898: NOS 3'. The 3' end of the *Agrobacterium tumefaciens* nopaline synthase gene which encodes the polyadenylation sites for the KAN gene (Depicker *et al.*, 1982).
Nucleotides 3899 to 4381: Left Border segment isolated from the octopine Ti plasmid, pTiA6 and contains the direct repeat sequence (bases 4199 to 4222) that delimits the T-DNA transferred.

All DNA located clockwise beyond the left border of pMON10117, Figure III.1, up to the right border is not transferred to plant cells. The nontransferred segment includes all of the genetic elements listed below up to the right border.
Nucleotides 4382 to 5170: ori-V. The vegetative origin of replication that permits plasmid replication in *Agrobacterium* and was originally isolated from plasmid RK2. The function of this origin in binary plasmid vectors such as pMON10117 is described (Rogers *et al.*, 1987).
Nucleotides 5171 to 8136: DNA from pBR322 containing rop (the replication of the primer region) and ori-322. Plasmid replication origin permitting propagation of DNA in bacterial hosts such as *E. coli* (Sutcliffe, 1979).

Nucleotides 8137 to 9197: Spc/Str. The bacterial gene encoding the Tn7 AAD 3' adenylyltransferase conferring spectinomycin and streptomycin resistance on bacterial cells that carry the plant vector (Fling *et al.*, 1985).
Nucleotides 9198 to 9526: Right Border. This segment contains the nucleotide direct repeat sequence (9451 to 9474) that acts as the initial point of DNA transfer into plant cells and was originally isolated from pTiT37 (Depicker *et al.*, 1982).

All of the DNA segments from the right border clockwise toward the left border sequence in Figure III.1 are present in plant cells.

Extensive restriction analysis of the plasmid PV-LERP07 demonstrated that all of the genetic elements and restriction fragments were correctly assembled and produced the correctly sized DNA fragments when digested and separated on a 1.0% agarose gel.

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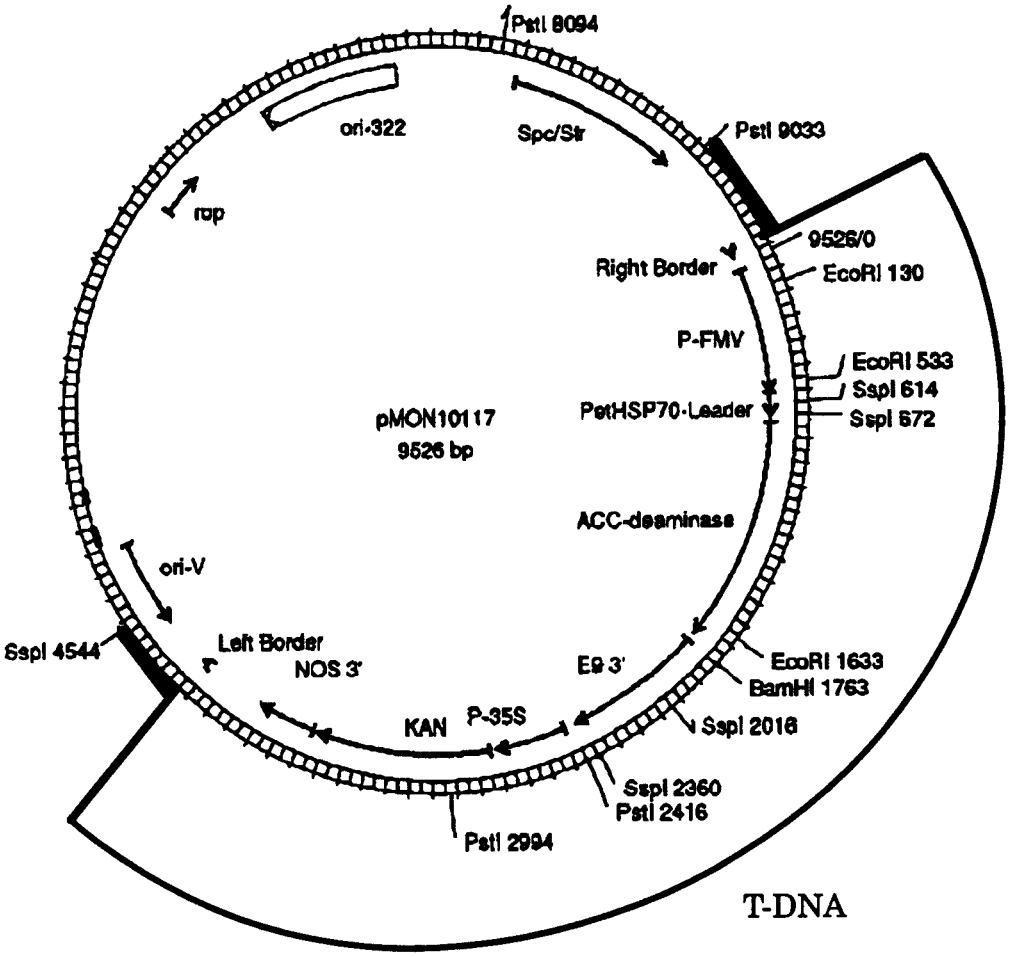
Figure III.1. Plasmid PV-LERP07 (pMON10117)

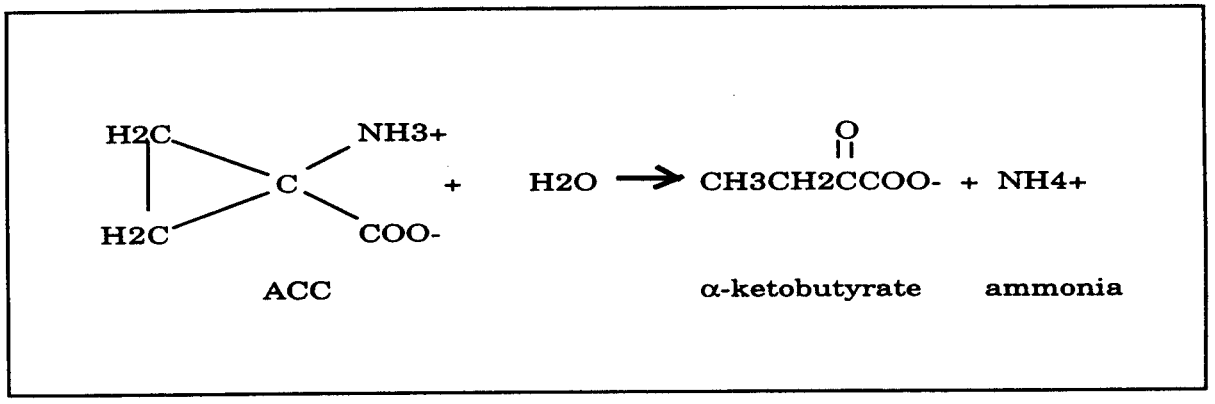


IV. The Genes Present in DR Tomato Line 8338.

A. *ACCd* and *NPTII*

Two genes were transferred from PV-LERP07 to the UC82B parent tomato line that encode the enzymes ACCd and NPTII. The *accd* gene, which confers the delayed ripening trait, was isolated from the soil bacterium *Pseudomonas chloroaphis*, strain 6G5 (Klee *et al.*, 1991). The ACCd protein contains the coenzyme pyridoxal phosphate, and catalyzes deamination of ACC to ammonia and α-ketobutyrate as shown below. Pyridoxal phosphate cofactor requiring enzymes such as transaminases are involved in amino acid biosynthesis. ACCd is found in several additional common soil bacteria including several *Pseudomonas sp.*, *Enterobacter cloacae, Enterobacter agglomerans*, *Xanthomonas maltophilia* and *Achromobacter sp.* (Tran and Kretzmer, 1993). The ACCd protein has also been found in the filamentous fungi *Paecilomyces variotii* and *Penicillium verrucosum* and the yeasts, *Hansenula saturnus* and *Hansenula polymorpha* which are in the same family as brewer's and baker's yeast (Tran and Kretzmer, 1993). The *Hansenula saturnus* protein cross-reacts with antiserum prepared against the *Pseudomonas chloroaphis*, strain 6G5 protein.

The biosynthetic pathway of the phytohormone ethylene, and the stimulatory effect of ethylene on tomato fruit ripening have been well characterized (Taiz and Zeiger, 1991; Grierson and Covey, 1988; Grierson and Kader, 1986). The amino acid methionine is converted to ethylene in a series of reactions involving S-adenosyl methionine (SAM) and ACC as metabolic intermediates.



Delayed ripening tomato plants were produced by stable insertion of the *accd* gene into the tomato chromosome (Klee *et al.*, 1991). ACCd metabolizes ACC making it unavailable for ethylene production in the tomato. Consequently, fruit ripening and over-ripening are delayed relative to controls (Klee *et al.*, 1991).

The *Pseudomonas chloroaphis*, strain 6G5 *accd* gene has been completely

sequenced and encodes a 36.8 kD protein consisting of a single polypeptide. *Pseudomonas chloroaphis* is commonly isolated from water (Palleroni, 1984) and is a saprophyte which is not associated with any human, animal or plant pathogenicity (Doudoroff and Palleroni, 1974). The products of ACCd metabolism, ammonia and α-ketobutyrate, are natural metabolic intermediates in plant amino acid biosynthesis (Goodwin and Mercer, 1990), and, based on all available information, are expected to be rapidly re-assimilated. The levels of ACC in tomato fruit are very low, increasing from 0.1 to 10 nmol/g fresh weight during ripening from green to red fruit (Hoffman and Yang, 1980). Consequently, endogenous levels of ammonia and α-ketobutyrate in tomato tissues are expected to be comparable in both delayed ripening and control plants.

The NPTII protein catalyzes the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics, thereby inactivating the antibiotics. Therefore, the presence of the NPTII protein in the plant genome allows selection of transformed tomato cells in the presence of the antibiotic kanamycin. The nucleotide sequence of the vector *nptII* gene (Keck, 1993) is identical to the *nptII* gene sequence reported by Beck *et al.*, 1982, and differs from that reported by Calgene (1993) for *aph(3')-II* by a single nucleotide at position 180. This third position change does not alter the encoded amino acid and the NPTII protein is identical to the APH(3')-II protein that is an approved food additive (Food and Drug Administration, 1994).

B. References

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V. Genetic Analysis, Agronomic Performance, and Compositional Analysis of Line 8338

A. Description, History, and Mendelian Transmission of the Gene Encoding ACCd in DR Tomato Line 8338

DR tomato line 8338 is a homozygous R_2 selection from an original R_0 transformant, 8338, which was obtained by *Agrobacterium* transformation of the public variety, UC82B with vector PV-LERP07, as described above. This vector contains a gene encoding ACCd and the gene encoding the NPTII marker protein, as shown in Figure III.1.

The original 8338 R_0 transformant was identified following evaluation for ACCd by western blot assay. The 8338 R_0 was selfed in the greenhouse to obtain R_1 seed. R_1 progeny of line 8338 were sown in the greenhouse on May 12, 1992 for a Jerseyville, IL field trial. R_1 progeny were screened for ACCd expression prior to transplanting in the field under USDA permit # 92-049-01. Plants negative for ACCd expression (assessed by western blot) were discarded leaving a mixture of homozygous and heterozygous expressing plantlets for transplanting in the field. The R_1 screening data showed a 3:1 segregation for ACCd expression (70.5% of the plantlets expressed) as expected for a single locus inserted into the tomato genome.

Seed were collected from individual ACCd expressing plants from the field and plants homozygous for ACCd expression were identified. Seed were generated by allowing the R_1 plant to self. These seed were designated homozygous R_2 progeny and are referred to as line 8338. Homozygous R_2 seed derived from the plant designated 8338 2-1, were tested in 1992-1993 regulatory field trials in Florida, under USDA-APHIS permit #92-176-01.

When line 8338 2-1 was backcrossed to nontransgenic commercial tomato varieties, 100% of the progeny expressed ACCd. On further backcrosses out to BC_6F_1 material from seven commercial lines, all data collected to date suggest the *accd* gene is stably integrated into the tomato genome and is present as a single copy. Progenies of crosses between other tomato lines and the heterozygous backcrossed lines derived from DR tomato line 8338 2-1 yielded the expected segregation ratio of approximately 1:1 with respect to ACCd expression (Table V.1).

This establishes that the 8338 insert behaves as a single dominant gene inherited in a Mendelian fashion. All of the data are consistent with there being a single, stable insert of the ACCd and marker genes in line 8338.

Table V.1. ACCd Segregation Data for Backcross Progeny of DR Tomato line 8338 with Different Nontransgenic Tomato Varieties.

Generation	Nontransgenic parental line	Number expressing	Number Negatives	X^{2*}
$BC1F_1$	FL 1B	15	13	0.14
$BC5F_1$	BHN Ax	165	191	1.9
$BC5F_1$	BHN Dx	159	133	2.3
$BC5F_1$	BHN Hx	177	193	0.69
$BC6F_1$	BHNA	7	7	0.0
$BC6F_1$	BHN D	9	7	0.25
$BC6F_1$	BHN E	4	5	0.11
$BC6F_1$	BHN I	8	3	2.27
$BC6F_1$	BHN J	4	2	0.67
$BC6F_1$	BHN K	6	8	0.29
$BC6F_1$	BHN L	7	3	1.6
			Total[1]	10.24

* = Uncorrected goodness-of fit-test for hypothesis of 1:1 segregation. None of the chi-square values are significant at a significance level of 95% ($X^2_{0.05,\ 1\ d.f.} = 3.8$).
[1] Total Chi-square value not significant at significance level of 95% ($X^2_{0.05,\ 10\ d.f.} = 18.3$).

B. DNA Analysis of Delayed Ripening Tomato Line 8338

As described in section III, line 8338 was derived from *Agrobacterium tumefaciens*-mediated transformation of UC82B tomato line with plasmid PV-LERP07 (Figure III.1). DNA analyses were performed to address three key points regarding the DNA insertion event(s) in line 8338: 1) the number of insertion sites where DNA derived from PV-LERP07 has integrated into the plant genomic DNA; 2) the identity of the DNA elements that are present in the inserted DNA; and 3) the stability of the DNA insert in the genome of line 8338 through backcrosses to nontransgenic tomato varieties. Genomic DNA of DR line 8338 and control lines were analysed by Southern blot (Southern, 1975). Hybridization probes (^{32}P-labelled) for the *accd*, *nptII*, and *spc/str* (*aad*,

spectinomycin/streptomycin resistance) genes and the origin of replication (ori-pUC) were used in these analyses. Examples of some Southern blots are shown in Figures V.1, V.2, and V.3, but not all Southern blots for these studies are presented. In these analyses of the DNA insert in DR tomato line 8338, differences between expected and observed fragment sizes are within the variability associated with fragment size determinations using Southern blot. Also, differences in fragment size determinations from blot-to-blot are within the variability associated with the method. As expected, there was no specific hybridization of the *accd, nptII, spc/str,* and origin of replication probes with restriction digest fragments of control tomato line UC82B (examples in Figures V.1, V.2, and V.3).

1. Number of insertion sites in line 8338

The number of DNA insertions in line 8338 was determined using SpeI and EcoRV, restriction enzymes that do not cut inside the plasmid PV-LERP07, (known restriction enzyme sites within the plasmid are shown in Figures III.1 and V.4). Since no internal fragmentation of PV-LERP07-derived DNA can occur with SpeI and EcoRV, the number of bands present in each Southern blot corresponds to the minimum number of inserts. Examples of Southern blots probed with *accd,* and *spc/str* are shown in Figures V.1 and V.2, respectively. Single unique DNA bands were present in the 8338 digest with SpeI and EcoRV, respectively, but not in the UC82B control digests, when probed with either *accd* or *nptII*. These results suggest that DNA derived from PV-LERP07 was inserted at a single site in the genomic DNA of line 8338. DNA fragments produced by SpeI and EcoRV digests did not hybridize with either the *spc/str* or origin of replication probes, showing the absence of the *spc/str* and origin of replication genes in the genome of line 8338.

2. Identity of the DNA elements present in the insertion of 8338.

The single DNA insert in line 8338 contains the *accd* and *nptII* genes, therefore, the only proteins encoded by PV-LERP07 DNA present in line 8338 are the ACCd and NPTII proteins. Genomic DNA preparations from DR line 8338 and control line UC82B were digested with EcoRI, SspI, PstI, and BamHI, restriction enzymes that cleave within the T-DNA borders of PV-LERP07. Examples of the Southern blots are shown in Figures V.1, V.2, and V.3. The size of the DNA fragments that hybridized with the *accd* and *nptII* probes were of the predicted size based on known restriction sites within the plasmid (Figure III.1 and V.4). These data show that the *accd* and *nptII* genes are present as single copies at a single locus in the genome of line 8338. There were no DNA fragments that hybridized with either the *spc/str* or origin of replication probes, confirming the absence of these genes in the genome of line 8338. A schematic diagram of the T-DNA insert in the genome of line 8338 summarizes these results (Figure V.4), which confirm the expected functioning of the two T-DNA border sequences. Only the *accd* and *nptII* genes within the T-DNA were transferred from vector PV-LERP07 to a single locus in the tomato genome.

Also, the structural integrity of the T-DNA was maintained during transfer.

3. Insert stability

Line 8338 was backcrossed to three different nontransgenic tomato varieties (designated BHN-B, BHN-H, BHN-N) through four generations to determine the stability of the DNA insert in the genome of line 8338. Genomic DNA of the backcross lines were analysed by Southern blot, using some of the same restriction enzymes and hybridization probes as described for line 8338 analysis. An example of Southern blot analysis of these lines using the *accd* hybridization probe is shown in Figure V.1. Patterns of hybridization for the backcrossed lines were identical to those observed for the parental 8338 line. These results verify the stable integration and transfer of the inserted DNA in line 8338 through four backcross generations with nontransgenic tomato varieties.

4. Summary of the DNA analysis of DR tomato line 8338

The single DNA insert in line 8338 contains only the *accd* and *nptII* genes, and, thus, the only proteins encoded by PV-LERP07 DNA present in line 8338 are the ACCd and NPTII proteins. This conclusion is based on the following data: 1) the positive detection of the *accd* and *nptII* genes by Southern blot analysis; and 2) the lack of ori-pUC and *spc/str* signals by Southern blot analysis. The ends of the 8338 insert have been mapped to within several hundred nucleotides. Based on extensive restriction analysis of line 8338 DNA, it was concluded that the maximal size of PV-LERP07 DNA contained in line 8338 is 5.04 kb and lies between the PstI site at nucleotide 9033 and the SspI site at nucleotide 4544 (Figures III.1 and V.4). This T-DNA insert is shown schematically in Figure V.4 and the complete T-DNA nucleotide sequence shown in Appendix I. The DNA terminates between the border sequences and the PstI or SspI restriction sites, most likely near the 25 bp border sequences. Computer translation of the nucleotide sequences between the borders and the PstI or SspI restriction sites (these sequences are potentially present in line 8338), revealed only one open reading frame capable of encoding a protein of molecular weight greater than 5 kD. This sequence (nucleotides 4395 to 4201) located outside the left border was derived from pTiA6 octopine Ti plasmid sequences located outside the T-DNA. Therefore, this is an *Agrobacterium* bacterial gene without plant gene transcription signals and is not expected to express in plants. The sequence of the T-DNA between the PstI and SspI restriction sites is shown in Appendix 1. In addition, the genetic insert in line 8338 remained stably integrated in the plant genome through four successive backcrosses to nontransgenic tomato varieties. In conjunction with *accd* gene inheritance data previously discussed, these results verify that there is a single, stable DNA insertion in line 8338, and the *accd* gene behaves as a single dominant gene inherited in Mendelian fashion.

C. DR Tomato line 8338 Field Tests for Analytical Evaluation

In order to generate plant material for protein expression and fruit quality analysis, four field tests were conducted in the State of Florida during the 1992-1993 growing season (USDA permit # 92-176-01). The tomato lines tested consisted of three genotypes: control line UC82B and DR tomato line 8338, as well as an additional DR tomato line designated 5673. DR tomato line 5673 is not of commercial significance, and analyses of this line are not included in this report. Plants of DR tomato line 8338 were grown from homozygous R_2 seed, selected from original R_0 transformants of the line. Seed of parental control line UC82B were obtained from Ferry Morse Seed Co., Modesto, CA. Seeds of each tomato line were germinated in minicell trays, transplants grown under greenhouse conditions at BHN-Joint Venture, Bonita Springs, FL, for approximately six to seven weeks, and tomato seedlings transplanted to four separate field sites in Florida. Field site locations and transplant dates were as follows: Field Site 1, Gulfcoast farm # 2, Greenway Rd N., Collier County, FL, transplanted 11/13/92; Field Site 2, Gulfcoast farm # 7, 15000 E. Tamiami Trail, Naples, FL, transplanted 11/23/92; Field Site 3, BHN-Joint Venture, 16750 Bonita Beach Rd., Bonita Springs, FL, transplanted 11/20/92; and Field Site 4, Gulfcoast farm # 11, Immokalee Road N., Collier County, FL, transplanted 12/31/92. Plant beds at all field sites were spaced as 6 ft center-to-center rows. Fertilizer was applied in the beds according to soil test recommendations, and beds were fumigated (67% methyl bromide, 33% chloropicrin) at 2.5 lbs per 100 ft. linear row. Seven or more days after fumigant application, plants were transplanted into the beds through the polyethylene mulch, and were spaced approximately 20 inches apart.

The plot design was completely random at Field Sites 1 and 2, and was a randomized complete block at Field Sites 3 and 4. There were four replicates of each tomato line at each of the field sites. Normal Florida fresh market tomato production practices were used for plant culture at each field test site. These practices included resetting weak transplants, plant staking, tying, seep irrigation, and use of registered pesticides within the labeled application rates for control of weeds, insects, and diseases. No adverse effects from environmental or other conditions occured during these studies.

Leaf tissue was harvested from plants grown at Field site 3, at approximately 2 weeks after transplanting, and at approximately 3-week intervals thereafter throughout the growing season. One healthy fully expanded terminal leaflet of the youngest fully expanded leaf was excised from six randomly selected plants per plot. The leaves were pooled by plot, immediately frozen on dry ice, and shipped frozen by overnight carrier to Monsanto Co. Leaves were stored frozen at -80°C prior to ACCd and NPTII expression analysis. Fruit were harvested at different stages of ripening from each of the four field sites. The fruit were analyzed for ACCd and NPTII expression, and for quality components.

D. Disease and Pest Characteristics

Line 8338, and backcross progeny, have been field tested in the U.S. in 1992, 1993, and 1994 (USDA-APHIS permit #s 92-049-01, 92-176-01, 93-054-01N,

93-063-04, 93-203-01, 94-014-01N, and 94-234-01N). Detailed monitoring for the disease and insect susceptibility of line 8338, versus the control UC82B line, was performed at the four Florida field sites listed in Table V.2. These field sites have been described in section V.C. No differences in insect infestation or severity were detected between the DR tomato lines (including line 8338) and the control line, UC82B (Table V.2). Susceptibility to *Fusarium* crown rot disease (*Fusarium oxysporum*, f.sp. *radici-lycopersici*) was noted in the California variety UC82B when grown under Florida conditions. Line 8338 showed a further increase in susceptibility to the disease under similar conditions. This susceptibility is overcome by introduction of a *Fusarium* crown rot resistance gene, *fcr*, into line 8338 (Appendix II, Attachment I). It is important to note that approximately 90% of the tomatoes grown commercially in Florida are susceptible to *Fusarium* crown rot disease (Appendix II, Attachment I), consequently there will be no increased application of crop protection chemicals during production of DR tomatoes. There were no differences between line 8338 and the control line UC82B in susceptibility to other diseases monitored in these studies. See Appendix II for USDA final reports, and Appendix III for example monitoring forms.

Private 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 his/her plots to decide on the acceptability of a new line for commercial release. Tomato breeders normally walk through a representative number of plots of the variety to be released to visually check for the appearance of possible disease symptoms such as spotted leaves, leaf necrosis, stunted or distorted plants, and wilting of the plants. They make notes on insect populations, including armyworms, loopers, fruitworms, and pinworms. They also make notes of any other undesirable characteristics that may be noticeable, for example flowering time and vegetative growth characteristics.

Table V.2. 1992-1993 DR Tomato Field Sites Reporting Line 8338 Status.

Site	Difference in Susceptibility versus UC82B Control	
	Disease	Insect
Field Site 1[1]	No*	No
Field Site 2	No*	No
Field Site 3	No*	No
Field Site 4	No*	No

* Differences in susceptibility to *Fusarium* crown rot disease between DR tomato line 8338 and control UC82B were observed. (See text above) There were no differences in susceptibility to other diseases between the lines.

[1] The location of each field site in Florida is described in section V.C.

E. Yield Characteristics of DR Tomato Line 8338

Yield of DR tomato line 8338 was statistically equivalent to yield of control line UC82B at Florida Field Sites 1 and 3, but was significantly lower than the control at Field Sites 2 and 4 (Table V.3). The lower yields of line 8338 at these sites were attributed to *Fusarium* crown rot disease pressure. Since susceptibility of line 8338 to *Fusarium* crown rot disease is overcome by introduction of a resistance gene, it is expected that commercial tomato lines derived from line 8338 germplasm will have yields equivalent to the parental control when hybridized with lines containing the resistance gene.

Table V.3. Tomato Fruit Yield of Lines 8338 and UC82B at Four Different Field Site Locations[a].

Field Site[1]	Yield per Plant (kg) 8338	UC82B
1	9.44	10.44
2	6.60*	10.45
3	6.14	5.40
4	5.08*	7.74

[a] Yields are reported as the average yield per plant across plots
[1] The location of each field site in Florida is described in section V.C.
* Statistically significant at the 5% level as determined by protected t-test.

F. Expression Levels of the ACCd and NPTII Proteins

Based on the Southern blot analyses described in section V.B, DR tomato line 8338 contains a single DNA insert, and this insert contains single copies of the *accd* and *nptII* genes. These genes are expressed in DR tomato line 8338. Levels of ACCd and NPTII proteins in the DR tomato lines and control line UC82B were determined in fruit and leaf samples by ELISA. The ACCd ELISA is a validated, direct double antibody sandwich assay, specific for the ACCd protein (Reed *et al.*, 1995). A protein-G purified polyclonal goat anti-ACCd antibody was used for antigen capture and the same goat antibody conjugated to alkaline phosphatase, used for detection. The precision, accuracy, and assay working limits of the ACCd ELISA for fruit and leaf tissues were determined and are summarized in Appendix IV. The variability of analysis for ACCd in red ripe tomato fruit tissue was 37.3% (data generated over more than a six month time period). Although this value appears high, it represents the "worst-case" of assay variability, and takes into account variability resulting from individual extractions and day-to-day assay variability. In any case, the variability of ACCd expression between field sites due to environmental conditions is greater, as expected, than the variabilities associated with the tomato fruit ELISA for ACCd. Assay of the red ripe fruit

quality control (QC) sample on different days yielded a lower variability, 19.9%. Sample variability and variability of analysis measurements for ACCd in leaf tissue were significantly less than those measured for red ripe fruit. The accuracy of the ACCd ELISA, as measured by spike and recovery of the protein, showed no apparent loss of ACCd during extraction and assay from fruit and leaf tissue. The high value for spike and recovery of ACCd from leaf tissue may be a result of several factors. These include assay variability and leaf extract loading levels in the ELISA that are greater than normally used for sample analysis. Accordingly, this result is not expected to have any impact on measurements of leaf ACCd expression, since sample loading levels in the ELISA are extremely low.

The NPTII ELISA is a validated, direct double antibody sandwich assay, specific for the NPTII protein (Rogan *et al.*, 1992). A protein-A purified polyclonal rabbit anti-NPTII antibody was used for antigen capture and the same rabbit antibody conjugated to horseradish peroxidase, used for detection. The precision, accuracy, and working limits of the assay were determined for measurement of NPTII in fruit and leaf tissues, and are summarized in Appendix V. The QC sample variability and variability of analysis for the tomato fruit and leaf NPTII ELISA were similar to the assay precision measurements for the ACCd ELISA, described above. The accuracy of the NPTII ELISA, measured as extraction efficiency and spike and recovery, was well within acceptable limits. The extraction efficiency of NPTII from fruit and leaf samples was greater than 90%, and NPTII spike and recovery values from both tissues approximated to 100%.

It is concluded that the ACCd and NPTII ELISAs are reliable methods for quantitation of ACCd and NPTII proteins, respectively, in fruit and leaf tissues of DR tomatoes.

1. Expression of ACCd in fruit and leaves

Fruit expression results of ACCd are shown in Table V.4 and Figure V.5. Expression of ACCd was measured in red ripe fruit collected from four Florida field sites (Table V.4). The enzyme was also measured in fruit at different stages of ripening (mature green, orange, red ripe, and 2-weeks post red ripe) collected from one field site (Figure V.5). The mean expression (across four field sites) of ACCd in red ripe tomato fruit of line 8338 was 39.4 µg/g fresh weight. Mean expression levels of ACCd in fruit at different stages of ripening ranged from 31.4 to 47.4 µg/g fresh weight (Figure V.5). The ACCd protein was not detected in red ripe fruit or fruit of different ripening stages of control line UC82B. Since a tomato is approximately 1% protein (Davies and Hobson, 1981), ACCd constitutes approximately 0.4% of the total fruit protein.

Table V.4. Expression of ACCd in Red Ripe Tomato Fruit Tissues Collected from Four Different Field Site Locations, Determined by ELISA.[a]

Field[b] Site		Mean[c] & Range[d] Values µg ACCd/ g Tissue Fresh Weight 8338	UC82B
1	mean	37.8	ND[e]
	range	27.6-53.1	ND
2	mean	47.1	ND
	range	14.2-123	ND
3	mean	47.4	ND
	range	30.1-66.1	ND
4	mean	25.3	ND
	range	20.4-36.3	ND
Mean across sites		**39.4**	**ND**
Range across sites		**14.2-123**	**ND**

[a] Duplicate extracts of fruit samples from each of the four plots at each field site were analyzed by ELISA
[b] The location of each field site in Florida is described in section V.C.
[c] Means shown here are the averages for each line across four plots.
[d] Range denotes the lowest and highest individual assay result for each plot.
[e] ND = Non-Detectable

The ACCd leaf expression levels were determined on tissue collected from plants at Field Site 3, throughout the growing season. Results are shown in Figure V.6. Results are presented as the mean and range of leaf ACCd expression across plots for DR line 8338 at each harvest date. The highest ACCd expression levels in leaves were observed during flowering and early fruit development. The highest mean value was 752 µg ACCd/g fresh wt., and the range of measured expression values throughout the growing season was 431 to 789 µg ACCd/g fresh wt. The ACCd protein was not detected in leaves of control line UC82B at any time during the growing season.

2. Expression of NPTII in fruit and leaves

Fruit expression results of NPTII protein are shown in Table V.5 and Figure V.7. Expression of NPTII was measured in red ripe fruit collected from four Florida field sites (Table V.5), and also measured in fruit at different stages of ripening, collected from one field site (Table V.7). The mean expression (across four field sites) of NPTII in red ripe tomato fruit of line 8338 was 0.437 µg/g fresh weight (approximately 0.004% of the total fruit protein) (Table V.5). Mean expression of NPTII in line 8338 fruit at different ripening stages ranged

from 0.260 to 1.14 µg/g fresh weight (Figure V.7). The NPTII protein was not detected in red ripe fruit or fruit of different ripening stages of control line UC82B.

Table V.5. Expression of NPTII in Red Ripe Tomato Fruit Tissues Collected from Four Different Field Site Locations, determined by ELISA[a]

Field[b] Site		Mean[c] & Ranged[d] Values µg NPTII/ g Tissue Fresh Weight 8338	UC82B
1	mean	0.637	ND[e]
	range	0.164-1.73	ND
2	mean	0.346	ND
	range	0.319-0.394	ND
3	mean	0.478	ND
	range	0.388-0.559	ND
4	mean	0.287	ND
	range	0.237-0.363	ND
Mean across sites		**0.437**	**ND**
Range across sites		**0.164-1.73**	**ND**

[a] Duplicate extracts of fruit samples from each of the four plots at each field site were analyzed by ELISA.
[b] The location of each field site in Florida is described in section V.C.
[c] Means shown here are the averages for each line across four plots.
[d] Range denotes the lowest and highest individual assay result for each plot.
[e] ND = Non-Detectable

The NPTII leaf expression levels were determined on tissue collected from plants at Field Site 3, throughout the growing season. Results are shown in Figure V.8. Results are presented as the mean and range of leaf NPTII expression across plots for DR line 8338 at each harvest date. The highest mean value was 16.6 µg NPTII/g fresh wt., and the range of measured expression values throughout the growing season was 1.73 to 19.9 µg NPTII/g fresh wt. The NPTII protein was not detected in leaves of control line UC82B at any time during the growing season.

G. Tomato Products and Human/Animal Consumption

To design a relevant food/feed safety assessment program for DR tomato, it was crucial to understand the production and uses of tomatoes. The key aspects of tomato food and feed production and use are summarized below.

1. Tomato production and export

Florida is the largest producer of fresh market tomatoes and accounts for 48% of the production in North America (U.S. Department of Agriculture, 1992). California and Mexico also represent significant production areas with 28% and 20% of the North American production, respectively (U.S. Department of Agriculture, 1992). Florida supplies tomatoes primarily to the east and midwest regions of the United States January through June and September through December. California produces tomatoes from April until November. Mexico primarily supplies the Western region with tomatoes during December through April. The Eastern seaboard is a major supplier of tomato fruit during July, August and September. Approximately 350,000 acres are used for production of processing tomatoes. California accounts for 85% of this acreage with an average harvest of 31 tons of tomato per acre. Most US tomatoes are produced for domestic consumption with small amounts (9%) of fresh market tomatoes being exported to Canada (Plummer, 1992).

2. Animal consumption

The primary use of tomato is as a human food with little being fed to animals (Redenbaugh *et al.*, 1992). The amounts fed to animals vary by geographical region and time of year. Overall the animal feed use of tomatoes and processing by-products does not provide a significant portion of the diet of commercially produced animals.

3. Human consumption

Approximately 60% of consumers purchase tomatoes year round. Highest purchase volumes occur during the summer months. Fresh tomato usage can be segmented into four categories - salads, 48%; sandwiches, 27%; cooked, 14%; and by themselves, 11%. An estimated 5% of consumer purchased tomatoes are wasted, primarily due to spoilage. Processing tomatoes are used to produce sauces (35% of total production), paste (18%), peeled tomatoes (17%), ketchup (15%), and juices (15%) (Florida Tomato Committee Annual Report, 1991-1992).

The mean daily consumption for tomatoes and tomato products is 23 grams (TAS, Inc. 1992) with approximately 26% of all individuals consuming tomatoes at least once a day. Based on Daily Reference Values, tomatoes are considered an important source of ascorbic acid (20% of the RDI) and folic acid (12% of the RDI) in the human diet. Tomatoes are also considered an important source of Vitamin A, and other carotenoids including ß-carotene and lycopene. This nutritional information is summarized in Attachment 17 to Reed *et al.* (1994).

H. Compositional Analyses of DR Tomatoes

Compositional (proximate) analyses were performed on red ripe tomato fruit from DR line 8338 and the UC82B control line. Compounds measured were total solids, protein, fat, and ash. Carbohydrates and calories were calculated from these values. There is a relatively wide range of analysis values for total solids and carbohydrates for tomatoes in the literature, as indicated below. There is a narrower range of analysis values for other proximate components of

the fruit.

To provide test material for compositional analyses, fruit of DR tomato line 8338 and control line UC82B were collected from plants grown at four Florida field sites, as previously described in section V.C. Fruit samples were analyzed separately by field site, and means of analysis calculated across field sites. The results of proximate analyses are shown in Table V.6. Results from each of the four sites of each line were statistically analyzed. The average levels of proximate components in DR line 8338 were statistically equivalent to levels in control line UC82B, and were within the ranges reported in the literature for tomatoes.

Additional tomato quality data collected on line 8338 and the control UC82B line include, in addition to the proximates shown in Table V.6: vitamins: A and C, folic acid, vitamin B-6, riboflavin, thiamin, niacin; minerals: calcium, iron, sodium, magnesium, phosphorus), flavor components (sugars: fructose, glucose, sucrose; organic acids: citric acid, malic acid, lactic acid; and volatiles: hexanal, trans-2-hexenal, cis-3-hexenal, 6-methyl-5-heptan-2-one, 2-isobutylthiazole, 1-penten-3-one, geranylacetone, acetaldehyde, acetone, methanol, ethyl caproate, cis-3-hexenol, octanol, t,t-2,4-decadienal, eugenol), and processing components: natural tomato soluble solids, pH, titratable acidity, lycopene). In addition to analyses for nutrients, flavor and processing components, tomato plant toxicants (tomatine, solanine and chaconine) were measured in mature green and red ripe fruit. Monsanto has provided this data to the Food and Drug Administration, and has consulted with the FDA concerning the animal and human food safety of delayed ripening tomato line 8338. FDA concluded the consultation and accepted our conclusion that the 8338 line is not altered significantly when compared to other tomato varieties with a history of safe use (FDA memorandum of conference, September 19, 1994, Appendix VIII).

Table V.6. Average Proximate Analysis Results for DR Tomato Line 1992 Field Tests

Component	Line UC82B	Line 8338	Literature Range
Total Solids[a] (%)	5.3	4.7	4.5 - 8.3[1,2]
Ash (%)[b]	0.59	0.57	0.5 - 0.7[2]
Fat (%)[c]	<0.25	<0.25	0.0 - 0.3[2,3]
Protein (%)[d]	1.2	1.1	0.9 - 1.1[2,3]
Carbohydrates (%)[e]	3.5	2.9	2.7 - 4.7[2,3]
Calories (Kcal /100g)[f]	18.7	16.3	14 - 22[2,3]

[1] Stevens and Scott, 1988.
[2] Davies and Hobson, 1981.
[3] Gould, 1993.
[a] Association of Analytical Chemists, 1990, method number 964.22.
[b] Association of Analytical Chemists, 1990, method number 940.26.
[c] Association of Analytical Chemists, 1990, method number 983.23.
[d] Association of Analytical Chemists, 1990, method number 920.152.
[e] calculation
[f] calculation

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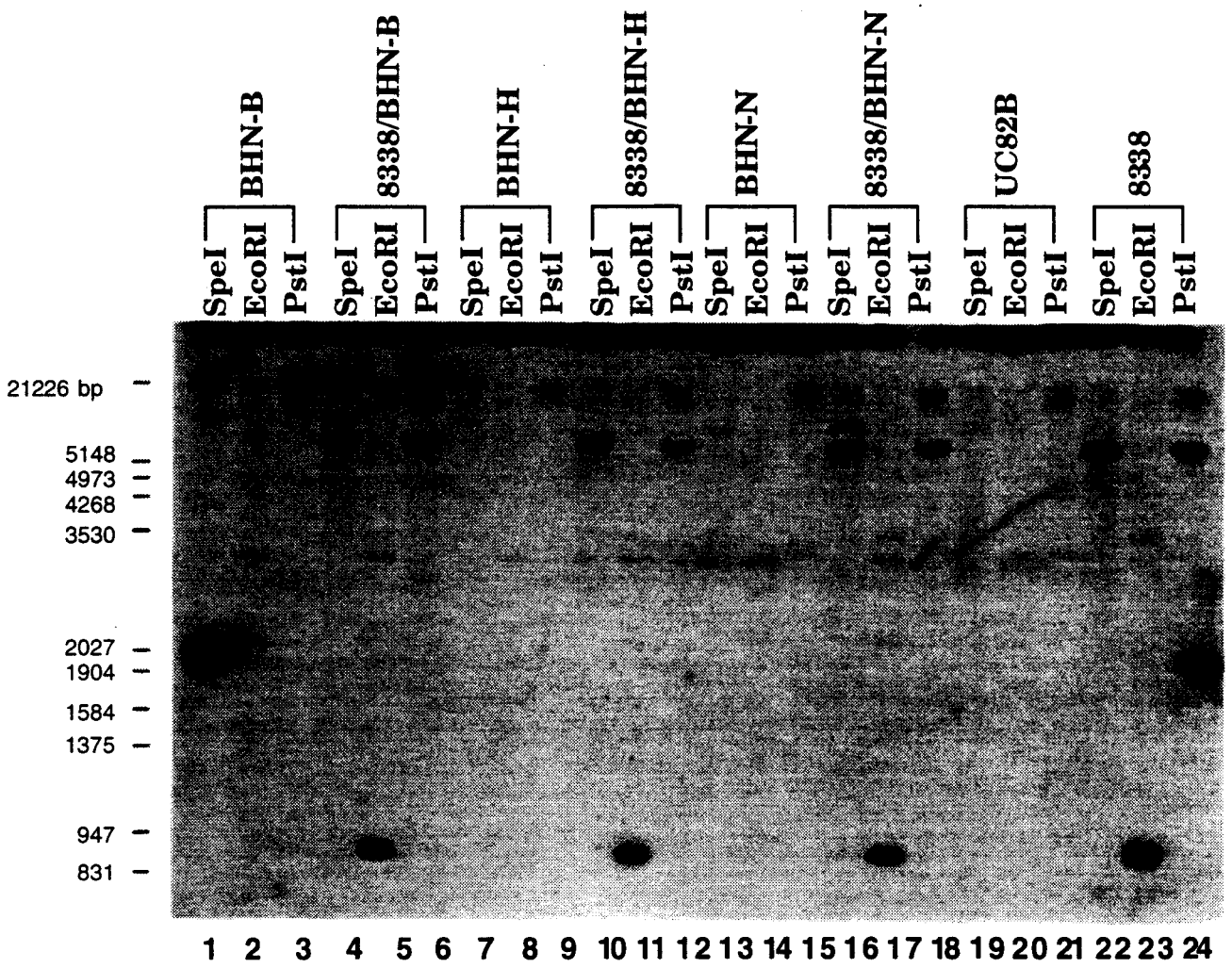


Figure V.1. Southern blot analysis of 8338 BC_3F_1 backcross lines probed for ACC deaminase.

Southern blot analysis was performed on line 8338, parental control UC82B, backcross lines (8338 with BHN-B, BHN-H, and BHN-N) and corresponding nontransgenic controls to determine insert stability. Genomic DNA for line BHN-B (lanes 1-3), 8338/BHN-B (lanes 4-6), BHN-H (lanes 7-9), 8338/BHN-H (lanes 10-12), BHN-N (lanes 13-15), 8338/BHN-N (lanes 16-18), UC82B (lanes 19-21), and 8338 (lanes 22-24) was restricted with SpeI, EcoRI, and PstI, respectively. The resulting hybridization membrane was probed with a ^{32}P labelled *accd* fragment.

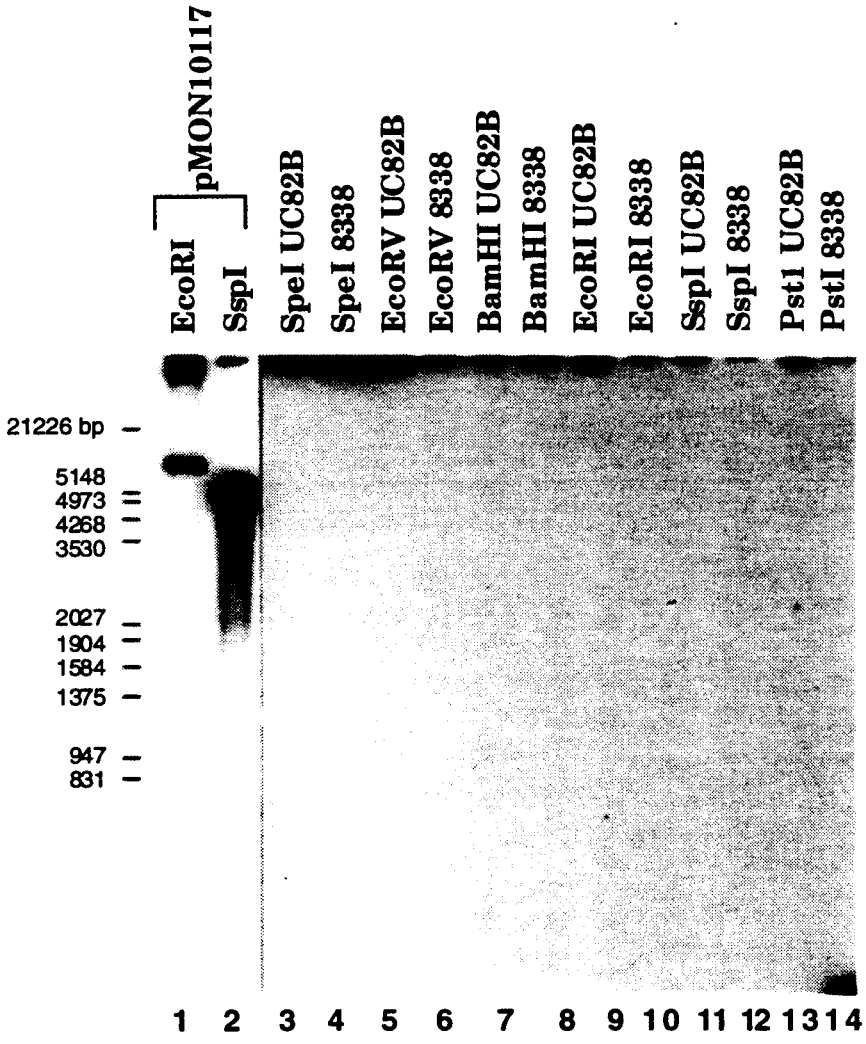


Figure V.2. Southern blot of line 8338 and pMON10117 probed for *Spc/Str*.

Genomic DNA from Delayed Ripening tomato line 8338 was digested with SpeI (lane 4), EcoRV (lane 6), BamHI (lane 8), EcoRI (lane 10), SspI (lane 12), and PstI (lane 14). Genomic DNA from control line UC82B was digested with SpeI (lane 3), EcoRV (lane 5), BamHI (lane 7), EcoRI (lane 9), SspI (lane 11), and PstI (lane 13). Plasmid pMON10117 DNA was digested with EcoRI (lane 1) and SspI (lane 2). The digested DNA was then subjected to electrophoresis in a 1% agarose gel, transferred to a nylon membrane and then probed with a ^{32}P labelled *Spc/Str* fragment. The blot is a composite of two exposures; lanes 1 and 2 were exposed for 30 minutes, lanes 3-10 were exposed for 20 hours. DNA standards are shown to the left of each blot.

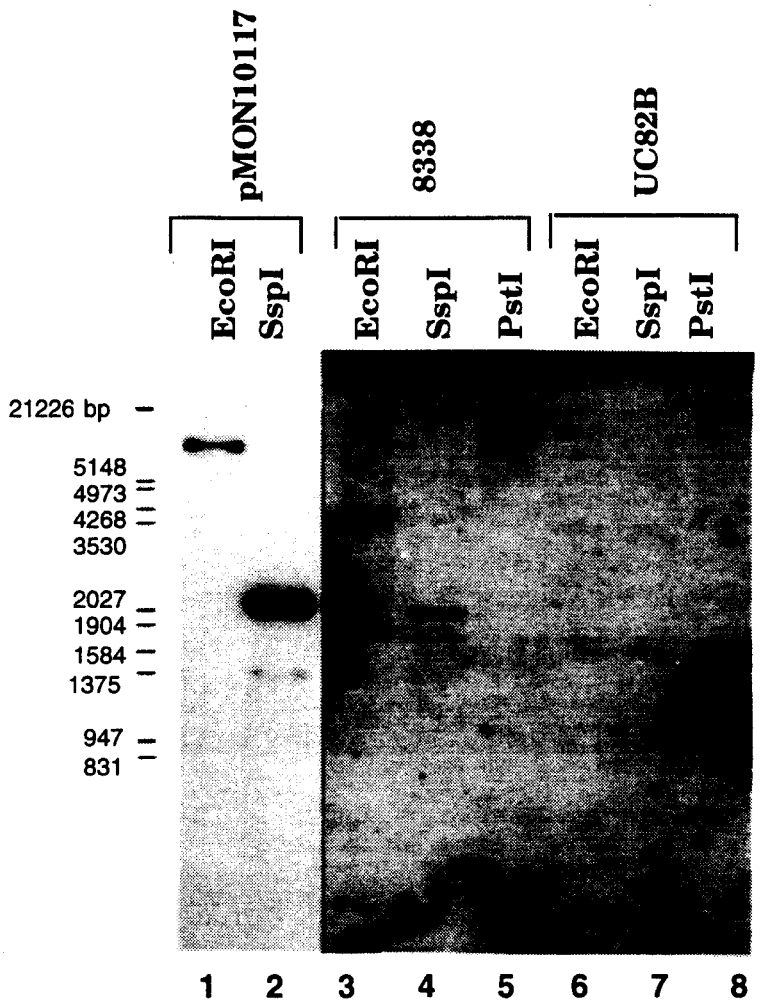


Figure V.3. Southern blot of line 8338 and pMON10117 probed for NPTII.

Genomic DNA from Delayed Ripening tomato line 8338 was digested with EcoRI (lane 3), SspI (lane 4), and PstI (lane 5). Genomic DNA from control line UC82B was digested with EcoRI (lane 6), SspI (lane 7), and PstI (lane 8). Plasmid pMON10117 DNA was digested with EcoRI (lane 1) and SspI (lane 2). The digested DNA was then subjected to electrophoresis in a 1% agarose gel, transferred to a nylon membrane and probed with a ^{32}P labelled NPTII fragment. The blot is a composite of two exposures; lanes 1 and 2 were exposed for 30 minutes and lanes 3-8 for 20 hours. DNA standards are shown to the left of each blot.

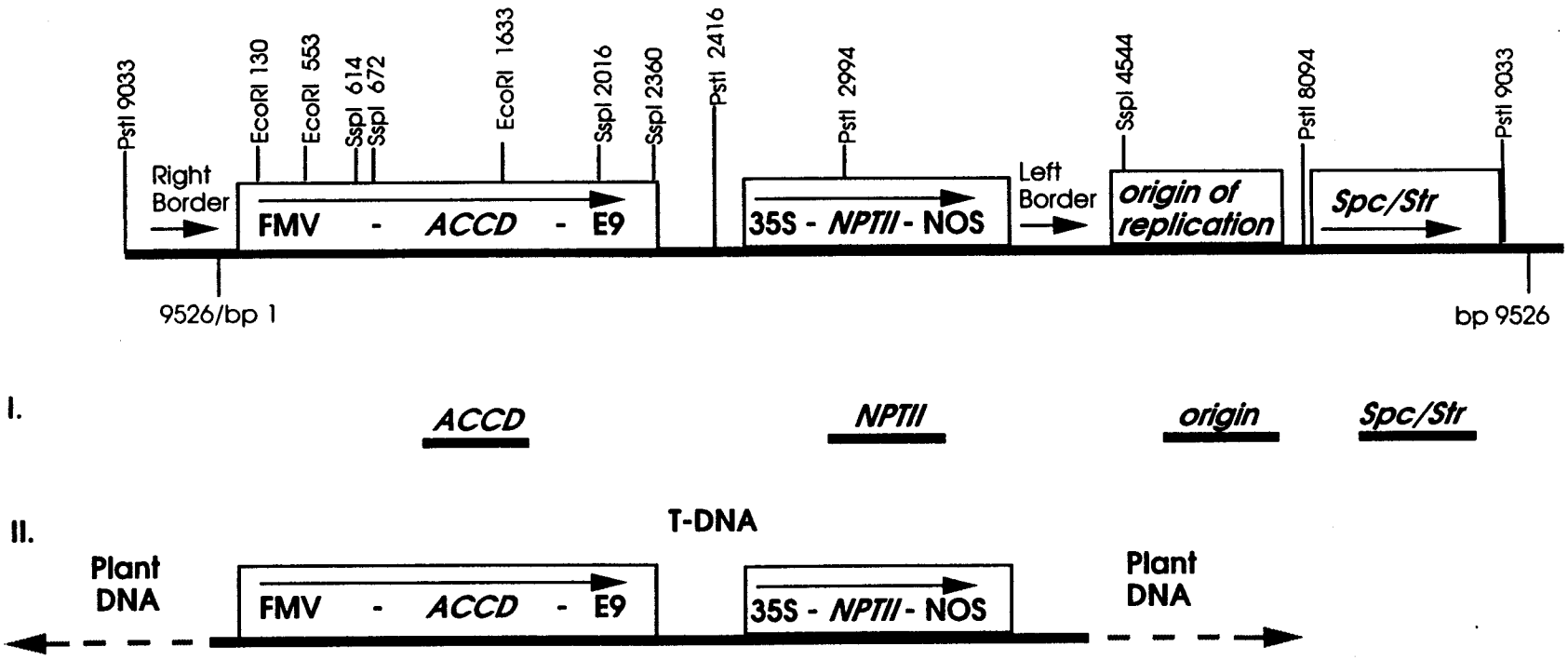
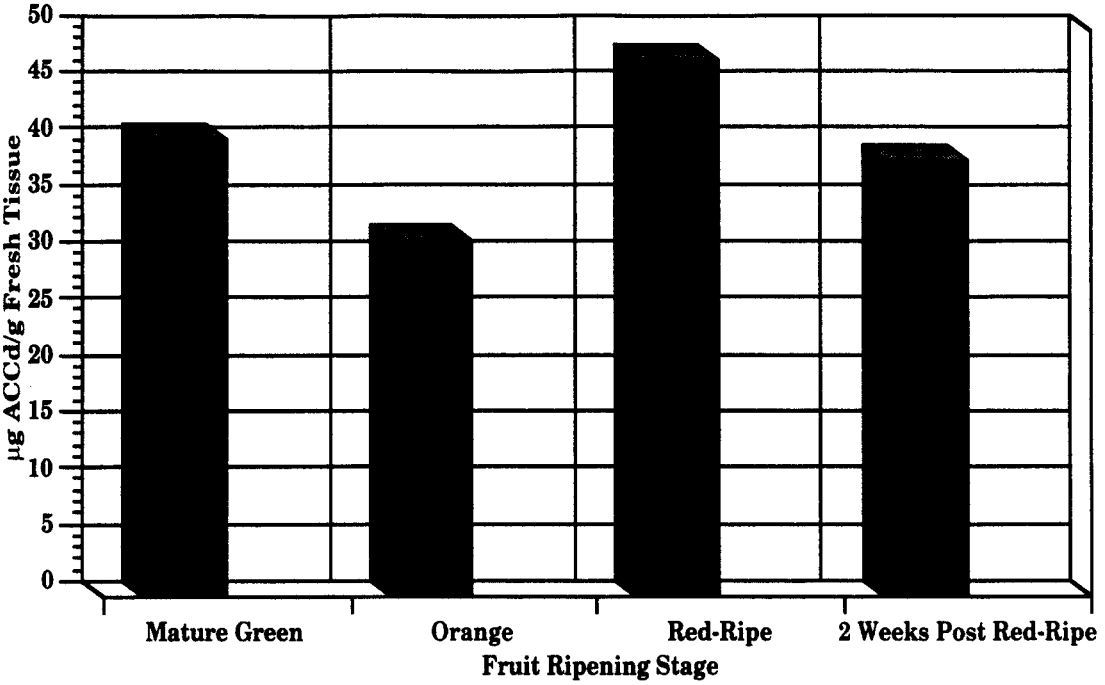


Figure V.4. Schematic diagram of plasmid vector pMON10117 used to produce DR Tomato line 8338, and deduced T-DNA structure in line 8338.

The diagram shows restriction sites within the plasmid. Shown below are 1) the probe fragments used for Southern blot analysis of DR Tomato line 8338, and 2) the deduced structure of T-DNA inserted into the genome of the plant line. Line 8338 contains a single copy of the *accd* and *nptII* genes.

Figure V.5. Expression of ACCd in tomato fruit Tissue[a] of Different Ripening Stages for DR Tomato Line 8338. Fruit were Harvested from Field Site 3[b].



Tomato Line	Fruit Ripening Stage			
	Mature Green	Orange	Red Ripe	2 Weeks Post Red-Ripe
8338 Mean† (µg/g)*	40.2	31.4	47.4	38.5
8338 Range# (µg/g)	33.2 - 46.1	25.9 - 39.1	30.1 - 66.1	21.7 - 49.2
UC82B Mean (µg/g)	ND^	ND	ND	ND
UC82B Range (µg/g)	ND	ND	ND	ND

[a] Duplicate extracts of fruit samples from each of the four plots at field site 3 were analyzed by ELISA.

[b] The location of each field site in Florida is described in section V.C.

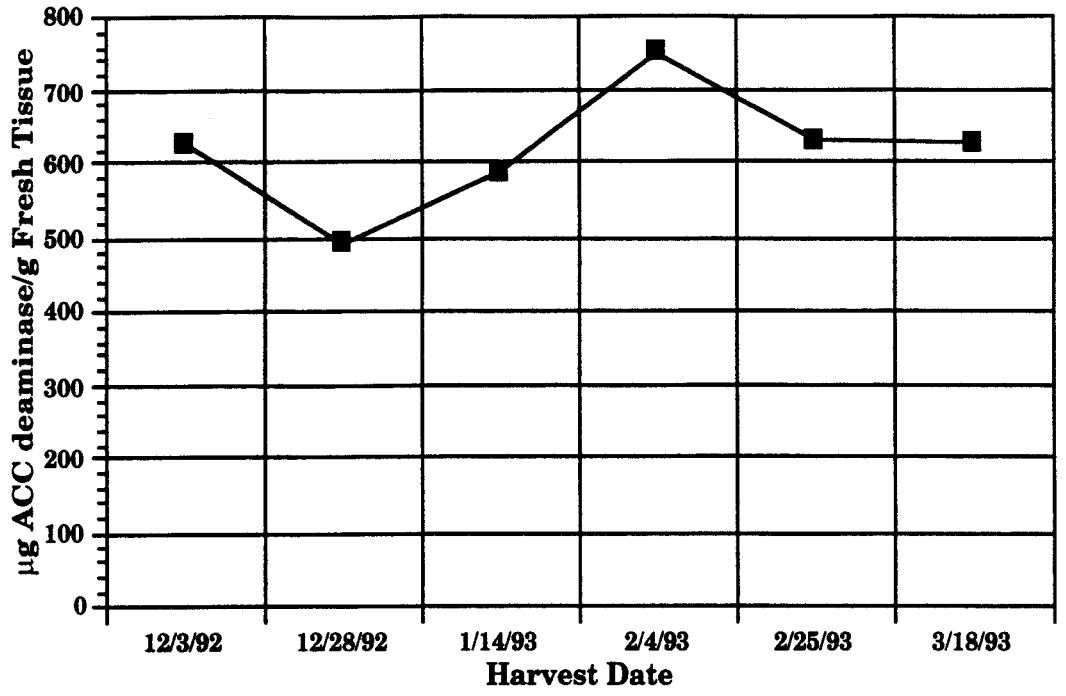
† Means shown here are the averages for each line across four plots.

Range denotes the lowest and highest individual assay result for each plot.

^ND = Non-Detectable

*µg ACC deaminase per g fresh tissue

Figure V.6. Expression of ACCd in Tomato Leaf Tissue[a] at Intervals throughout the Growing Season for DR Tomato Line 8338. Fruit were Harvested from Field Site 3[b].



	Harvest Date					
Tomato Line	**12/3/92**	**12/28/92**	**1/14/93**	**2/4/93**	**2/25/93**	**3/18/93**
8338 Mean† (µg/g)*	628	491	586	752	631	628
8338 Range# (µg/g)	524-714	431-571	559-644	715-789	607-652	590-661
UC82B Mean (µg/g)	ND^	ND	ND	ND	ND	ND
UC82B Range	ND	ND	ND	ND	ND	ND

[a] Duplicate extracts of leaf samples from each of the four plots at field site 3 were analyzed by ELISA.

[b] The location of each field site in Florida is described in section V.C.

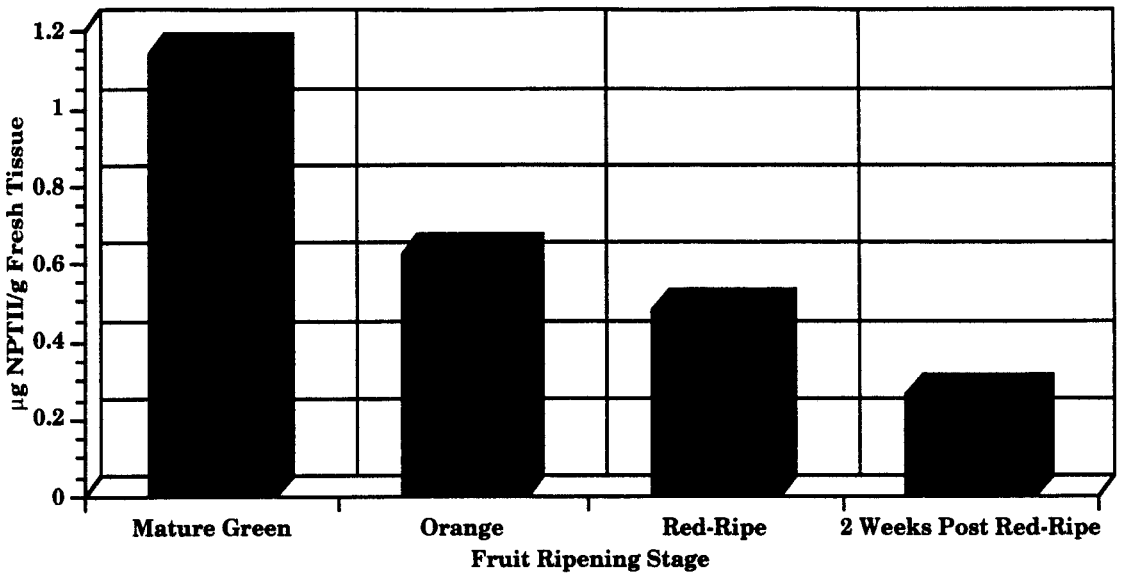
† Means shown here are the average for each line across four plots.

Range denotes the lowest and highest individual assay result for each plot.

^ND = Non-Detectable

*µg ACC deaminase per g fresh tissue

Figure V.7. Expression of NPTII in tomato fruit Tissue[a] of Different Ripening Stages for DR Tomato Line 8338. Fruit were Harvested from Field Site 3[b].



	Fruit Ripening Stage			
Tomato Line	**Mature Green**	**Orange**	**Red-Ripe**	**2 Weeks Post Red-Ripe**
8338 Mean† (µg/g)*	1.14	0.623	0.478	0.26
8338 Range# (µg/g)	0.959 - 1.24	0.521 - 0.832	0.388 - 0.559	0.205 - 0.288
UC82B Mean (µg/g)	ND^	ND	ND	ND
UC82B Range (µg/g)	ND	ND	ND	ND

[a] Duplicate extracts of fruit samples from each of the four plots at field site 3 were analyzed by ELISA.

[b] The location of each field site in Florida is described in section V.C.

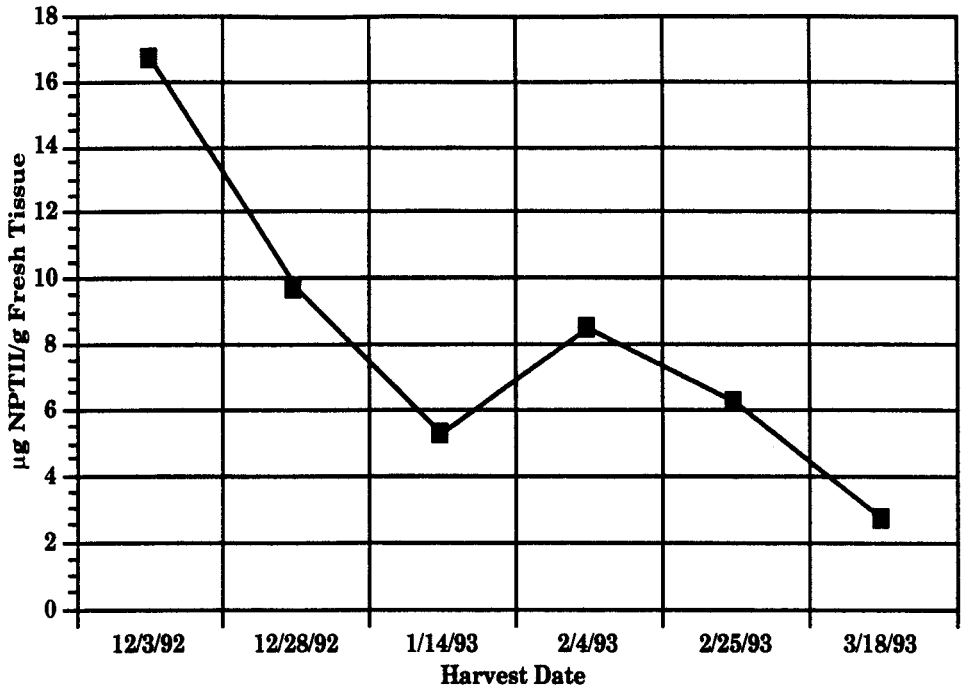
† Means shown here are the averages for each line across four plots.

Range denotes the lowest and highest individual assay result for each plot.

^ND = Non-Detectable

*µg NPTII per g fresh tissue

Figure V.8. Expression of NPTII in Tomato Leaf Tissue[a] at Intervals throughout the Growing Season in DR Tomato Line 8338 . Fruit were Harvested from Field Site 3[b].



	Harvest Date					
Tomato Line	**12/3/92**	**12/28/92**	**1/14/93**	**2/4/93**	**2/25/93**	**3/18/93**
8338 Mean† (µg/g)*	16.60	9.68	5.30	8.44	6.19	2.65
8338 Range# (µg/g)	14.5 - 19.9	4.59 - 12.6	4.63 - 7.02	4.89 - 12.0	4.60 - 7.12	1.73 - 3.78
UC82B Mean (µg/g)	ND^	ND	ND	ND	ND	ND
UC82B Range (µg/g)	ND	ND	ND	ND	ND	ND

[a] Duplicate extracts of leaf samples from each of the four plots at field site 3 were analyzed by ELISA.

[b] The location of each field site in Florida is described in section V.C.

† Means shown here are the averages for each line across four plots.

Range denotes the lowest and highest individual assay result for each plot.

^ ND = Non-Detectable

* µg NPTII per g fresh tissue

VI. Environmental Consequences of Introduction of DR Tomato Line 8338.

A. The Delayed Ripening Trait in Tomatoes

Consumption of fresh tomatoes in the United States is relatively high among vegetable crops (U.S. Department of Agriculture, 1992). However, tomatoes are generally considered by the consumer as having poor taste quality (Stevens, 1986). The poor taste quality of fresh tomatoes can be attributed to a production system which is based on fruit harvest at the mature green stage of development. Mature green fruit have the firmness and market life attributes necessary for a national distribution system. However, fruit harvest typically consists of both mature green and immature green fruit (the two green fruit types are externally indistinguishable), and immature green fruit do not develop full flavor qualities when ripened to red ripe (Grierson and Kader, 1986). To avoid contamination with inferior immature green fruit, many growers harvest fruit that have developed some red color. Although these "vine-ripened" fruit develop good flavor quality, the fruit typically have a relatively short market life and do not maintain the physical characteristics necessary for national distribution. To prolong the life of a vine ripened or mature green fruit, the retailer and/or consumer may refrigerate the tomato, which has been shown to destroy tomato flavor (Kader *et al.*, 1978; Buttery *et al.*, 1987).

Tomato plants with delayed fruit ripening traits have been described (Tigchelaar *et al.*, 1978; Hamilton *et al.*, 1990; Oeller *et al.*, 1991; Klee *et al.*, 1991). Introduction of a delayed ripening trait into fresh market tomatoes will allow the following benefits to be realized:

- Growers will be able to harvest fruit at the breaker stage (first break of color) eliminating the inferior immature green fruit from the harvest.
- Packers, shippers and retailers will be able to transport and store tomatoes at higher temperatures thereby saving energy and preserving flavor qualities.
- Packers, shippers and retailers will reduce fruit loss due to soft and over-ripe fruit thereby increasing the yield of marketable fruit.
- Packers and shippers will be able to expand the geographical distribution of the tomato product.

All of which provide the consumer with a better tasting tomato.

Monsanto has developed tomato lines that are delayed in fruit ripening. These tomato lines have been modified to express the enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd), which catalyzes metabolism of ACC to ammonia and α-ketobutyrate (Honma and Shimomura, 1978). Because ACC is an essential precursor for ethylene biosynthesis (Adams and Yang, 1979; Yang, 1981), and levels of ethylene initiate and control the rate of tomato fruit ripening (Taiz and Zeiger, 1991), removal of ACC in these lines reduces ethylene

production and delays ripening (Klee *et al.*, 1991; Klee, 1993).

B. Weediness Potential of DR Tomato Line 8338

The introduction of a delayed ripening gene 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) adaption for short- and long-distance dispersal; 9) if perennial, vegetative production or regeneration from fragments and brittleness (so not easily removed from the ground); and 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 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 delayed ripening trait into this cultivar has not imparted any new "weedy" characteristics. No increase was noted with the transformed cultivar with respect to the number of seeds produced (yield data), and no changes were noted with respect to the germination characteristics of seeds or final stands. Average percent seed germination under field conditions was 57.8% for line 8338 compared to 56.4% for parental line UC82B. Seeds of both lines germinated mostly within 2 weeks after sowing, and there was no apparent difference between the lines with respect to seed dormancy. 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. Average time-to-flowering for plants of line 8338 was 32.1 days compared to 34.9 days for plants of line UC82B. Although time-to-flowering of line 8338 was 2.8 days shorter than that of the control line, fruit maturation of both lines occured at approximately the same time. Average seed number per fruit for lines 8338 and UC82B was 71 and 68, respectively, and the difference between the lines was not statistically significant. The average seed weight of tomato lines 8338 and UC82B was 0.233 and 0.249 g/100 seed, respectively, and the difference between the lines was statistically significant at the 5% level. However, the slightly lower seed weight of line 8338 did not affect germination and seedling vigor of this line (Appendix VI. An assessment of the weediness potential of delayed ripening tomato line 8338).

No differences in insect infestation or severity were detected between the DR tomato lines (including line 8338) and the control line, UC82B. Susceptibility to *Fusarium* crown rot disease (*Fusarium oxysporum*, f.sp. *radici-lycopersici*) was

noted in the California variety UC82B when grown under Florida conditions. Line 8338 showed a further increase in susceptibility to the disease under similar conditions. This susceptibility is overcome by introduction of a *Fusarium* crown rot resistance gene, *fcr*, into line 8338 (Appendix II, Attachment I). Therefore, it is expected that commercial tomato lines derived from line 8338 germplasm will not be susceptible to *Fusarium* crown rot disease when hybridized with lines containing the resistance gene. It is important to note that approximately 90% of the tomatoes grown commercially in Florida are susceptible to *Fusarium* crown rot disease (Appendix II, Attachment I), so there will be no increased application of crop protection chemicals during production of DR tomatoes. There were no differences between line 8338 and the control line UC82B in susceptibility to other diseases monitored in these studies. See Appendix II for USDA final reports, and Appendix III for example monitoring forms.

C. Effects of Delayed Ripening Tomatoes on Nontarget Organisms
Delayed ripening tomato line 8338 has been field tested at seven sites (two in California, four in Florida and one in Illinois) in the U.S. since 1992 and the plants show no toxicity towards insects, birds, or other species that frequent tomato production fields (Appendix II, USDA Final Reports; Appendix III, Example Monitoring Forms). As discussed earlier in section IV, the ACCd enzyme is present in common water or soil microorganisms and therefore is ubiquitous in nature and may be present as a contaminant in food derived from plant sources. Monsanto has consulted with the Food and Drug Administration concerning the animal and human food safety of delayed ripening tomato line 8338. FDA concluded the consultation and accepted our conclusion that the 8338 line is not altered significantly when compared to other tomato varieties with a history of safe use (FDA memorandum of conference, September 19, 1994, Appendix VIII).

D. Indirect Plant Pest Effects on Other Agricultural Products
The only route of exposure to ACCd protein will be via oral ingestion but it must survive the hostile environment of the gastrointestinal tract. The gastrointestinal tract is designed to digest ingested dietary proteins by conversion to amino acids and small peptides, which are absorbed by the intestinal tract. This is accomplished through the combined action of acid conditions and pepsin in the stomach and further action of bile acids and enzymes (trypsin, chymotrypsin, carboxypeptidases, etc.) in the intestinal tract. Our own studies have shown that ACCd protein is digested readily by trypsin. We have experimentally confirmed the digestibility of ACCd by examining the rate of degradation *in vitro* using simulated gastric and intestinal fluids (*The United States Pharmacopeia, 1990*). Purified ACCd has also been fed to rodents, with no dose-related effects observed.

Therefore, based on 1) the specificity of the ACCd enzyme and 2) the rapid degradation of ingested proteins, no adverse effects are predicted if this enzyme is ingested as a minor constituent in food.

Furthermore, extensive studies of the composition of tomato fruits produced by line 8338 (summarized in Table VI.1) show that the DR tomato line is not materially different from the control line in essential nutrients, flavor and processing components, and toxicant.

Table VI.1. Summary of compositional analyses performed on DR tomato line 8338 (Reed *et al*, 1994)

Component	Tomato fruit
Proximate analysis (total solids, ash, fat, protein, carbohydrates, and calories)	NMD
Minerals (Ca, Fe, Na, Mg, and P)	NMD
Vitamins (A, C, B-6, Folic Acid, Thiamin, Niacin, Riboflavin)	NMD
Lycopene	NMD
Sugars (fructose, glucose, sucrose)	NMD
Organic acids (malic, citric, and lactic)	NMD
Natural soluble solids	NMD
Volatile compounds	NMD
Tomatine, solanine, chaconine	NMD

NMD = not materially different from the parental control

Although we focused these analyses on the fresh tomato, we also manufactured several selected, important tomato products for additional analyses. Paste, juice and wet pomace were produced and analyzed to provide data on several of the most important processed commercial tomato products.

Compositional analyses were conducted on juice and paste processed fruit fractions, derived from tomato fruit processed at The National Food Laboratory, Processing Department, CA. The components analyzed were as follows: processing traits, sugars and organic acids as listed above for whole tomatoes. Analysis results for the processed fruit fractions paralleled those for whole tomatoes and are not presented in this summary. Analysis results for the processed fruit fractions are detailed in Reed *et al.*, 1994. We conclude from the results of these analyses that processed products of DR tomato lines and the control are not materially different, and that DR tomato lines meet processing quality standards.

Based upon the foregoing information and the application of the criteria provided in the FDA Policy for "Foods Derived from New Plant Varieties" (United States Food and Drug Administration, 1992), we conclude that we have established the compositional equivalence of DR tomato line 8338 with its traditional counterpart. FDA accepted our assessment that the 8338 line is not altered significantly when compared to other tomato varieties with a history of safe use in their summary and conclusion of consultation (FDA memorandum of conference, September 19, 1994, Appendix VIII) provided to the Food Advisory and Veterinary Medicine Advisory Committees at their

November 2, 1994 joint meeting, Appendix VIII.

E. Potential for Outcrossing

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 are limited to Latin America. (Rick, 1976; Appendix VII, Letters from Drs. Charles M. Rick, Raymond L. Clark and Jay W. Scott.)

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. (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 ACCd gene to a microbe would not pose any plant pest risk. ACCd genes are naturally found in microorganisms. These microbes are not plant pests and one of them, *Pseudomonas chloroaphis*, was the source of the ACCd gene introduced into the delayed ripening tomato. Based on these considerations transfer to microbes is unlikely and of no significant consequence from a plant pest point of view.

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VII. Statement of Grounds Unfavorable

DR tomato line 8338 has been field tested since 1992 at seven locations in commercial tomato growing areas under field release permits granted by USDA/APHIS (United States Department of Agriculture / Animal and Plant Health Inspection Service) (USDA# 92-049-01, 92-176-01, 93-054-01N, 93-063-04, 93-203-01, 94-014-01N, 94-234-01N). Susceptibility to *Fusarium* crown rot disease (*Fusarium oxysporum*, f.sp. *radici-lycopersici*) was noted in the California variety UC82B when grown under Florida conditions. Line 8338 showed a further increase in susceptibility to the disease under similar conditions. This susceptibility is overcome by introduction of a *Fusarium* crown rot resistance gene, Fcr, into line 8338 (Appendix II, Attachment I). It is important to note that approximately 90% of the tomatoes grown commercially in Florida are susceptible to *Fusarium* crown rot disease (Appendix II, Attachment I), so there will be no increased application of crop protection chemicals during production of DR tomatoes. The absence of increased use of crop protection chemicals is further supported by the fact that the delay in fruit ripening in line 8338 and other lines expressing ACCd is only seen after removal of fruit from the plant (Klee, 1993). Fruit will be harvested at breaker stage (first appearance of external fruit color) [Grierson and Kader, 1986], at most one to two days later than current harvest practice.

Monsanto and our partner, N.T. Gargiulo, plan to commercialize DR tomato line 8338 and other tomato varieties exhibiting the delayed ripening phenotype derived through traditional breeding methods. Standard tomato breeding requires the evaluation of the progenies of the original crosses over several years before selecting the commercial lines. Indeed, it takes eight backcrosses to transfer the 8338 delayed ripening gene from the processing tomato background into a commercial freshmarket tomato variety with continuous field observation and evaluation during the breeding process. Accumulated data collected from field trials of the 8338 line and progenies from the breeding program (data including yield, agronomic characteristics, vigor, disease and insect susceptibility), literature references, and expert opinion letters demonstrate that a DR tomato line: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than the non-modified parental varieties; 3) shows no potential to increase the weediness of any other cultivated plant or native wild species; 4) does not negatively impact processed agricultural commodities; and 5) shows no potential to harm other organisms that are beneficial to agriculture. Therefore, we request that the line and any progenies derived from crosses between line 8338 and traditional tomato varieties no longer be regulated under 7 CFR part 340.6 in order to provide the necessary flexibility required for continued commercial development.

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Klee, H.J. 1993. Ripening physiology of fruit from transgenic tomato (*Lycopersicon esculentum*) plants with reduced ethylene synthesis. *Plant Physiol* 102:911-916.

VIII. Appendices

Appendix I. Maximum Nucleotide Sequence of PV-LER07 in DR Tomato line 8338.

The nucleotide sequence of plasmid PV-LERP07 (pMON10117) T-DNA from PstI restriction site 9033 to SspI restriction site 4544, as shown in Figure III.1. Based on restriction enzyme and Southern blot analysis of line 8338 DNA, it was concluded that the maximal size of PV-LERP07 DNA contained in line 8338 lies between the PstI and SspI restriction sites described above. The extent of each genetic element that comprises the T-DNA is shown by solid horizontal lines. Enzyme restriction sites are labeled and shown as vertical lines. Open reading frames encoding the ACCd and NPTII proteins are shown by double dash lines. An open reading frame between SspI restriction site 4544 and the left border is also shown by double dash lines. Because this open reading frame is derived from a bacterial gene, it is not expected to express in line 8338 plants.

[

CBI DELETED

]

1992 Modified Ripening Tomato Trial

USDA PERMIT #92-049-01 (Mons # 92-014)
Jerseyville, IL and Davis, CA

FINAL REPORT

Bernard Sammons
Monsanto Co.

The objective of trials using genetically modified tomatoes was to evaluate delayed ripening properties in plants transformed with a gene which decreases ethylene production. The trials were conducted at the Monsanto research farm located in Jerseyville, IL and at Davis, California in collaboration with [CBI DELETED]

Experimental Layout

Planting material consisted of transplants of tomatoes transformed with a gene that degrades ACC and non-transgenic controls at both locations.

Jerseyville, IL

Seed were sown in greenhouses at the Monsanto Research Facility located in Chesterfield, MO. Seed were sown May 12 and 19, 1992. Plants generated from embryo rescue were put to soil on May 26 and June 14, 1992. Seedlings were held in greenhouses until they were large enough to transplant in the field. Seedlings were transported to Jerseyville, IL in agreement with USDA permit # 92-052-04M. Field planting dates were June 10 and June 30, 1992. Major operations related to the field trial are listed in Table 1.

A randomized complete block with four blocks were planted. Plants were a mixture of several genotypes: CBI [

](Table 2). Each genotype was represented in each block except in cases where availability of plant material was limited. In particular, all embryo rescue lines were limited to a single block. If plant material was not limiting, up to 20 plants of each genotype were planted for a total of 80 plants per genotype across the four blocks. Agronomic practices of pest control and irrigation typical of irrigated tomatoes in the area were followed.

Davis, CA

Seed were shipped to Davis, CA on May 13, 1992 in agreement with USDA permit number 92-052-04M. Seed were sown in the greenhouse and held until plantlets attained adequate growth for transplanting. Transplanting in the field was on June 18, 1992.

A block design was used with 75 plants per block with two replicates (for a total of 150 plants of each genotype). CBI [

]. Agronomic practices typical of tomato production in California were followed. Major operations conducted in the field trial are listed in Table 2.

Responses to Specific Issues:

1. Horizontal Movement:

Immunoblotting and ethylene generation assays were performed on fruit samples collected from the field. The **CBI [ ] gene was detected in transgenic fruit onlyand not detected in non-transformed control fruit. No evidence of movement of the delayed ripening gene was observed at either location.

2. Changes In Survival Characteristics:

Jerseyville, IL

Plots were mowed and disked on October 7, 1992. The area was left fallow until spring 1993 at which time soybeans were planted on May 19. Plots were observed in spring and late summer 1993 for volunteer tomatoes, but none could be found. There was no evidence of changes in survival characteristics of the transgenic tomato plants.

Davis, CA

Plots were disked on November 9, 1992. The area was left fallow through the fall and winter and observed for volunteers in the spring and summer of 1993. The plot area received adequate moisture to allow for germination of tomato seed during the observation period for volunteers. There were no volunteers found in spring 1993. The plot area was disked again in April of 1993. The area was monitored for volunteers during the summer of 1993, once again, none could be found. There was no evidence of changes in survival characteristics of the transgenic tomato plants.

3. Stability And Pattern Of Inheritance:

GUS assays for line 8301 showed the expected of 1:2:1 segregation ratio. Breeding programs involving several tomato lines transformed with the introduced gene show normal inheritance patterns in resulting progeny. Continual testing of selected lines indicates trait stability over several generations.

4. Protein Expression Level:

**CBI [

]**

5. Published Data:

At this point, we are not aware of any published data by Monsanto for the specific test.

Table 1. Schedule of major operations (all dates 1992).

Jerseyville, Illinois

May 12 and 19	Greenhouse Sowing Dates
May 26 and June 14 (embryo rescue)	Seedlings put to soil in greenhouse.
June 10	Transplanting in field (lines generated from seed).
June 30	Transplanting in field (lines generated from embryo rescue).

DATE OF FRUIT HARVEST	APPROX WEIGHT OF FRUIT TRANSPORTED TO CV
September 9	21. 5 kg
September 16	32 kg
September 22	35 kg
September 24	16 kg
September 28	30 kg
October 7	25 kg (fruit transported from Jerseyville, IL to Chesterfield, MO under USDA permit # 92-052-04M)
October 7	Test was terminated (completed)

TABLE 2. LIST OF LINES UNDER TEST JERSEYVILLE---1992

[**FRESH MARKET:**

LINE	VECTOR
	CBI
[CBI DELETED	
	]

PROCESSING:

	CBI
[CBI DELETED	
	CBI
	]

1992 Delayed Ripening GLP
Tomato Field Trials

USDA PERMIT #92-176-01
MONS #92-075
Fall 1992 Planting

Bernie Sammons

The purpose of the field trials was to evaluate agronomic performance, gene expression of introduced proteins, quality traits and food safety of two tomato lines genetically modified for delayed ripening. The field trials were conducted at four sites located in Florida in collaboration with BHN-Joint Venture, located in Bonita Springs, FL. Data collected from the field trials will be used for regulatory approval of delayed ripening tomatoes containing the delayed ripening gene.

Experimental Layout:

Planting material consisted of transplants of tomatoes transformed with a gene that delays ripening and non-transgenic controls. Trials were conducted at four sites. The approximate acreage under test at each location was 0.8 acres:

Site #1. Experiment #92-449-701, located at [CBI DELETED]

Site #2. Experiment #92-449-702, located at [CBI DELETED]

Site #3. Experiment #92-449-703, located at BHN-Joint Venture.

Site #4. Experiment #92-449-704. located at [CBI DELETED]

Seed were shipped from Monsanto Co., Chesterfield, MO to BHN-Joint Venture, Bonita Springs, FL on September 21 and again on September 23, 1992 in agreement with USDA permit number 92-190-02M. Seed were sown in the greenhouse and held until plantlets attained adequate size for transplanting. Transplanting in fields occurred on the following dates: Site #1 on November 13; Site #2 on November 23; Site #3 on November 2; and Site #4 on December 31, 1992.

Plot design varied slightly at test locations. A completely random design was used at test sites #1 and #2. A complete randomized block design was used at sites #3 and #4. Three genotypes were evaluated: lines 5673 (transformed with construct PV-LERP01), 8338 (transformed with construct PV-LERP07) and non-transgenic UC82B control plants. Four replicates of each genotype were grown at each location. At sites #1 and #2, each plot consisted of a single row of 19-20 plants approximately 21 inches apart in the row. At site #3, each plot consisted of three

1993 Modified Ripening Tomato Trials

USDA PERMIT #93-063-04
and #93-054-01N
Jerseyville, IL and Huron, CA
MONS #93-001N, and
MONS #93-036R

Bernie Sammons

The purpose of the field trials was to evaluate agronomic performance and determine efficacy of selected genetically modified tomato lines with genes that delay ripening by decreasing levels of ethylene, the plant hormone that controls ripening. Plants expressing all but one of the delayed ripening genes were evaluated at two locations: at the Monsanto Research Farm located in Jerseyville, IL and at [CBI DELETED] located in Huron, CA under USDA permit #93-063-04. Plants containing one of the transgenes were evaluated only at the Jerseyville, IL location under USDA permit #93-054-01.

Experimental Layout

Jerseyville, IL

Planting material consisted of transplants of tomatoes transformed with either one of two delayed ripening genes. In addition to transgenic plants, non-transformed plants were evaluated in the field test for comparison purposes. Seed were sown in greenhouses at the Monsanto Research Facility located in Chesterfield, MO. Seed were sown over the following dates in 1993: April 17, 18, 19, 20, and May 5, 7, 10, and 14. Plants generated from embryo rescue (hybrids generated from line 8681) were put on media on May 11 and put to soil on May 20, 1993. Seedlings were held in the greenhouse until they were large enough to transplant in the field. Seedlings were transported to Jerseyville, IL in agreement with USDA permit #93-069-06M and USDA permit #93-075-07M. Field planting dates were May 27, June 8, and June 24, 1993. Major operations related to the field test are listed in Table 1. The lines evaluated in this test are shown in Table 2.

A randomized complete block design consisting of four blocks were planted on the full bed plastic mulch system. Each genotype was represented in each block by a single row plot of 25 plants for a total of 100 plants per genotype across all 4 blocks. Plants were spaced 24 in. apart within rows with a 5 foot spacing between rows. Fresh market tomato production practices typical of those used in Florida were adapted to Jerseyville, IL conditions: i.e, staking, tying, pest control, and drip irrigation.

Huron, CA

Planting material consisted of tomatoes transformed with a delayed ripening gene and non-transgenic controls. Seed were shipped to [CBI DELETED] located in Gustine, CA on April 15, 1993 in agreement with USDA permit #93-069-01M. Seed were sown at [CBI DELETED] on May 26, 1993. Seedlings were held in the greenhouse until they were large enough to transplant in the field. Seedlings were transported to [CBI DELETED] in Huron, CA and were transplanted in the field on July 15, 1993. Major operations related to the field test are listed in Table 2; lines evaluated in Huron are shown in Table 3.

Plants were grown in single linear rows containing approximately 200 plants per genotype. Plants were spaced 18 inches apart within the row with 5 foot spacing between rows. Production practices typical of California were used: i.e., plants were grown on raised open beds with drip irrigation and routine monitoring for plant pests.

Data Collection/Analysis

Jerseyville, IL

In total, approximately 1,727 kg of fruit was harvested over 9 dates during the months of August and October, 1993. Fruit were transported from Jerseyville, IL to Chesterfield, MO in accordance with USDA permit #93-207-01N. Fruit were harvested at either mature green, breaker, orange/pink, or red. Shelf-life studies, sensory panel and analytical analyses were performed on fruit harvested from the field trial.

Huron, CA

Approximately 94 kg of fruit was shipped from Huron, CA to Chesterfield, MO on October 20, 1993. Fruit were shipped in accordance with USDA permit #93-207-01N. Fruit were harvested at stages 2/3 (breaker/turning) with the majority of fruit at stage 3. Apparently fruit were damaged either in transit from California or in storage prior to delivery to Chesterfield, MO. Inspection of fruit upon arrival showed deterioration of tissue making the fruit unsuitable for shelf-life studies, the fruit were discarded and the experiment was terminated.

Plant Growth And General Observations

Jerseyville, IL

Survival of transplants and overall vigor of plants in the field were excellent. Differences in plant development between tomato lines were observed. Differences noted were delayed maturity, variation in plant height and overall vigor between lines. In some cases, homozygous inbred plants showed reduced vigor (stunting and more variation in growth) in comparison to heterozygous F_1 hybrids and non-transgenic control plants. In contrast, other homozygous inbred lines showed no apparent

**Table 2. Schedule Of Major Operations
Huron, CA Site. (All Dates 1993)**

April 15	Seed shipped to [CBI DELETED] located in Gustine, CA under UDSA permit #93-069-01M
May 26	Greenhouse sowing dates.
July 15	Field transplanting date
October 20	Fruits shipped from Huron, CA to Chesterfield, MO under USDA permit #93-207-01N. Approx. 206 lbs of fruit were shipped to Missouri.
November 1	Plot area disked (trial terminated). Plot area was left fallow.

Appendix VII

Tomatoes with a
Delayed Ripening gene

To: Glenn D. Austin, Monsanto

Re: Field evaluation of delayed ripening tomato trial (exp.# 92-449-703)

On March 2, 1993 I evaluated three tomato breeding lines at the BHN Research Facility in Bonita Springs, FL. The three genotypes examined were UC82B, 5673, and 8338. Some general observations of the trial as a whole might be an appropriate starting point.

The varieties appeared to all be processing tomatoes and the size and density of the bush was somewhat sub-standard to what I am used to seeing in a production field. I wrote this off to the fact that they were "grown for research" and researchers tend to be better scientists then farmers. I was very impressed, and am always relieved, to find an extremely high rate of consistency of observable traits across the repetitions. This lack of variation due to reps gave me a high degree of certainty that variation between treatments was due to the genetics and not the environment.

The following is a synopsis of the characteristics I observed for each of the varieties:

UC82B: This variety exhibited a short plant height, with an unusually dense bushy terminal growth habit on some of the plants. The fruit set was heavy, and consisted of a high percentage of red ripe fruit from the top to the bottom of the plant. Fruit size was very uniform in size and firmness. Some uneven ripeness was present, there were essentially no flowers on the plant, and 66% of fruit was trilocular.

5673: This variety had a similar plant height as UC82B, but did not show the bushy terminal growth habit. Fruit were a bit smaller, but had a heavy set, and 66% were bilocular. These fruit characteristics may have been a function of being less mature than UC82B. Only a small percentage had turned red. Fruit tended to have more interlocular air space. There were no flowers present and some of the outer most leaves exhibited a distinct purple cast. This was not due to any frost injury, but may have been a function of phosphorus deficiency.

8338: This variety had by far the tallest bush and the most immature fruit (no red fruit at all). Fruit was 66% trilocular and exhibited a lot of interlocular air space. Flowers were present on many of the plants, and there was a high incidence of dead or dying plants (most likely due to crown rot or a vascular wilt disease).

Please let me know if I can answer any questions or be of further assistance.

[CBI DELETED]

3. FTE-12
4. Duke

no comparisons were made between cultivars, only harvest maturity.

Harvest maturity:

A. Immature green (breaker/turning at 72 hrs in ethylene)
B. Mature-green (breaker/turning at 24 hrs in ethylene)
C. Vine-ripe (breaker/turning at harvest)
D. Pink (advanced turning to minimum pink at harvest)

As in the preliminary study, only fruits of the 4 harvest maturities were used which reached the table-ripe stage on the same day.

Edible quality was evaluated by the Triangle tests test. Composition was evaluated by standard laboratory methodology.

Taste Test Results:

Comparisons	Statistical significance
Maturity B vs C	ns
Maturity B vs D	ns
Maturity A vs B	*
Maturity A vs C	**
Maturity A vs D	**

There was a flavor difference between fruits harvested immature green and those of the more advanced stage of maturity. In general, the panel rated fruits harvested immature green as being more sour and lacked flavor intensity. However, the panel could not detect flavor differences (could not pair correctly) between fruits harvested mature-green and the two more advanced maturities. All samples ripened to a good red color.

[CBI DELETED]

[

CBI DELETED

]

Pages 155-183

Appendix II. USDA Final Reports

1992 Modified Ripening Tomato Trial

USDA PERMIT #92-049-01 (Mons # 92-014)
Jerseyville, IL and Davis, CA

FINAL REPORT

Bernard Sammons
Monsanto Co.

The objective of trials using genetically modified tomatoes was to evaluate delayed ripening properties in plants transformed with a gene which decreases ethylene production. The trials were conducted at the Monsanto research farm located in Jerseyville, IL and at Davis, California in collaboration with [CBI DELETED]

Experimental Layout

Planting material consisted of transplants of tomatoes transformed with a gene that degrades ACC and non-transgenic controls at both locations.

Jerseyville, IL

Seed were sown in greenhouses at the Monsanto Research Facility located in Chesterfield, MO. Seed were sown May 12 and 19, 1992. Plants generated from embryo rescue were put to soil on May 26 and June 14, 1992. Seedlings were held in greenhouses until they were large enough to transplant in the field. Seedlings were transported to Jerseyville, IL in agreement with USDA permit # 92-052-04M. Field planting dates were June 10 and June 30, 1992. Major operations related to the field trial are listed in Table 1.

A randomized complete block with four blocks were planted. Plants were a mixture of several genotypes: CBI [

](Table 2). Each genotype was represented in each block except in cases where availability of plant material was limited. In particular, all embryo rescue lines were limited to a single block. If plant material was not limiting, up to 20 plants of each genotype were planted for a total of 80 plants per genotype across the four blocks. Agronomic practices of pest control and irrigation typical of irrigated tomatoes in the area were followed.

Davis, CA

Seed were shipped to Davis, CA on May 13, 1992 in agreement with USDA permit number 92-052-04M. Seed were sown in the greenhouse and held until plantlets attained adequate growth for transplanting. Transplanting in the field was on June 18, 1992.

A block design was used with 75 plants per block with two replicates (for a total of 150 plants of each genotype). CBI [

]. Agronomic practices typical of tomato production in California were followed. Major operations conducted in the field trial are listed in Table 2.

Data Collection / Analysis

Jerseyville, IL

In total, approximately 160 kg of fruit was harvested over six dates in the months of September and October, 1992. Fruit were transported from Jerseyville, IL to Chesterfield, MO in accordance with USDA permit #92-052-04M. Fruit were harvested at either mature green, breaker, orange/pink, or red. A taste panel was done on these fruit and analytical work was performed to determine basic quality parameters.

Davis, CA

All data collection and analysis were conducted at Davis, CA (i.e., no fruit were shipped to Chesterfield, MO). Fruit were harvested at several stages of maturity and were subjected to various analytical analyses including firmness of fruit, ethylene production, enzyme analysis, aroma volatile analysis, decay and other storage parameters.

Plant Growth and General Observations

Jerseyville, IL

Survival of transplants and overall vigor of plants in the field were excellent. Differences in plant development between tomato lines were observed. Differences noted were delayed maturity, variation in plant height and overall vigor between lines. In early September, early blight disease (caused by the fungus *Alternaria solani*) was detected in plots. Since older plant tissue is more susceptible to the fungus, differences in levels of infection were observed due to varying stages of plant maturity in the field. Routine sprays with fungicides commonly used in tomatoes were initiated and served to protect plants from new infections by *Alternaria*. No increased incidence of other diseases or insect damage relative to controls was observed.

The plots were regularly monitored for *Agrobacterium* infection symptoms. None could be found.

Davis, CA

Plant stand was uniform except for transgenic line 8301 which was slower to develop. The population of 8301 plants was segregating for the transgene, yet, the slow development was uniform within this genotype. Thus, the reduced rate of development was not related to the transgene and was within normal variation seen for different seed lots. In general, plant growth and development of lines was normal and all surviving plants bore fertile fruit. However, reduced plant vigor was noted in homozygous 8301 plants when compared to heterozygous 8301 or non-transformed Flora Dade control plants. No increased incidence of diseases or insect damage relative to controls was observed.

Responses to Specific Issues:

1. Horizontal Movement:

Immunoblotting and ethylene generation assays were performed on fruit samples collected from the field. The **CBI [ ] gene was detected in transgenic fruit onlyand not detected in non-transformed control fruit. No evidence of movement of the delayed ripening gene was observed at either location.

2. Changes In Survival Characteristics:

Jerseyville, IL

Plots were mowed and disked on October 7, 1992. The area was left fallow until spring 1993 at which time soybeans were planted on May 19. Plots were observed in spring and late summer 1993 for volunteer tomatoes, but none could be found. There was no evidence of changes in survival characteristics of the transgenic tomato plants.

Davis, CA

Plots were disked on November 9, 1992. The area was left fallow through the fall and winter and observed for volunteers in the spring and summer of 1993. The plot area received adequate moisture to allow for germination of tomato seed during the observation period for volunteers. There were no volunteers found in spring 1993. The plot area was disked again in April of 1993. The area was monitored for volunteers during the summer of 1993, once again, none could be found. There was no evidence of changes in survival characteristics of the transgenic tomato plants.

3. Stability And Pattern Of Inheritance:

GUS assays for line 8301 showed the expected of 1:2:1 segregation ratio. Breeding programs involving several tomato lines transformed with the introduced gene show normal inheritance patterns in resulting progeny. Continual testing of selected lines indicates trait stability over several generations.

4. Protein Expression Level:

**CBI [

]**

5. Published Data:

At this point, we are not aware of any published data by Monsanto for the specific test.

Table 1. Schedule of major operations (all dates 1992).

Jerseyville, Illinois

May 12 and 19	Greenhouse Sowing Dates
May 26 and June 14 (embryo rescue)	Seedlings put to soil in greenhouse.
June 10	Transplanting in field (lines generated from seed).
June 30	Transplanting in field (lines generated from embryo rescue).

DATE OF FRUIT HARVEST	APPROX WEIGHT OF FRUIT TRANSPORTED TO CV
September 9	21. 5 kg
September 16	32 kg
September 22	35 kg
September 24	16 kg
September 28	30 kg
October 7	25 kg (fruit transported from Jerseyville, IL to Chesterfield, MO under USDA permit # 92-052-04M)
October 7	Test was terminated (completed)

TABLE 2. LIST OF LINES UNDER TEST JERSEYVILLE---1992

[**FRESH MARKET:**

LINE	VECTOR
	CBI
[CBI DELETED	
	]

PROCESSING:

	CBI
[CBI DELETED	
	CBI
	]

Table 3. Schedule of major operations (all dates 1992).

Davis, California

May 13	Seed mailed to Davis, CA in accordance with USDA permit # 92-052-04M.
June 18	Transplanting in field
November 9	Trial was terminated (completed)

1993 Modified Ripening Tomato Trial

USDA PERMIT #92-176-01 Amend
MONS #92-075
Spring 1993 Planting

Bernie Sammons

The purpose of the field trial was to evaluate agronomic performance and determine efficacy of selected genetically modified tomato lines with a delayed ripening gene. This spring trial was conducted at Bonita Springs, FL in collaboration with BHN under an amendment to USDA Permit 92-176-01 to allow a spring planting.

Experimental Layout

Planting material consisted of tomatoes transformed with a gene that delays ripening and non-transgenic controls. The initial shipment of seed to BHN occurred on January 5, with sowing of seed in BHN's greenhouses on January 20 and transplanting in the field on March 3, 1993. Plants in this first planting were lost due to sandblasting. There was only vegetative material that was disked into the ground. The second shipment of seed to BHN was made on March 16, seed were sown in the greenhouse on March 22, and transplanting in the field occurred on April 5, 1993. Seed shipment to BHN on both dates was in agreement with USDA permit number 92-190-02M. Field test sites were located at the BHN Research experimental farm in Bonita Springs, Florida. Major operations conducted in the field trial are listed in Table 1.

A randomized complete block design with four blocks of each line was planted on the full bed plastic mulch system. Lines evaluated are shown in Table 2; 8301 is a transgenic line transformed with PV-LERP04 and derived from Flora Dade, 8338 is a transgenic line transformed with PV-LERP07 and derived from UC82B, 8495 is a transgenic line transformed with PV-LERP04 and derived from Flora Dade, and 8681 is a transgenic line transformed with PV-LERP05 and derived from Hayslip. Each line was represented in each block by a plot of approximately 48 plants (3 rows of 16 plants) with a total of about 192 plants per genotype across all 4 blocks. Normal Florida fresh market tomato production practices were used, i.e., staking, tying, and pest control. Flora Dade and Hayslip lines were pruned removing the first four suckers. UC82B lines were not pruned.

Data Collection / Analysis

Approx. 14 kg of fruit of line 8495 were shipped from BHN to Chesterfield, MO on June 28, 1993. Fruit were shipped in accordance with USDA permit #92-294-01M.

Plant Growth And General Observations

Plants from the first planting were killed by sandblasting within two weeks of transplanting in the field. Plants generated from the later planting were exposed to high temperatures which reduced fruit number and quality. In addition, this later planting suffered from a high infestation of insects, particularly sweet potato whitefly, and a high incidence of tomato mottle gemini virus. Due to growing conditions that were substandard, fruit quality was judged to be so low that no meaningful shelf life or fruit quality evaluations could be initiated. These effects were seen equally in both the control and transgenic tomatoes.

Responses To Specific Issues:

1. Horizontal Movement:

The trial was conducted under the separation distance specific for tomato which is a self-pollinated crop. Due to the poor quality of fruit available from this field trial, quality analyses, enzyme expression, and shelf life studies were not conducted. While no definitive statement can be made on evidence of horizontal movement of the delayed ripening gene based on data generated from this field trial, data collected from other field trials where control fruit were analyzed for the presence of transgenes show no evidence of horizontal movement.

2. Changes In Survival Characteristics:

There was no evidence of changes in the survival characteristics of the transgenic tomato plants. Under normal cultural practices in Florida, fallow fields are routinely disked every two weeks during the summer when good moisture promotes seed germination, and disking is performed every 4-5 weeks after that. After regular diskings, beds are typically covered in plastic and fumigated. The areas between beds are typically maintained weed free with regular sprays of herbicides. No volunteer tomatoes have been detected in the area.

3. Stability And Pattern Of Inheritance

Breeding programs involving tomato lines transformed with the delayed ripening gene show normal inheritance patterns in resulting progeny. Continual testing of selected lines indicates trait stability over several generations.

4. Published Data

No publications are possible from this specific test. This test was terminated due to poor plant growth and substandard quality fruit.

Table 1. Schedule of major operations (all dates 1993).

Bonita Springs, Florida

January 5	First seed shipment date to BHN. Shipment in accordance with USDA permit #92-190-02M.
January 18	Seed of line 8681 shipped to BHN.
January 20	First seed sowing date in BHN research greenhouses.
March 3	First transplanting date in the field. Trial destroyed by windstorm.
March 16	Second seed shipment date to BHN. Shipment in accordance with USDA permit #92-190-02M.
March 22	Second seed sowing date in BHN research greenhouses.
April 5	Second transplanting date in the field.
June 28	Approx. 30 lbs of fruit shipped to Chesterfield, MO. Fruit were shipped in accordance with USDA permit #92-294-01M. Poor yield due to poor growing conditions (high heat); high infestation with insects; and high incidence of tomato mottle gemini virus.

1992 Delayed Ripening GLP
Tomato Field Trials

USDA PERMIT #92-176-01
MONS #92-075
Fall 1992 Planting

Bernie Sammons

The purpose of the field trials was to evaluate agronomic performance, gene expression of introduced proteins, quality traits and food safety of two tomato lines genetically modified for delayed ripening. The field trials were conducted at four sites located in Florida in collaboration with BHN-Joint Venture, located in Bonita Springs, FL. Data collected from the field trials will be used for regulatory approval of delayed ripening tomatoes containing the delayed ripening gene.

Experimental Layout:

Planting material consisted of transplants of tomatoes transformed with a gene that delays ripening and non-transgenic controls. Trials were conducted at four sites. The approximate acreage under test at each location was 0.8 acres:

Site #1. Experiment #92-449-701, located at [CBI DELETED]

Site #2. Experiment #92-449-702, located at [CBI DELETED]

Site #3. Experiment #92-449-703, located at BHN-Joint Venture.

Site #4. Experiment #92-449-704. located at [CBI DELETED]

Seed were shipped from Monsanto Co., Chesterfield, MO to BHN-Joint Venture, Bonita Springs, FL on September 21 and again on September 23, 1992 in agreement with USDA permit number 92-190-02M. Seed were sown in the greenhouse and held until plantlets attained adequate size for transplanting. Transplanting in fields occurred on the following dates: Site #1 on November 13; Site #2 on November 23; Site #3 on November 2; and Site #4 on December 31, 1992.

Plot design varied slightly at test locations. A completely random design was used at test sites #1 and #2. A complete randomized block design was used at sites #3 and #4. Three genotypes were evaluated: lines 5673 (transformed with construct PV-LERP01), 8338 (transformed with construct PV-LERP07) and non-transgenic UC82B control plants. Four replicates of each genotype were grown at each location. At sites #1 and #2, each plot consisted of a single row of 19-20 plants approximately 21 inches apart in the row. At site #3, each plot consisted of three

parallel rows of approximately 25-26 plants per row. Spacing within the row was 19 inches. At site #4, each plot consisted of a single row of 20 plants approximately 20 inches apart. Normal Florida fresh market tomato production practices were used, i,e., staking, tying, and pest control. Major operations conducted at the test sites are listed in Table 1.

Data Collection/Analysis

In total, approximately 2,300 pounds of fruit were shipped. Fruit were shipped from BHN to Monsanto Co. and the National Food Labs (NFL) located in Dublin, CA. In addition, after receipt of fruit from BHN at the NFL, shipments were also made from the NFL back to Chesterfield, MO (Monsanto Co.). Dates of fruit shipments are shown in Table 1. All shipments were made in accordance with USDA permit #92-294-01M. Fruit were harvested at several stages. Several analyses were conducted on fruit from field trials such as yield determinations, ethylene assays, firmness, rat feeding, and nutrient and toxicant determinations.

Plant Growth and General Observations

Survival of transplants and overall vigor of plants in the field initially were excellent. After planting however, several plants at each location showed symptoms of *Fusarium* crown rot, caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici.* The transformed line 8338 as a homozygous inbred clearly showed increased susceptibility to the disease across all four sites under test in comparison to UC82B control plants. Subsequent greenhouse screening showed that by classical breeding, genetic resistance in the presence of the delayed ripening transgene remains functional (Attachment I, A). Specifically, two rounds of backcrosses were made from line 8338 into a BHN inbred possessing crown rot resistance. Progeny from the backcrossed line were resistant to crown rot. No differences in disease susceptibility were observed between backcrossed progeny that expressed the delayed ripening transgene and the non-transgenic BHN inbred with resistance to crown rot. Thus, susceptibility was not related to the presence of the delayed ripening gene.

Furthermore, none of the eight most common commercial tomato varieties grown in Florida, which account for approximately 90% of the tomato acreage in the state, are resistant to *Fusarium* crown rot. Thus, line 8338 is typical of commercial Florida tomato varieties with respect to *Fusarium* crown rot susceptibility, and the 8338 germplasm is not expected to have any impact on current tomato production and production practices in Florida (Attachment I, B).

The plots were regularly monitored for *Agrobacterium tumefaciens* infection symptoms. None could be found.

Responses To Specific Issues:

1. Horizontal Movement:

Analytical assays and ethylene determinations were performed on fruit samples collected from the field. The products of the transgenes were detected in transgenic fruit only. No evidence of movement of the delayed ripening gene or the marker gene was observed.

2. Changes In Survival Characteristics:

There was no evidence of changes in the survival characteristics of the transgenic tomato plants. Under normal cultural practices in Florida, fallow fields are routinely disked every two weeks during the summer when good moisture promotes seed germination, and disking is performed every 4-5 weeks after that. As such, disking typical for the area has been conducted in the plot area at each site. A few volunteers were observed initially after harvest. These volunteers were treated as weeds and were destroyed by disking. No differences were observed in survival of volunteers between transgenic and control plants. Plots were left fallow for 1 year (12 months) after termination of the study at each test site.

3. Stability And Pattern Of Inheritance:

Breeding programs involving several tomato lines transformed with the delayed ripening gene show normal inheritance patterns in resulting progeny. Continual testing of selected lines indicates trait stability over several generations.

4. Published Data:

Results from field trials will be published in Plant Physiology (abstract).

Table 1. Schedule of major operations

September 21 and 23, 1992	Seed mailed to BHN in accordance with USDA permit #92-190-02M.
September 23, 1992	Seed sown in BHN research greenhouse for test site #1.
September 25, 1992.	Seed sown in BHN research greenhouse for test site #2.
October 1, 1992	Seed sown in BHN research greenhouse for test site #3.
November 9, 1992	Seed sown in BHN research greenhouse for test site #4.
November 13, 1992	Transplanting, site #1.
November 23, 1992	Transplanting, site #2.
November 20, 1992	Transplanting, site #3.
December 31, 1992	Transplanting, site #4.

In total, approximately 2300 pounds of fruit were shipped from BHN to Monsanto Co. and the National Food Laboratory (NFL), or from the NFL back to Monsanto. All shipments were made in accordance with USDA permit #92-294-01M on the following dates:

February 15, 1993	From site #1, mature green and orange fruit shipped to NFL. Orange fruit shipped to Monsanto.
March 3, 1993	From site #2, mature green and orange fruit shipped to NFL. Orange fruit shipped to Monsanto.

Table 1 (cont). Schedule of major operations

February 22, 1993	From site #3, mature green and orange fruit shipped to NFL. Mature green and orange fruit shipped to Monsanto.
March 1, 8, 9, 15, 17, 18, 1993	From site#3: shipment of fruit and 22, from BHN to Monsanto.
March 15, 16, and 17, 1993	From site #3: shipment of fruit from BHN to NFL, Dublin, CA
March 17, 18, and 25, 1993	From site #3: shipment of fruit from NFL to Monsanto Co.
April 19, 1993	From site #4, mature green and orange fruit shipped to NFL. Orange fruit shipped to Monsanto.

Each Trial Was Terminated Approx. 1 Week After 2nd Harvest:

March 24, 1993	Second harvest, site #1.
April 6, 1993	Second harvest, site #2.
April 8, 1993	Second harvest, site #3.
May 3, 1993	Second harvest, site #4.

Attachment I

A. Summer 1993 greenhouse *Fusarium* crown rot evaluation of Monsanto delayed ripening genotypes, by Dr. David Linde and Dr. J. Augustine, BHN-Joint Venture, Bonita Sprngs FL.

B. Letter from Dr. J. Scott, The University of Florida, Gulfcoast Research and Education Center, FL stating that most commercial tomato varieties grown in Florida are *Fusarium* crown rot susceptible.

RESEARCH REPORT

TITLE Summer 1993 Fusarium Crown Rot Evaluation of Monsanto ACC-Deaminase Genotypes

AUTHORS David Linde and Jim Augustine
BHN-Joint Venture
16750 Bonita Beach Rd
Bonita Springs
FL 33923

ABSTRACT

Tomato plants that express the enzyme 1-aminocyclopropane-1-carboxylic acid (ACCd) have been developed at Monsanto Co., St Louis. Fruit of these plants have reduced rates of ethylene synthesis and delayed fruit ripening compared to controls. In field trials with two Delayed Ripening (DR) tomato lines (designated 5673 and 8338) and the parental control line UC82B, some plants were affected by the disease, *Fusarium* crown rot. A higher incidence of the disease was observed in DR line 8338 compared to the control. Differences in disease incidence between line 5673 and the control UC82B were minimal. In the current study, susceptibility of tomato lines 5673, 8338, and UC82B to *Fusarium* crown rot disease was examined under controlled environment growth conditions. The purpose of the study was to a) confirm the field observation that line 8338 is more susceptible to *Fusarium* crown rot disease than the control line, b) determine if 8338 plants lacking disease in the field studies are selections away from disease susceptibility, and c) determine if disease susceptibility of line 8338 can be overcome by backcross to a *Fusarium* crown rot resistant cultivar.

Under controlled environment conditions, DR line 8338 was more susceptible to *Fusarium* crown rot disease than control line UC82B, confirming observations made under field conditions. However, susceptibility of line 8338 to *Fusarium* crown rot was overcome by backcross to a resistant cultivar. Since *Fusarium* crown rot resistance germplasm can be used in tomato breeding programs, susceptibility of line 8338 to *Fusarium* will be eliminated in the commercial

development breeding program with this line. Accordingly, there is no concern with *Fusarium* susceptibility of line 8338. There was no significant difference in disease susceptibility between DR tomato line 5673 and control line UC82B, in this study. Disease susceptibility of line 8338 single plant selections (no disease observed in field studies) was either equivalent or greater than that of line 8338 derived from the original seed lot. This suggests that the absence of disease in these plants in the field trials was due to the absence of disease pressure, and was not due to selection away from factors that affect susceptibility of this line to *Fusarium* crown rot disease.

INTRODUCTION

Tomato plants that express the enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) have been developed at Monsanto Company, St Louis, MO (1). The enzyme ACCd catalyzes metabolism of ACC to α-ketobutyrate and ammonia (2). Because ACC is the immediate precursor to ethylene in the synthesis of ethylene from methionine in plants (3, 4), and ethylene initiates and controls the rate of fruit ripening (5), these plants have reduced rates of ethylene synthesis and delayed fruit ripening (1).

Two tomato lines that express ACCd (lines designated 5673 and 8338) and the parental control line UC82B, were grown at four field sites in Florida during the 1992-1993 season, as part of a food safety assessment of delayed ripening (DR) tomato lines (6). Some plants in these field studies were affected by *Fusarium* crown rot disease, and a higher incidence of the disease was observed in DR line 8338 compared to the control (6). Differences in disease incidence between line 5673 and the control UC82B were minimal. The disease is caused by the soil borne fungus *Fusarium oxysporum*, f.sp. *radici-lycopersici* and can be controlled through the use of resistant cultivars (7).

In the current study, susceptibility of tomato lines 5673, 8338, and UC82B to *Fusarium* crown rot disease was examined under controlled environment growth conditions. The purpose of the study was to a) confirm the field observation that line 8338 is more susceptible to *Fusarium* crown rot disease than the control line, b) determine if 8338 plants lacking disease in the Florida Regulatory field trials are selections away from disease susceptibility, and c) determine if disease susceptibility of line 8338 can be eliminated by backcross to a *Fusarium* crown rot resistant cultivar. Results of this study will be used to support the safety assessment and regulatory approval of DR tomato line 8338, and provide key information for commercial variety selection.

MATERIALS and METHODS

Plant lines

Tomato lines examined in the study are divided into three groups, as follows:
Group A. Plants of DR lines 5673, 8338, and control line UC82B were grown from the same seed lot used to grow plants for the 1992-1993 Florida Regulatory field trials (6).
Group B. Plants from seed of single 8338 plants grown in 1992-1993 Florida Regulatory field trials that showed no *Fusarium* crown rot disease symptoms in the field study. These plants are designated 8338-1, 8338-2, 8338-3, 8338-4, 8338-5, and 8338-6.
Group C. CRl is a crown rot resistant line. BC2F1-Fcr is the second backcross generation of line 8338 into a BHN inbred which possesses crown rot resistance. BC2F1-Fcr+ is the second backcross generation of 8338 into a BHN inbred which does not possess crown rot resistance. BC2F1-Fcr and BC2F1-Fcr+ have similar parental pedigree.

Plant culture, and plant disease assessment

Two separate experiments were conducted to determine susceptibility of the tomato lines described above to *Fusarium* crown rot disease. The two experiments differed in age of plants used, and associated tolerance to the pathogen.

Experiment 1

Seed of the lines described above (plant groups A, B, and C) were sown in coal flats on June 8, 1993. The soil plug mix was washed off the roots, and plants were root dip inoculated on June 23, 1993 with a mixture (approximately 1:1) of two *Fusarium* crown rot isolates, FOR-1 and FOR-2. Isolate FOR-1 was obtained from Gulfcoast Farm #11, Collier Co, FL, and FOR-2 isolate was obtained from Manley Farm, Bonita Springs, FL., and were collected from tomato plants grown during Spring 1993. Plants were grown in a growth chamber at 72°F and a 12-hour photoperiod for the first 12 days of the experiment. For the last week of the experiment, plants were transferred to a greenhouse. Plants were fertilized with 100 ppm Nutrileaf fertilizer solution during the course of the experiment. The experimental design was a randomized complete block with 3 blocks of each tomato line. Blocks 1 and 2 consisted of 240-celled Speedling type flats filled with a 50:50 mixture of peatmoss and vermiculite adjusted to pH 4.56 with calcium carbonate. Block 3 consisted of flats filled with the same planting mix adjusted to pH 4.45. Each tomato line was represented in each block by 36 plants. Disease susceptibility of each tomato line was determined on July 12, and was measured as the number of plants surviving from pathogen inoculation.

Experiment 2

Seed of the tomato lines described above (plant groups A, B, and C) were sown in 128-celled Speedling trays on May 7 and 10, 1993. The soil plug mix was washed off the roots and plants were root-dip inoculated with *Fusarium* crown rot isolates, FOR-l and FOR-2, on July 2, 1993. The experiment was a randomized complete block design with 3 blocks of each tomato line. Block 1 consisted of commercial bulb flats filled with a 50:50 mixture of peatmoss and vermiculite adjusted to pH 4.50 with calcium carbonate. Block 2 consisted of bulb flats filled with the same planting mix adjusted to pH 4.92, and block 3 consisted of bulb flats filled with the same soil mix adjusted to pH 4.42. Each tomato line was represented in each block by 12 plants. Plants were fertilized with 100 ppm Nutrileaf fertilizer, that contains high ammonium nitrogen. Plants were grown in a growth chamber set at 72°F with a 12 hour photoperiod. Plants were rated for crown rot damage on July 26, 1993 using the following scale:

O = no vascular discoloration in the crown.
1 = light brown vascular discoloration in the crown.
2 = dark brown vascular discoloration in less than 50% of the crown.
3 = dark brown vascular discoloration in more than 50% of the crown.
4 = plant dead.

Statistical analyses
A 2 x 5 chi-square test of homogeneity was used to make specific comparisons of disease severity between tomato lines. The test detects differences between lines with respect to the distribution of disease ratings. The disease ratings were pooled across all blocks, since analysis of variance (ANOVA) showed no block effects with respect to line and disease.

RESULTS and DISCUSSION

Experiment 1
The average percent of plants surviving from *Fusarium* crown rot infection across all 3 experimental blocks for each tomato line are shown in Table 1. The data show that the *Fusarium* crown rot disease pressure was too high in this experiment, with no survival of most tomato lines, and some plant death among resistant lines. Therefore, detection of small differences in disease susceptibility between lines was not possible in this experiment. Accordingly, the experiment was repeated using older plants, that are generally more tolerant of the disease.

Experiment 2
The disease ratings for each tomato line averaged across individual plants in all 3 experimental blocks are shown in Table 2. Statistical comparisons of disease ratings among lines (based on the data presented in Table 2) are shown in Table 3. DR line 8338 was slightly more susceptible to *Fusarium* crown rot disease than control line UC82B (statistically significant at the 5% level), consistent with the observation from 1992-1993 Florida Regulatory field trials (6). There was no significant difference in *Fusarium* crown rot disease susceptibility between DR line 5673 and control UC82B in this study. *Fusarium* crown rot disease susceptibilities of line 8338 single plant selections (8338-1 to -6) were equivalent to line 8338 (grown from the same seed lot used for 1992-1993 Florida Regulatory field trials), except for 8338-2 that showed significantly greater disease susceptibility. *Fusarium* crown rot disease susceptibility of line 8338 was overcome when backcrossed to a resistant cultivar (BC_2F_1-Fcr). Disease susceptibilities of the control resistant line (CR1) and line BC_2F_1-Fcr were negligible in this experiment. Line BC_2F_1-Fcr+ (line 8338 backcrossed into a *Fusarium* crown rot susceptible cultivar) was disease susceptible (Table 2). This showed that elimination of line 8338 *Fusarium* crown rot disease susceptibility was specific to the resistance germplasm of the backcross line.

CONCLUSIONS

In the current study, DR tomato line 8338 was slightly more susceptible to *Fusarium* crown rot disease than control line UC82B, confirming observations made in the 1992-1993 Florida Regulatory field trials (6). Among the eight most common commercial tomato varieties grown in Florida (these varieties account for approximately 90% of the tomato acreage in the state), none have *Fusarium* crown rot resistance (personal communication, Dr. J. Scott, The University of Florida, Institute of Food and Agricultural Sciences, FL., in a

letter included as Attachment 1 to this report). Therefore, line 8338 is typical of commercial Florida tomato varieties with respect to *Fusarium* crown rot resistance, and the 8338 germplasm is not expected to have any impact on current tomato production and production practices in Florida. In addition, the *Fusarium* crown rot resistance germplasm can be used in tomato breeding programs, and we have demonstrated that susceptibility of line 8338 to *Fusarium* crown rot disease is readily overcome by backcross to a resistant line. Accordingly, *Fusarium* susceptibility of line 8338 is of no concern.

Disease susceptibility of line 8338 single plant selections was either equivalent or greater than that of line 8338 derived from the original seed lot. This suggests that the absence of disease in the single plant selections in the 1992-1993 Florida Regulatory field trials was due to the absence of disease pressure, and was not due to selection away from factors that affect susceptibility of this line to *Fusarium* crown rot disease.

There was no significant difference in disease susceptibility between DR tomato line 5673 and control line UC82B. Since line 5673 expresses ACCd and ethylene synthesis is reduced in this line (6), the data suggest that line 8338 susceptibility to *Fusarium* crown rot disease is unrelated to ACCd expression and inhibition of ethylene synthesis. However, further studies with 8338 backcross lines and additional Delayed Ripening tomato lines are required to determine if inhibition of ethylene synthesis, ACCd expression and susceptibility to *Fusarium* crown rot disease are related for line 8338.

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TABLE 1. Effect of *Fusarium* crown rot infection on plant survival of Delayed Ripening tomato lines 8338 and 5673, control line UC82B, line 8338 single plant selections (8338-1 to 8338-6), resistant line CR1, and 8338 backcross lines to disease resistant and susceptible inbreds, BC_2F_1-Fcr and BC_2F_1-Fcr+, respectively. Identity of all lines and experimental methods are described in Materials and Methods for Experiment 1.

Tomato line	Plant survival (%)
UC82B	0
5673	0
8338	0
8338-1	0
8338-2	0
8338-3	0
8338-4	0
8338-5	0
8338-6	0
CR1	83
BC_2F_1-Fcr	65
BC_2F_1-Fcr+	1

TABLE 2. Disease rating for Delayed Ripening and control tomato lines after *Fusarium* crown rot infection. Disease ratings are averages for individual plants of a line across experimental blocks. The lines are identified in the legend of Table 1. The disease rating scale ranges from 0 (no disease) to 4 (plant death), and is described in Materials and Methods for Experiment 2.

Tomato line	Average disease rating
UC82B	2.9
5673	3.3
8338	3.4
8338-1	3.5
8338-2	3.8
8338-3	3.5
8338-4	3.4
8338-5	3.3
8338-6	3.7
CR1	0.1
BC_2F_1-Fcr	0.1
BC_2F_1-Fcr+	2.9

TABLE 3. Statistical comparisons of disease severity ratings for Delayed Ripening and control tomato lines. The statistical comparisons are for results from Experiment 2. Average disease ratings for each line are shown in Table 2. Experimental details and a descriptioin of statistical methods are presented in Materials and Methods.

Statistical Comparison	chi-square value	Significance[1]
UC82B vs. 5673	7.26	ns
UC82B vs. 8338	11.14	*
8338 vs. 8338-1	4.06	ns
8338 vs. 8338-2	10.78	*
8338 vs. 8338-3	4.04	ns
8338 vs. 8338-4	1.82	ns
8338 vs. 8338-5	4.16	ns
8338 vs. 8338-6	4.38	ns
CRl vs. BC2F1-Fcr	1.02	ns
BC2F1-Fcr vs. BC2Fl-Fcr+	65.40	**

[1] ns = not significant, * = significant at 5% level, ** = significant at 1% level.

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June 6, 1994

Dr. Glen Austin
Monsanto
Mailzone BB40
700 Chesterfield Village Parkway
Chesterfield, MO 63198

Dear Glen;

The major tomato varieties in Florida in descending order of acreage are: Agriset 761, Sunny, Solar Set, Bonita, Sunbeam, BHN 26, Merced, and Cobia. These varieties account for about 90% of the acreage in the state. None are resistant to Fusarium crown rot (F. oxysporum f sp. radicus lycopersici).

See you in Asheville.

Sincerely yours,

J. W. Scott
Professor,
Vegetable Breeding

JWS:dmb

1993 Modified Ripening Tomato Trials

USDA PERMIT #93-063-04
and #93-054-01N
Jerseyville, IL and Huron, CA
MONS #93-001N, and
MONS #93-036R

Bernie Sammons

The purpose of the field trials was to evaluate agronomic performance and determine efficacy of selected genetically modified tomato lines with genes that delay ripening by decreasing levels of ethylene, the plant hormone that controls ripening. Plants expressing all but one of the delayed ripening genes were evaluated at two locations: at the Monsanto Research Farm located in Jerseyville, IL and at [CBI DELETED] located in Huron, CA under USDA permit #93-063-04. Plants containing one of the transgenes were evaluated only at the Jerseyville, IL location under USDA permit #93-054-01.

Experimental Layout

Jerseyville, IL

Planting material consisted of transplants of tomatoes transformed with either one of two delayed ripening genes. In addition to transgenic plants, non-transformed plants were evaluated in the field test for comparison purposes. Seed were sown in greenhouses at the Monsanto Research Facility located in Chesterfield, MO. Seed were sown over the following dates in 1993: April 17, 18, 19, 20, and May 5, 7, 10, and 14. Plants generated from embryo rescue (hybrids generated from line 8681) were put on media on May 11 and put to soil on May 20, 1993. Seedlings were held in the greenhouse until they were large enough to transplant in the field. Seedlings were transported to Jerseyville, IL in agreement with USDA permit #93-069-06M and USDA permit #93-075-07M. Field planting dates were May 27, June 8, and June 24, 1993. Major operations related to the field test are listed in Table 1. The lines evaluated in this test are shown in Table 2.

A randomized complete block design consisting of four blocks were planted on the full bed plastic mulch system. Each genotype was represented in each block by a single row plot of 25 plants for a total of 100 plants per genotype across all 4 blocks. Plants were spaced 24 in. apart within rows with a 5 foot spacing between rows. Fresh market tomato production practices typical of those used in Florida were adapted to Jerseyville, IL conditions: i.e, staking, tying, pest control, and drip irrigation.

Huron, CA

Planting material consisted of tomatoes transformed with a delayed ripening gene and non-transgenic controls. Seed were shipped to [CBI DELETED] located in Gustine, CA on April 15, 1993 in agreement with USDA permit #93-069-01M. Seed were sown at [CBI DELETED] on May 26, 1993. Seedlings were held in the greenhouse until they were large enough to transplant in the field. Seedlings were transported to [CBI DELETED] in Huron, CA and were transplanted in the field on July 15, 1993. Major operations related to the field test are listed in Table 2; lines evaluated in Huron are shown in Table 3.

Plants were grown in single linear rows containing approximately 200 plants per genotype. Plants were spaced 18 inches apart within the row with 5 foot spacing between rows. Production practices typical of California were used: i.e., plants were grown on raised open beds with drip irrigation and routine monitoring for plant pests.

Data Collection/Analysis

Jerseyville, IL

In total, approximately 1,727 kg of fruit was harvested over 9 dates during the months of August and October, 1993. Fruit were transported from Jerseyville, IL to Chesterfield, MO in accordance with USDA permit #93-207-01N. Fruit were harvested at either mature green, breaker, orange/pink, or red. Shelf-life studies, sensory panel and analytical analyses were performed on fruit harvested from the field trial.

Huron, CA

Approximately 94 kg of fruit was shipped from Huron, CA to Chesterfield, MO on October 20, 1993. Fruit were shipped in accordance with USDA permit #93-207-01N. Fruit were harvested at stages 2/3 (breaker/turning) with the majority of fruit at stage 3. Apparently fruit were damaged either in transit from California or in storage prior to delivery to Chesterfield, MO. Inspection of fruit upon arrival showed deterioration of tissue making the fruit unsuitable for shelf-life studies, the fruit were discarded and the experiment was terminated.

Plant Growth And General Observations

Jerseyville, IL

Survival of transplants and overall vigor of plants in the field were excellent. Differences in plant development between tomato lines were observed. Differences noted were delayed maturity, variation in plant height and overall vigor between lines. In some cases, homozygous inbred plants showed reduced vigor (stunting and more variation in growth) in comparison to heterozygous F_1 hybrids and non-transgenic control plants. In contrast, other homozygous inbred lines showed no apparent

differences in the field when compared to control and F_1 hybrid lines. Such variability is common in primary transformants and initial progeny and inbreds.

The plots were regularly monitored for *Agrobacterium tumefaciens* infection symptoms. None could be found.

Huron, CA

The overall vigor of plants was judged moderate to fair at best. Fruit from all lines were smaller than expected when compared to fruit produced by these same lines at other locations (previous trials in Bonita Springs, FL and Jerseyville, IL). Although all fruit were smaller in size than expected, no differences in size were observed between transgenic lines 8495, 8680 and control Flora Dade fruit. However, transgenic fruit from line 8338 were smaller than control UC82B fruit. It must be stressed that fruit across all lines were smaller in size than expected and were of marginal quality.

The plots were regularly monitored for *Agrobacterium tumefaciens* infection symptoms. None could be found.

Responses To Specific Issues:

1. Horizontal Movement - Jerseyville IL:

Ethylene generation and analytical assays were conducted on fruit samples collected from the field. The delayed ripening genes were detected in transgenic fruit only. No evidence of movement of delayed ripening genes was observed.

2. Changes In Survival Characteristics - Jerseyville IL:

The plot area was disked on October 21, 1993. The area was planted with wheat on October 26, 1993. The plot area was observed for volunteer tomatoes on March 16, 1994. None could be found. The plot area will be monitored through summer and fall of 1994 for the presence of volunteers. To date, there is no evidence of changes in survival characteristics of the transgenic tomato plants.

Huron, CA:

The plot area was disked on November 1, 1993. The area was left fallow after termination of the trial. The plot area was observed for volunteer tomatoes on April 4, 1994. None could be found. The plot area will be monitored through summer and fall of 1994 for the presence of volunteers. To date, there is no evidence of changes in survival characteristics of the transgenic tomato plants.

3. Stability And Pattern Of Inheritance:

Breeding programs involving several tomato lines transformed with delayed ripening genes show normal inheritance patterns in resulting progeny. Continual testing of selected lines indicates trait stability over several generations.

4. Published Data
At this point, we are not aware of any published data by Monsanto for this specific test.

Table 1. Schedule Of Major Operations Jerseyville Site. (All Dates 1993)

April 17, 18, 19, 20 and May 5, 7, 10, 14,	Greenhouse sowing dates
May 20 (embryo rescue plants)	Seedlings put to soil in greenhouse.
May 27, June 8 and 24	Field transplanting dates
August 5, 11, 17, 24, 31; September 9, 15, 28; and October 11	Fruit transported from Jerseyville, IL to Chesterfield, MO. In total, approx. 3,800 lbs of fruit were transported from IL to Chesterfield, MO. All transport of fruit was performed under USDA permit #93-207-01N.
October 23	Plot area disked (trial terminated). Wheat planted in plot area Oct. 26.

**Table 2. Schedule Of Major Operations
Huron, CA Site. (All Dates 1993)**

April 15	Seed shipped to [CBI DELETED] located in Gustine, CA under UDSA permit #93-069-01M
May 26	Greenhouse sowing dates.
July 15	Field transplanting date
October 20	Fruits shipped from Huron, CA to Chesterfield, MO under USDA permit #93-207-01N. Approx. 206 lbs of fruit were shipped to Missouri.
November 1	Plot area disked (trial terminated). Plot area was left fallow.

Table 3. Lines Evaluated in Jerseyville, IL and Huron, CA 1993

Jerseyville, IL:

Line	Vector
8301	PV-LERP04
8338	PV-LERP07
8495	PV-LERP04
8680	PV-LERP04
8681	PV-LERP05
9143	PV-LERP08
9250	PV-LERP04
9465	PV-LERP05
9535	PV-LERP08
9543	PV-LERP08
9699	PV-LERP08
9710	PV-LERP09
9711	PV-LERP09
9714	PV-LERP08
10136	PV-LERP09
10419	PV-LERP09
10422	PV-LERP09

Huron, CA

8338	PV-LERP07
8495	PV-LERP04
8680	PV-LERP04

Fall 1993 Modified Ripening Tomato Trial
USDA PERMIT #93-203-01N
MONSANTO #93-085-RB
Bonita Springs FL / BHN

Bernie Sammons

The purpose of the field trials was to evaluate agronomic performance and determine efficacy of selected genetically modified tomato lines with a delayed ripening trait resulting from decreased ethylene production. Plants were evaluated under USDA permit #93-203-01N in a trail conducted at Bonita Springs, FL in collaboration with BHN.

Experimental Layout

Planting material consisted of transplants of tomatoes transformed to express the trait of delay in fruit ripening. In addition to transgenic plants, nontransformed plants were evaluated in the field test for comparison purposes. With the exception of line 8338 backcrossed material, seed were shipped to BHN on July 26 and August 4, 1993 in agreement with USDA permit number 93-187-02N. Seed generated from backcrosses with line 8338 were generated at BHN located in Bonita Springs, FL. All seed were sown in BHN's greenhouses on August 9, 1993. Field transplanting occurred on September 28, 1993. Field test sites were at the BHN research facility (Field 1) and NT Gargiulo Farm 8, both located in Bonita Springs, FL. Major operations conducted in the field trial are listed in Table 1. Lines that were evaluated are shown in Table 2.

A randomized complete block design was used at both locations. At the BHN site (Field #1), there were 4 blocks x 30 plants/block for a total of 120 plants per genotype. At the NTG Farm 8 location, where possible, there were 2 blocks with 30 plants/block for each genotype. Fresh market tomato production practices typical for Florida were used for field trials (staking, tying, pruning and pest control).

Data Collection/Analysis

In total, approximately 880 lbs of fruit was harvested and shipped to Chesterfield, MO. Fruit were shipped to Monsanto Co. in accordance with USDA permit #93-203-01N/Monsanto #93-085-RB. Fruit were harvested at stages 1, 2, 3, 4, and 5. Fruit were sent to Monsanto Co. research laboratories for ethylene analysis, gene expression assays and sensory panel/flavor life evaluation. Of fruit kept at BHN, lead lines picked at different stages were either exposed to ethylene or left untreated and subjected to shelf-life evaluations.

Plant Growth And General Observations

Survival of transplants was generally very good and few resets were necessary.

Vigor was good in all lines except 9465 which was somewhat stunted. Overall, transgenics and non-transgenics were generally indistinguishable for plant type. Several diseases typical of the Florida production area were observed during the field trials: virus diseases (potato virus Y and/or tomato mottle geminivirus), bacterial spot, early blight, and *Fusarium* crown rot (Fcr).

As a followup to a previous field trial and green house study where plants of the parental line 8338 showed statistically significantly greater infection by *Fusarium* crown rot than those of the UC82B non-transgenic control, three 8338 backcrossed lines were selected for evaluation in this field trial. The three recurrent lines used as parents were: Line 22 (resistant to crown rot), Line 24 (highly susceptible to crown rot) and 26 (moderately susceptible to crown rot) and the resulting BC_2F_1 backcross progeny lines were 21, 23 and 25 respectively. The results show that in the susceptible backgrounds, plants positive for the 8338 insert are more susceptible than negative segregants or the non-transformed recurrent parent. However, in the presence of a Fcr resistance gene in line 21, transgenic plants show equivalent resistance ratings to negative segregants and the non-transformed parent. That is, in the resistant backcross line, there was no increase in the level of disease in the positive plants (those expressing the transgene) over the negative plants (those without the transgene). As noted in the attached letter (Attachment I), none of the eight most common commercial tomato varieties grown in Florida, which account for approximately 90% of the tomato acreage in the state, are resistant to *Fusarium* crown rot. Thus, line 8338 is typical of commercial Florida tomato varieties with respect to *Fusarium* crown rot susceptibility, and the 8338 germplasm is not expected to have any impact on current tomato production and production practices in Florida.

There were no differences observed between genotypes for the other diseases scored.

There was no evidence of *Agrobacterium tumefaciens* or crown gall in the field.

Responses To Specific Issues:

1. Horizontal Movement:
Ethylene generation and analytical assays were conducted on fruit samples collected from the field. The delayed ripening genes and trait were detected in transgenic fruit only. No evidence of pollen movement of a delayed ripening gene to other tomatoes was observed.

2. Changes In Survival Characteristics:
The BHN site (Field #1) was disked on February 12 and the NTG Farm #8 site was disked in March 1, 1994. Fields were disked 3 additional times after the initial disking operation. Fields were left fallow until September 1994. Fields were planted with tomatoes on September 6, 1994. Direct observations for volunteers found none. To date, there is no evidence of changes in survival characteristics of the delayed ripening tomato plants.

3. Stability And Pattern Of Inheritance:

Breeding programs involving tomato lines transformed with a delayed ripening gene show the expected inheritance patterns in resulting progeny. Continual testing of selected lines indicates trait stability over several generations.

4. Published Data:
At this point, we are not aware of any published data by Monsanto for this specific test.

**Table 1. Schedule Of Major Operations
Bonita Springs, Florida 1993**

July 26 and August 4, 1993	Seed shipped to BHN in agreement with USDA #93-187-02N
August 9, 1993	Greenhouse sowing date.
September 28, 1993	Field transplanting date
January 19, 22, 23 and February 3, 1994	Dates of harvest.
January 19 and 24, 1994	Approx. 880 lbs of fruit shipped to Chesterfield, MO. Fruit were shipped in accordance with USDA permit #93-203-01N.
February 12, 1994	Termination of BHN field site. Field disked.
March 1, 1994.	Termination of NTG farm 8 site. Field disked.

**Table 2. Lines Evaluated in Bonita Springs, FL.
Fall 1993**

Line	Vector
Progeny Line 21: (BC2F1 from line 8338)	PV-LERP07
Progeny Line 23: (BC2F1 from line 8338)	PV-LERP07
Progeny Line 25: (BC2F1 from line 8338)	PV-LERP07
8495	PV-LERP04
8680	PV-LERP04
8681	PV-LERP05
9143	PV-LERP08
9437	PV-LERP05
9465	PV-LERP05
9535	PV-LERP08
9543	PV-LERP08
9710	PV-LERP09
9711	PV-LERP09
10136	PV-LERP09

Attachment I

Letter from Dr. J. Scott, The University of Florida, Gulfcoast Research and Education Center, FL stating that most commercial tomato varieties grown in Florida are *Fusarium* crown rot susceptible.

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June 6, 1994

Dr. Glen Austin
Monsanto
Mailzone BB40
700 Chesterfield Village Parkway
Chesterfield, MO 63198

Dear Glen;

The major tomato varieties in Florida in descending order of acreage are: Agriset 761, Sunny, Solar Set, Bonita, Sunbeam, BHN 26, Merced, and Cobia. These varieties account for about 90% of the acreage in the state. None are resistant to Fusarium crown rot (F. oxysporum f sp. radicus lycopersici).

See you in Asheville.

Sincerely yours,

J. W. Scott
Professor,
Vegetable Breeding

JWS:dmb

Appendix III. Example Monitoring Forms

1992 Tomato Ripening TRIAL
Jerseyville, IL

USDA # 92-049-01
MONS# 92-014
PS-89

Field Monitoring for Disease, Insect, Weediness and Plant Growth Characteristics

Appendix III

Tomatoes with a Delayed Ripening gene

- Make observations at least once every 2 weeks during the growing season.

- Compare control vs. transgenic lines for obvious differences by the following criteria:

Disease:	resistance/susceptibility to diseases
Insects:	abundance of non-target species and resistance/susceptibility to arthropod feeding not specifically engineered to resist
Plant growth:	plant morphology and growth similar for both transgenic and non-transgenic plants
Weediness:	germination, flowering, seed production, etc. similar for both transgenic and nontransgenic plants

- Record observations on the attached forms.

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases?

Date 6/15/92 yes ☐ no ☒ No. of plants obs. ~1,400 (ENTIRE PLOT AREA) % of plants affected N/A

Comments: No Diseases Apparent

Date 6/24 yes ☐ no ☒ No. of plants obs. ~1,400 ENTIRE PLOT AREA % of plants affected N/A

Comments: No Diseases Apparent

Date 6/30 yes ☐ no ☒ No. of plants obs. ~1,400 ENTIRE PLOT AREA % of plants affected _____

Comments: No Diseases Apparent

Date 7/7 yes ☐ no ☒ No. of plants obs. ~1,400 ENTIRE PLOT AREA % of plants affected _____

Comments: No Diseases Apparent

Date 7/20 yes ☐ no ☒ No. of plants obs. ~1,400 ENTIRE PLOT AREA % of plants affected _____

Comments: No Diseases Apparent

Date 8/10 yes ☐ no ☒ No. of plants obs. 1,400 ENTIRE PLOT AREA % of plants affected _____

Comments: No Diseases Apparent

Date 8/27 yes ☐ no ☒ No. of plants obs. 1,400 ENTIRE PLOT AREA % of plants affected _____

Comments: No Diseases Apparent

Date 9/9 yes ☐ no ☒ No. of plants obs. 100 % of plants affected _____

Comments: Alternaria blight - present ; uniform infection across all lines

Date 9/16 yes ☐ no ☒ No. of plants obs. _____ % of plants affected _____

Comments: SAME - NEW Infections held in Check w/ fungicide sprays

Date 9/28/92 yes ☐ no ☒ No. of plants obs. 100 % of plants affected _____

Comments: SAME

Bernard Hammond — Individual recording observations

Jerseyville, IL — Study Location

DR-JV 1992 — Study Title

Field Monitoring for Insect Susceptibility:

Do transgenic plants have a higher incidence of non-target species than nontransgenic plants? If yes, which species are more prevalent?

Date 6/15/92 yes ☐ no ☒ No. of plants obs. ~1,400 ENTIRE PLOT AREA % of plants affected _____

Comments: NO INSECT DAMGE EVIDENT

Date 6/24 yes ☐ no ☒ No. of plants obs. ~1,400 % of plants affected _____

Comments: SAME

Date 6/30 yes ☐ no ☒ No. of plants obs. ~1,400 % of plants affected _____

Comments: SAME

Date 7/7 yes ☐ no ☒ No. of plants obs. ~1,400 % of plants affected _____

Comments: SAME

Date 7/20 yes ☐ no ☒ No. of plants obs. ~1,400 % of plants affected _____

Comments: SAME

Date 8/10 yes ☐ no ☒ No. of plants obs. ~1,400 % of plants affected _____

Comments: SAME

Date 8/27 yes ☐ no ☒ No. of plants obs. ~1,400 % of plants affected _____

Comments: SAME

Date 9/9 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: FEW TOMATO FRNITWORM / RANDOM IN PLOTS

Date 9/16 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: SAME

Date 9/28/92 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: SAME

Bernard Hammond
Individual recording observations

Jerseyville, IL
Study Location

DR-JV-1992
Study Title

Field Monitoring for Plant Growth Characteristics:

Is there a difference in the general appearance and growth of transgenic and nontransgenic plants? If yes, describe the differences (vigor, bushiness, leaf morphology, plant height, etc.).

Date 6/15/92 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: _____

Date 6/24 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: _____

Date 6/30 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: _____

Date 7/7 yes ☐ no ☒ No. of plants obs. ~100 % of plants affected _____

Comments: _____

Date 7/20 yes ☒ no ☐ No. of plants obs. ~1,000 % of plants affected 30%

Comments: Delayed maturity in some transgenic lines; variation in height & vigor

Date 8/10 yes ☒ no ☐ No. of plants obs. ~1,000 % of plants affected _____

Comments: SAME

Date 8/27 yes ☒ no ☐ No. of plants obs. ~1,000 % of plants affected _____

Comments: SAME

Date 9/9 yes ☒ no ☐ No. of plants obs. ~1,000 % of plants affected _____

Comments: SAME

Date 9/16 yes ☒ no ☐ No. of plants obs. ~100 % of plants affected _____

Comments: SAME

Date 9-28-92 yes ☒ no ☐ No. of plants obs. ~100 % of plants affected _____

Comments: SAME

Bernard Sammons — Individual recording observations

Jerseyville, IL — Study Location

DR-JV 1992 — Study Title

Field Monitoring for Weediness Characteristics:

Is the germination of transgenic plants in any way different than nontransgenic plants?
yes ☐ no ☒ If yes, describe differences and potential causes.

number of plants observed ~2,000 - Greenhouse sowings % of plants affected _____ Date _____

Is the number of days from planting until flowering (first flowers bloomed) the same for transgenic and nontransgenic plants? yes ☒ no ☐ If no, describe differences and potential causes.

OBSERVED NO DIFFERENCE IN TIME TO FIRST BLOOM

number of plants observed ~100 % of plants affected _____ Date _____

Does it appear that the number of flowers or fruiting bodies produced by transgenic and nontransgenic plants is the same? yes ☒ no ☐ If no, describe differences and potential causes.

number of plants observed _____ % of plants affected _____ Date _____

BERNARD SAMMONS — Individual recording observations

Jerseyville, IL — Study Location

DR-JV 1992 — Study Title

Monitoring for Volunteer Plants

- After harvest and all data is collected, destroy unwanted vegetative material/ seed/tubers by the method(s) specified in the permit.

- If appropriate for your crop and area, irrigate after harvest to encourage germination.

- One month later, make the initial observation for the number of volunteers (estimates will suffice if the numbers are substantial). During the rest of the offseason, monitor on a monthly basis whenever the weather conditions are favorable for germination. Continue to monitor on a monthly basis for the period specified in the permit or until another transgenic test is planted in the same area.

- Record observations below.

- Remove any volunteer plants by hand weeding, herbicide treatments, or mechanical cultivation.

- Return form to Monsanto ___ **months** after harvest.

Number of volunteers observed NONE

Method used to destroy volunteers N/A

Comments PLOT AREA WAS OBSERVED FOR VOLUNTEERS in SPRING AND LATE SUMMER - 1993 - NONE COULD BE FOUND

BERNARD SAMMONS
Individual reporting observations

LAST OBSERVED 10-11-93
Date

Jerseyville IL
Study Location

DR-JV-1992
Study Title

Signed 10-14-94 Bernard Sammons

Unintentional Release of Transgenic Material

If transgenic materials are unintentionally released into the environment (eg. - planting before release permits are obtained; planting or spillage in an area not designated for the release; movement of seed outside of test area by natural causes or vandals), notify Monsanto and the USDA/APHIS Regional Biotechnologist within 24 hours of your knowledge of the release. Record information about the release below.

What was released (seed, leaf tissue, tubers, etc.) N/A

How was it released _____

_____

Quantities released _____

Date and time of release (if known) _____

Steps taken to rectify unintentional release _____

_____

_____

N/A
Individual reporting the release

Date

Study Location

Study Title

Memorandum

USDA # 92-176-01

Monsanto #:92-075

Protocol #:92-01-38-01

Experiment #:92-449-701, 92-449-702, 92-449-703

Subject:1992 GLP Delayed Ripening Tomato Field Trial: Field Monitoring for Disease, Insect, Weediness and Plant Growth Characteristics

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The attached report contains a representitive sample from each of the four field sites for disease, virus, and insect susecpibility as determined by Keith Jackson, BHN-Joint Venture, Naples, Fl. This report is included as a supplement to the complete field monitoring tables for disease, insect, and weediness and plant growth characteristics because it contains descriptions of the report, sampling methods and a detailed listing of the parameters evaluated.

Study Title: Evaluation of Delayed Ripening Tomato Lines in 1992 - 1993: Florida Regulatory Field Tests

Study #: 92-01-38-01

Subject: Disease/Insect Biweekly Reports
Evaluated by Keith Jackson

Date: May 19, 1993

CONTENTS

DESCRIPTION OF REPORT FORMATS, SAMPLING METHODS, AND ABBREVIATIONS

General Format

Each site was checked biweekly for overall disease/insect activity and differences between plots evaluated on an as needed basis if there was a discernible difference in disease/insect activity. The right half of the report, subdivided into diseases and insects, is for the overall activity, and the left side of the report, listing individual plot numbers, shows the differences between plots.

A stand count, the number of plants in each plot, was taken on every check. This number was recorded as a ratio of affected/normal for each plot until around the middle of March. After this time the count was shown as a ratio only if there was a difference between the number of plants being currently counted and the original stand count. Otherwise the count was recorded as a single number. Also, in some cases, a column was added to the left of the plot numbers showing the original number of plants in each plot. The reason for doing this was to distinguish between the number of plants initially planted in the plot and the number evaluated at a later sampling date. Some plants died early in the experiment and were no longer recognizable later and it could have been misleading to include them in later counts.

Disease Evaluation

Diseases were rated on the following subjective basis:

0 none
1 very low
2 low
3 moderate
4 high
5 severe - plants dead or the disease is so high that fruit unmarketable.

Diseases were evaluated by external symptoms, except for once at site #2 in the 5th week in which I cut the crowns on some dead plants to check for internal stem discoloration in an area where plants had also been damaged by oil drops. I sent no plants or plant parts to a diagnostic lab. In some cases I did collect leaf spots to look at under a microscope for fungal spores or bacterial streaming.

Following is a list of common names used on the reports and the associated species from southwest Florida. Additional comments on sampling are also made. If a name was abbreviated on the field reports, it is shown in parenthesis next to the full name.

Bacterial spot *Xanthomonas campestris pv. vesicatoria*
Pseudomonas syringae pv. tomato

The second disease, bacterial speck, was not distinguished from bacterial spot because the symptoms are so similar.

Alternaria/ various *Alternaria spp.*
Target Spot *Corynespora cassiicola*

Not distinguished between each other on the reports.

Fusarium - Fusarium Crown and Root Rot (FCR)
Fusarium oxysporum f. sp. radicis - lycopersici

An additional summary sheet which shows the counts of FCR symptoms is provided for each site.

Virus Symptoms various spp.

Any distorted, stunted plants were assigned to this category.

Insects

Loopers, armyworms, and fruitworms were sampled by walking along each row, on one side of the row, and counting the number of plants which had live larvae. Live larvae were found by first finding their feeding damage to leaves or fruit then looking more closely for the larvae. The number of plants with larvae was recorded as a percentage. If the larvae count not be found and the feeding recent, I noted that as "damage". Also the presence of eggs was noted.

All lepidopteran larvae were rated by their relative size, regardless of the number of instars, as follows:

I	very small - recently hatched
II	intermediate
III	intermediate
IV	large - nearing pupation

Following is a list of common names used on the reports, with the associated species form southwest Florida, and description of specific sampling methods.

Loopers *Trichoplusia ni*

Armyworms - Beet Armyworm (BAW) *Spodoptera exigue*

Fruitworms *Heliothis zea*
Heliothis virescens

Pinworms - Tomato Pinworm (TPW) *Keiferia lycospersicella*

Since pinworms are small and require closer inspection to find than other lepidopteran caterpillars, their occurrence is quantified as present or absent; and, very low, low, moderate. And high instead of a percentage.

Whiteflies - Sweet Potatoe Whitefly (SPWF) *Bemisia tabaci*
Also referred to in the literature as the B strain, Florida strain, Poinsettia strain, or Silverleaf Whitefly.
Banded Winged Whitefly (BWWF) *Trialeurodes abutilonea*

Whiteflies are sampled two ways.

Adults/Leaf One compound leaf on the upper third of the plant is examined, top and bottom, and the number of adults counted on ten leaves from different plants.

Immatures/Trifoliate The trifoliate, terminal 3 leaflets, on the seventh leaf from the top of the plant is pinched off and the number of immatures on the underside is counted on five trifoliates from different plants.

Aphids various spp.

Are quantified by the this relative scale.

Colony Size	Frequency of Colonies
singles	few
small colonies	intermediate
moderate colonies	intermediate
large colonies	common

Leafminers *Liromyza trifolii*
Liromyza sativae

Are quantified three ways.

Active larvae/Trifoliate The same trifoliate is used as for the whitefly sample. The ratio is the number of live larvae per five trifoliates.

Adults The presence of adults on the foliage is rated from 0 (none) to 5 (extremely numerous).

Leaf Stippling Stippling is tiny spots on the leaves caused by adult feeding and egg laying activity and is also rated 0 (none) to 5 (extremely numerous).

Thrips *Frankliniella bispinosa* and other spp.

The number of thrips, (adults and immatures) is counted per bloom by pulling back the sepals on five blooms.

Tomatoes with a Delayed Ripening gene

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases? ~~1 plant at 1~~

Date 11/13/92 yes ☐ no ☒ No. of plants obs. 80 UC82B, 80 8338, 80 5673 % of plants affected UC82B 0, 8338 0, 5673 0

Comments: Virus, bacterial spot, alternaria Fusarium tested

Date 11/23/92 yes ☐ no ☒ No. of plants obs. 80 UC82B, 80 8338, 80 5673 % of plants affected UC82B = 0, 8338 = 1.25% (virus), 5673 = 0

Comments: 1 plant for line 8338 showed symptoms same as 11/13/92, all plots at this site esp pronounced terminal most foliage slightly yellowed few near petrioles

Date 12-8-92 yes ☐ no ☒ No. of plants obs. 80 UC82B, 80 8338, 80 5673 % of plants affected UC82B = 0, 8338 = 1.25%, 5673 = 0

Comments: Virus, bacterial spot alternaria + Fusarium tested Affected plant is same as on 11/23/92

Date 12-23-92 yes ☐ no ☒ No. of plants obs. 80 UC82B, 80 8338, 80 5673 % of plants affected UC82B = 0, 8338 = 1.25%, 5673 = 0

Comments: Virus, bacterial spot, alternaria and Fusarium tested affected plant is same as on 11-23-92

Date 1/5/93 yes ☐ no ☒ No. of plants obs. 80 UC82B, 80 8338, 80 5673 % of plants affected UC82B = 0, 8338 = 1.25%, 5673 = 0

Comments: Virus, bacterial spot, alternaria and Fusarium tested affected plant is same plant as above; alternaria rated 0-1 few lesions

Date 1/20/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected 0, 0, 0

Comments: Bacterial spot, alternaria, Fusarium tested overall alternaria rated 0-1

Date 2/3/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected 0

Comments: Bacterial spot, alternaria, Fusarium tested overall alternaria rated 1 (very low incidence); Plant 7 wilted, but not yellow

Date 2/17/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 0 % of plants affected 0, 0, 0

Comments: Bacterial spot = 0; alternaria = 1 (low) Fusarium - newly wilted plants resent

Date 3/2/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 78, 5673 = 78 Symptoms of FCR % of plants affected UC82B = 2.5%, 8338 = 25%, 5673 = 75%

Comments: Bacterial spot = 0; Alternaria = 1 (low) Fusarium listed above

Date 3/8/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 75, 5673 = 24 Fusarium Symptoms % of plants affected UC82B = 4%, 8338 = 5%, 5673 = 2%

Comments: Bacterial spot 2-3 foliage, 0-1 fruit, Alternaria 2-3 wind damage to plants from 3-13-93 storm; considerable decline in plants

Keith Jackson / transcribed by Kim Macfn — Individual recording observations

Naples, FL Farm 2, Site 1 — Study Location

see below — Study Title

Field Monitoring for Disease, Insect, Weediness and Plant Growth characteristics

Tomatoes with a Delayed Ripening gene

Field Monitoring for Insect Susceptibility:

Do transgenic plants have a higher incidence of non-target species than nontransgenic plants? If yes, which species are more prevalent?

Date 11/13/92 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected all 0

Comments: leafminers=0, Thrips=0 Loopers=0, Armyworms=0, Fruitworms=0 Pinworms=0 Whiteflies=0 Aphids=0

Date 11/23/92 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: Thrips=0 loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=0, Aphids=0, Armyworms=0 Fruitworms=0 leafminers=moderate

Date 12/8/92 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: Thrips=0 loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=0, Aphids=0 (few adults) moderate leafminers=mites

Date 12/23/92 yes ☐ no ☒ No. of plants obs. 8338=80 UC82B=80 5673=80 % of plants affected See below

Comments: Thrips=0 loopers=0 Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=0, Aphids=0, leafminers=0

Date 1/5/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: 12 known 10/19/94 leafminers=26, Thrips=0 loopers=0 Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=0 Aphids=0

Date 1/20/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=1, Thrips=0 - some fruit w/ white spots, yet none seen loopers 0 Armyworms 0, Fruitworms=0, Pinworms=0, Whiteflies=0, Aphids=0

Date 2/3/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=0, Thrips=0 loopers=0 Armyworms=1% Fruitworms=0, Pinworms=0, Whiteflies=0, Aphids=0

Date 2/17/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=0, Thrips=0 loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=0, Aphids=0

Date 3/2/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=1 Thrips=0 loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=1, Aphids=0

Date 3/18/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=3/5 Thrips=0 loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, Whiteflies=0 12/16 adults/leaf 9/15 immature/leaf Aphids=0

Keith Jackson / transcribed by Kim Magin

Individual recording observations

BHN Naples, FL Farm GCF#2 Site 1

Study Location

Study#92-01-38-0 Exp#92-449-701→7

Study Title

H. Field Monitoring & Incidence Insect, Weediness and Plant

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases?

Date 11/13/92 yes ☐ no ☒ No. of plants obs. ~~UC82B 80~~ 5673 80 8338 79 % of plants affected ~~UC82B 0~~ 5673 0 8338 0

Comments: ~~Virus, bacterial spot, alternaria, fusarium~~ (TR) KMM 10/17/94

Date 11/27/92 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B 0 5673 0 8338 0

Comments: Virus, Bacterial spot Fusarium all tested & negative

Date 12/8/92 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B 0 5673 0 8338 0

Comments: some plants damaged by oil spill and may not recover
Virus, Bacterial spot & Fusarium all negative

Date 12-23-93 (TR) KMM 10/19/94 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B 6.25% 5673 0% 8338 17.5%

Comments: difficult to tell if Fusarium or oil spill damage
Bacterial spot = 0 Alternaria = 0 Fusarium listed above

Date 1/5/93 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B = 7.5% 5673 = 0 8338 = 20%

Comments: difficult to distinguish Fusarium symptoms from Fusarium see above
Virus symptoms = 0, bacteria / spot = 0, alternaria = 0

Date 1/19/93 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B = 7.5% 5673 = 0 8338 = 20%

Comments: largest fruit are 1½" - 2" long
Bacterial spot = 0; Alternaria = 0-1; No new Fusarium activity

Date 2/3/93 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected Bacterial spot UC82B = 6 5673 = 2.5% 8338 = 2.5%

Comments: (Apr 4/19/93) one 8338 plant wilted but not yellowing
No new Fusarium symptoms; Alternaria = 0-1 low amt overall on lower inner Foliage

Date 2/17/93 yes ☐ no ☒ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected Bacterial Spot UC82B = 3.8% 5673 = 2.5% 8338 = 6.3%

Comments: Alternaria overall = 1
overall - Fusarium plants have begun to wilt since 2/3/93 Fusarium UC82B = 0% 5673 = 0% 8338 = 17.5%

Date 3/2/93 yes ☒ no ☐ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B = 47% 5673 = 5% 8338 = 34%

FCR line 8338 FCR

Comments: Alternaria = 1 Virus symptoms = 0
Bacterial spot seen on all lines but very low overall

Date 3/20/93 yes ☒ no ☐ No. of plants obs. UC82B 80 5673 80 8338 80 % of plants affected UC82B = 26% 5673 = 1% 8338 = 49%

FCR 8338

Comments: Virus symptoms 0, Alternaria 2-3, Bacterial spot Foliage = 2 Fruit = 0-1

Keith Jackson / transcribed by Kim Magie
Individual recording observations

Farm 7 (site 2) BHN. FL
Study Location

Field Monitoring for Disease, Insect, Weed and Plant Growth characteristics
Study Title

92-449-701
92-449-702
92-449-703

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases? (KE) KWN 10/18/94

Date ~~11/13/92~~ yes ☐ no ☒ No. of plants obs. ~~50 UC528~~ ~~50 5330~~ ~~50 5673~~ % of plants affected ~~UC528 0~~ ~~5330 0~~ ~~5673 0~~

Comments: ~~Virus, bacterial spot, alternaria, Fusarium~~

Date 4/2/93 yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: Bacterial spot: Foliage = 2, Fruit = 1 Alternaria = 3 present in all plots

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Keith Jackson / transcribed by Kim Magin
Individual recording observations

Site 2 BHN-Farm 7 FL
Study Location

92-449-701
92-449-702
92-449-703
Study Title

Title: Field Monitoring for Disease, Insect, weediness and plant

Field Monitoring for Insect Susceptibility:

Do transgenic plants have a higher incidence of non-target species than nontransgenic plants? If yes, which species are more prevalent?

Date 11/27/92 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected 0

Comments: leafminers = 0, Thrips = 0 Loopers = 0, Armyworms = 0, Fruitworms = 0 Pinworms = 0 Whiteflies = 0 Aphids = 0

Date 12/8/92 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected

Comments: Thrips = 6 Loopers = 0, Armyworms = 0, Fruitworms = 0, Pinworms = 0, Whiteflies = 0, Aphids = 0 leafminers = 5 moderate stripping few adults

Date 12/23/92 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected 0

Comments: Thrips = 0 Loopers = 0, Armyworms = 0, Fruitworms = 0, Pinworms = 0, Whiteflies = 0, Aphids = 0, leafminers = larvae/trifoli 0-1 adults

Date 1/5/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected

Comments: Thrips = 0 few fruit present; oil burn Loopers = 0 Armyworms = 0, Fruitworms = 0, Pinworms = 0, Whiteflies = 0, Aphids = common, leafminers = 45

Date 1/19/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected See below

Comments: leafminers = 0, thrips = 0, but few with white spots Loopers = 0 Armyworms = 0, Fruitworms = 0, Pinworms = 0, Whiteflies = 0 Aphids = few

Date 2/3/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected See below

Comments: leafminers = 0, Thrips = 0 but few fruit w/ white spots Loopers = 0 Armyworms = 0 Fruitworms = 0 Pinworms = 0, Whiteflies = 0, Aphids = few

Date 2/17/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected See below

Comments: leafminers = 0/5, Thrips = 0/5 Blooms Loopers = 0 Armyworms = 1, Fruitworms = 0, Pinworms = 0 Whiteflies = 0, Aphids = few

Date 3/2/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected See below

Comments: leafminers = 0, Thrips = 1/5 thrips/bloom Loopers = 0, Armyworms = I-II, Fruitworms = 0, Pinworms = 0, Whiteflies = 1/10, Aphids =

Date 3/20/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5673=80 % of plants affected See below

Comments: leafminers = Thrips - none but no worms seen Loopers = 0, Armyworms = damage, Fruitworms = 0, Pinworms = 0, Whiteflies = 1/5, Aphids =

Date 4/2/93 yes ☐ no ☒ No. of plants obs. UC82B=80, 8338=80, 5672=80 % of plants affected See below

Comments: leafminers = 2, Thrips = 0, but no blooms Loopers = 0, Armyworms = II, Fruitworms = 0, Pinworms = 0, Whiteflies = 1/5 Aphids =

Keith Jackson / transcribed by Kim Megan

Individual recording observations

BHN Naples, FL
Farm 7
site 2

Study Location

Study #92-01-35-01
GH# 92-449-701

Study Title

Title: Field Monitoring for Disease, Insect, Weediness and Plant

Tomatoes with a Delayed Ripening gen

FCR- fusarium Crown Rot

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases?

(E) Kmm 10/18/94

Date 11/19/92 yes ☐ no ☒ No. of plants obs. ~~50 UC82B~~ ~~50 8338~~ ~~50 5673~~ % of plants affected ~~UC82B = 0~~ ~~8338 = 0~~ ~~5673 = 17~~

Comments: ~~Virus, bacterial spot~~ alternaria Fusarium

Date 12/2/92 yes ☐ no ☒ No. of plants obs. UC82B = 98, 8338 = 99, 5673 = 99 % of plants affected FCR

Comments: Bacterial spot, Alternaria, Fusarium negative

Date 12/9/92 yes ☐ no ☒ No. of plants obs. UC82B = 98, 8338 = 99, 5673 = 99 % of plants affected

Comments: Bacterial Spot, Alternaria, and FCR negative

Date 12/24/92 yes ☐ no ☒ No. of plants obs. _____ % of plants affected Virus symptoms

Comments: Bacterial spot, Alternaria FCR were negative

Date 1/8/93 yes ☐ no ☒ No. of plants obs. UC82B = 98, 8338 = 99, 5673 = 99 % of plants affected Virus symptoms FCR

Comments: Bacterial spot, Alternaria negative

Date 1/21/93 yes ☐ no ☒ No. of plants obs. UC82B = 98, 8338 = 99, 5673 = 99 % of plants affected Virus symptoms

Comments: Bacterial spot = 0, alternaria = 1 (Fungal def spot on lower foliage) Fusarium - no new activity

Date 2/4/93 yes ☐ no ☒ No. of plants obs. UC82B 98, 8338 99, 5673 99 % of plants affected Virus symptoms

Comments: Bacterial spot 0-1 seen on 1 plant for line 8338; Alternaria = 2; FCR possible on overall, slight burn on upper foliage - Possibly from wind? 8338 plants

Date 2/9/93 yes ☐ no ☐ No. of plants obs. UC82B = 98, 8338 = 99, 5673 = 99 % of plants affected FCR

Comments: Bacterial spot = 1, alternaria = 1

Date 2/19/93 yes ☒ no ☐ No. of plants obs. UC82B = 198, 8338 = 197, 5673 199 % of plants affected FCR

Comments: 1 row count, previous counts only counted 1 row; here = 3 rows

Date 3/2/93 yes ☐ no ☐ No. of plants obs. UC82B = 294, 5673 = 295, 8338 = 293 % of plants affected FCR

Comments: Purple to blue ish color on leaves | Virus symptoms UC82B = 0, 5673 = 0, 8338 = 6% | Purple color UC82B = 0, 5673 = 27, 8338 = 27

Keith Jackson / transcribed
Individual recording observations

BHN Farm Site 3
Study Location

138

92-01-38-01
92-449-201-70
Study Title

Kim Wagin

"H" Field Monitoring for disease, insect, Weed Resistant plant growth characteristics

FCR = Fusarium Crown Rot

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases?

(XR) KMM 10/18/94

Date 11/13/92 yes ☐ no ☒ No. of plants obs. 50 UC82B, 40 8338, 50 5673 % of plants affected UC82B 0, 8338 0, 5673 0

Comments: Virus, bacterial spot, alternaria, Fusarium

Date 3/19/93 yes ☒ no ☐ No. of plants obs. UC82B = 296, 8338 = 293, 5673 = 296 % of plants affected FCR wilt sym. UC82B = 1%, 8338 = 4%, 5673 = 7%

no new virus symptoms; wind damage to plants

Comments: Bacterial spot 2-3 foliage, 0-1 on fruit; Alternaria = 3

Date 4/2/93 yes ☒ no ☐ No. of plants obs. UC82B = 293, 8338 = 293, 5673 = 296 % of plants affected FCR wilt symptoms UC82B = 18%, 5673 = 25%, 8338 = 73%

Comments: Bacterial spot foliage = 2-3 fruit = 1; Alternaria = 3 no new virus symptoms

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

Date ______ yes ☐ no ☐ No. of plants obs. ______ % of plants affected ______

Comments: ______

______ Individual recording observations

______ Study Location

______ Study Title

Field Monitoring for Insect Susceptibility:

Do transgenic plants have a higher incidence of non-target species than nontransgenic plants? If yes, which species are more prevalent?

Date 12/2/92 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0 Pinworms=0 Whiteflies=0 Aphids=0 leafminers=0-1, Thrips=0

Date 12/9/92 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0 w/White Flies=0, Aphids=, leafminers=0 Thrips=0 Tekum 12/9/92 adults=moderate larvae=1/5

Date 12/24/92 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0, Pinworms=I-II, White Flies=, Aphids=0 leafminers=0 Thrips=0 Tekum 10/19/94 adults=0 immature=0

Date 1/8/93 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0 Armyworms=0, Fruitworms=0, Pinworms=I-II, Whiteflies=0, Aphids=0, leafminers=inactive leafmine larvae, Mines on lower foliage Thrips=0 adults=1/5 immature=1/5

Date 1/21/93 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0, Pinworms=I-II, Whiteflies=, Aphids=few wingless (0 larvae) leafminers=1-3 thrips=0 but few white spots on fruit 4/10 adults/leaf 4/5 immatures/trifolate

Date 2/4/93 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0 Armyworms, Fruitworms=, Pinworms=I-II, White Flies=, Aphids=0 leafminers=0, Thrips=none seen, Blooms not checked 1/10 adults/leaf 4/5 immatures/trifolate Tekum 10/19/94 2/4/93

Date 2/4/93 yes ☐ no ☒ No. of plants obs. UC82B=98, 8338=99, 5273=99 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0, Pinworms=I-II, White Flies=, Aphids=0 leafminers=1/5 Thrips=0 1/10 adult 4/5 immature/trifoliate

Date 3-2-93 yes ☐ no ☒ No. of plants obs. UC82B=198, 8338=197, 5273=199 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0, Pinworms=II-III, White Flies=1/5, Aphids=0 leafminers=4/5 large Thrips=0 but no blooms 1/5 immatures/trifoliate adults=0-1

Date 3-19-93 yes ☐ no ☒ No. of plants obs. UC82B=295, 8338=293, 5273=296 % of plants affected See below

Comments: Loopers=0, Armyworms=0, Fruitworms=0, Pinworms=II-III, White Flies=, Aphids=few leafminers=5/6 larvae Thrips none, no blooms more common on edge of plots 1/10 adults 4/5 immatures old damage to fruit

Date 4-2-93 yes ☐ no ☒ No. of plants obs. UC82B=295, 8338=293, 5273=296 % of plants affected See below

Comments: Loopers=0, Armyworms=few, Fruitworms=0, Pinworms=I-II, White Flies=↑, Aphids= leaf miners= Thrips= 2/10 adults/leaf 4/5 immature/trifoliate

BHN Naples, FL
Farm BHN
Site 3

Study #92-01-35-C
Exp # 92-449-701-R

Keith Jackson / transcribed by Kim Magan

Individual recording observations

Study Location

Study Title

Field Monitoring & Disease Trial - Weediness and Plant

FCR = Fusarium Crown Rot

Field Monitoring for Disease Susceptibility:

Do transgenic plants have a higher incidence of disease than nontransgenic plants? If yes, to which diseases?

Date 11/3/92 yes ☐ no ☒ No. of plants obs. UC82B 50, 8338 50, 5673 50 % of plants affected UC82B 0, 8338 0, 5673 0

Comments: ~~Virus, bacterial spot, alternaria Fusarium~~

Date 1/8/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected UC82B = 0, 8338 = 0, 5673 = 0

Comments: Bacterial spot, very windy day Alternaria Fusarium negative

Date 1/20/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 5673 = 80, 8338 = 80 % of plants affected UC82B = 0, 8338 = 0, 5673 = 1%

Comments: Bacterial spot = 0; Alternaria = 0; one 5673 plant w/ FCR symptom

Date 2/2/93 yes ☐ no ☐ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected Virus symptoms UC82B = 17%, 8338 = 5%, 5673 = 1%

Comments: Slight yellowing on inside of terminal leaves Bacterial spot, Alternaria, FCR negative

Date 2/16/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected Virus symptoms UC82B = 1%, 8338 = 6%, 5673 = 1%

Comments: fruit are 3/4 inch long Bacterial spot, Alternaria and FCR negative

Date 3/1/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected Virus symptoms UC82B = 1%, 8338 = 6%, 5673 = 1%

Comments: Bacterial spot 0; Alternaria = 0-1; FCR = 0

Date 3/18/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected Virus symptoms UC82B = 0, 8338 = 9%, 5673 = 1%

Comments: wind damage on west edges TOPS of plants + leaves Slightly scorched Bacterial spot = 1; Alternaria = 0-1 FCR = 0

bacterial spot UC82B = 3%, 8338 = 5%, 5673 = 5%

Date 3/30/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 5673 = 80, 8338 = 80 % of plants affected Virus symptoms UC82B = 7%, 5673 = 1%, 8338 = 9%

Comments: Bacterial spot = 3 UC82B = 100%, 8338 = 100%, 5673 = 100% | Alternaria = 1-2; FCR = 0

Date 4/13/93 yes ☐ no ☒ No. of plants obs. UC82B = 80, 8338 = 80, 5673 = 80 % of plants affected Virus symptoms UC82B = 0, 8338 = 37%, 5673 = 1%

symptoms 8% 30% 87%

Comments: Bacterial spot = 3-4 Spots on fruit common; Alternaria = 2-3

Date 4/27/93 yes ☒ for 8338 FCR no ☐ No. of plants obs. UC82B = 80, 5673 = 80, 8338 = 80 % of plants affected FCR symptoms UC82B = 31%, 8338 = 93%, 5673 = 48%

Comments: Bacterial spot 3-4; Alternaria = 2-3

Study 92-01-38-W
92-449-761-703

Keith Jackson / transcribed by Kim Magin
Individual recording observations

Farm 11-Site 4
Study Location

Study Title

#6: Field Monitoring for Disease, Insect, Weediness

Field Monitoring for Insect Susceptibility:

Do transgenic plants have a higher incidence of non-target species than nontransgenic plants? If yes, which species are more prevalent?

Date 1/8/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=0, Thrips=0 Loopers=0, Armyworms=0, Fruitworms=0 Pinworms=0 whiteflies=0 Aphids=0

Date 1/20/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: Thrips= loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, WhiteFlies=↑, Aphids=few, leafminers=0 12/10 adults/leaf 1/5 larvae/trifoliate 3 adults

Date 2/2/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: Thrips=0 loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, WhiteFlies=↑, Aphids=↑, leafminers= 1/10 adults/leaf very few 1/5 larvae/trifoliate no adults

Date 2/16/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: Thrips loopers=0 Armyworms=0, Fruitworms=0, Pinworms=0, WhiteFlies=↑, Aphids=0, leafminers=0 0/10 adults/leaf 0/5 immatures/trifoliate

Date 3/1/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=0, Thrips=0 Spidermites found: loopers=0, Armyworms=0, Fruitworms=0, Pinworms=0, WhiteFlies=↑, Aphids=few 2/5 immatures/trifoliate 2/10 adults/leaf few

Date 3/18/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=0, Thrips=0 loopers=0 Armyworms=0 Fruitworms=0, Pinworms=0, WhiteFlies=↑, Aphids=0 1/10 adults/leaf few 1/5 immatures/trifoliate

Date 3/30/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=adults Thrips=0 loopers=0 Armyworms=0 Fruitworms=0 Pinworms=0, WhiteFlies=↑, Aphids=few small colonies 0/10 adults/leaf 0/5 immatures/trifoliate 1/5 larvae/trifoliate

Date 4/13/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers=adults Thrips=0 - but no blooms loopers=0, Armyworms=1%, Fruitworms=0 Pinworms=0, WhiteFlies=↑, Aphids=few single 2/10 adults/leaf 3/5 immatures/trifoliate 1/5 larvae/trifoliate

Date 4/27/93 yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers= Thrips 0 - but no blooms loopers=IV Armyworms= damage found no worms found Fruitworms=0, Pinworms=II, whiteflies=↑, Aphids=2 common colonies 2/10 adults/leaf 100/5 immatures <1% damage but no worms found on 10% plants

Date ____ yes ☐ no ☒ No. of plants obs. UC82B=80 8338=80 5673=80 % of plants affected See below

Comments: leafminers= Thrips=0 loopers= Armyworms= Fruitworms= Pinworms= whiteflies= Aphids=

Keith Jackson / transcribed by Kim Magin

Individual recording observations

BHN Naples, FL Farm 11 site 4

Study Location

Study#92-01-38-C Exp#92-449-701

Study Title

Hi, Field Monitoring for Disease, Insect, Weediness and Plant

Monitoring for Volunteer Plants

- After harvest and all data is collected, destroy unwanted vegetative material/ seed/tubers by the method(s) specified in the permit.

- If appropriate for your crop and area, irrigate after harvest to encourage germination.

- One month later, make the initial observation for the number of volunteers (estimates will suffice if the numbers are substantial). During the rest of the offseason, monitor on a monthly basis whenever the weather conditions are favorable for germination. Continue to monitor on a monthly basis for the period specified in the permit or until another transgenic test is planted in the same area.

- Record observations below.

- Remove any volunteer plants by hand weeding, herbicide treatments, or mechanical cultivation.

- Return form to Monsanto X **months** after harvest.

Number of volunteers observed Volunteer plants were not monitored

Method used to destroy volunteers

Comments

Individual reporting observations

Date

Study Location

Study Title

Study #92-C1-3871

Exp # 92-1-F1-FC1-FC3

Unintentional Release of Transgenic Material

If transgenic materials are unintentionally released into the environment (eg. - planting before release permits are obtained; planting or spillage in an area not designated for the release; movement of seed outside of test area by natural causes or vandals), notify Monsanto and the USDA/APHIS Regional Biotechnologist within 24 hours of your knowledge of the release. Record information about the release below.

What was released (seed, leaf tissue, tubers, etc.) No Release occured

How was it released

Quantities released

Date and time of release (if known)

Steps taken to rectify unintentional release

Individual reporting the release

Date

Study Location

Study Title

Study: 92-01-38-01

Experiment: 92-449-701

92-449-702

92-449-703

USDA # 92-176-01

Monsanto # 92-075

Field Monitoring for Weediness Characteristics:

Is the germination of transgenic plants in any way different than nontransgenic plants?
yes ☒ no ☐ If yes, describe differences and potential causes.

Transgenic plant (line 8338) germinated overall more quickly with a greater percent of seeds germinating

number of plants observed 8338-500 UC82B-500 % of plants affected Germination UC82B=76.6 8338=87.6 Date 5-18-93 to 5-25-93

Is the number of days from planting until flowering (first flowers bloomed) the same for transgenic and nontransgenic plants? yes ☐ no ☒ If no, describe differences and potential causes.

Statistical analysis shows an increase of 2-3 days from planting until flowering over the control. Because ethylene inhibits the onset of flowering, it is expected that Delayed Ripening lines with reduced ethylene synthesis, will flower slightly later than control

number of plants observed 8338-500 UC82B-500 % of plants affected NA Date 1/24/92 - 2/4/92

Does it appear that the number of flowers or fruiting bodies produced by transgenic and nontransgenic plants is the same? yes ☒ no ☐ If no, describe differences and potential causes.

number of plants observed — % of plants affected — Date —

Keith Jackson / transcribed by Ann Magill — Individual recording observations

Davis CA — Study Location

Field Monitoring for Disease, Insect Weediness and Plant Growth Characteristics — Study Title

INSECT/DISEASE REPORT

Study# 92-01-38-01
Exp# 92-449-701
Site# 1

Farm 2
Blk #308 Plt. Date 11/9/92

N ↑

4	3	2	1
8	7	6	5
12	11	10	9

Date 12/8/92

Stage Bloom / 1st Tic

Week# 5

Plot#	Plant Loss	#Plts Blooming (A)	Virus Symptoms
1	0/20	6	0
2	0/20	10	0
3	0/20	5	0
4	0/20	16	0
5	0/19	16	(B) 1
6	0/19	15	0
7	0/19	7	0
8	0/19	8	0
9	0/20	7	0
10	0/20	14	0
11	0/20	4	0
12	0/20	4	0

(A) #plts with one or more blooms on the 1st flower cluster.

(B) 2nd plant still abnormal.

DISEASES:

Bacterial Spot	0
Alternaria/ Target Spot	0
Fusarium	0

INSECTS:

Loopers	0
Armyworms	0
Fruitworms	0
Pinworms	0
Whiteflies	0/10 lvs adults 0/5 trifoliates immatures
Aphids	few adults (winged)
Leafminers	0/5 Larvae/trifoliate
Thrips	0/5 Blooms

Keith Overman

Tomatoes with a Delayed Ripening gene

INSECT/DISEASE REPORT

Study# 92-01-38-01
Exp# 92-449-702
Site# 2
Farm 7

N ↑

24	23	22	16	17	18
21	20	19	13	14	15

Date 1/5/93
FUK
Stage Bloom-Frt/2nd Tier
Week# 7

Plt. Date 11/23/92

Plot#	Plant Loss (A)	Virus Symptoms	
13	0/20	0	
14	2/20	0	
15	2/20	0	
16	0/20	0	
17	0/20	0	
18	1/20	0	
19	0/20	0	
20	4/20	0	
21	4/20	0	
22	0/20	0	
23	9/20	0	
24	0/20	0	

(A) Plant Loss from old oil burn and FCR.

DISEASES:

Bacterial Spot	0
Alternaria/ Target Spot	0
Fusarium	See (A)

INSECTS:

Loopers	0
Armyworms	0
Fruitworms	0
Pinworms	0
Whiteflies	0/10 Adlts/Leaf 0/5 Immatures/trifoliate
Aphids	dead winged adults are common.
Leafminers	6/5 larvae/Trifoliate Stippling - 0-1 Adults - 0-1
Thrips	0/5 Blooms

Few fruit are present. Largest fruit are approximately 1-1½ inches long.

INSECT/DISEASE REPORT

Study# 92-01-38-01
Exp# 92-444-703
Site# 3

BHN
Field 2W

Plt. Date 11/20/92

36	25
35	26
34	27
33	28
32	29
31	30

N ↑

Date 3/19/93

Stage Fruit/4th Tic

Week# 18

Plot#	Wilt Symptoms		
25	3/14		
26	10/15		
27	30/16		
28	4/11		
29	0/13		
30	29/11		
31	29/12		
32	0/12		
33	3/14		
34	5/16		
35	1/11		
36	30/14		

DISEASES:

Bacterial Spot	2-3 foliage 0-1 fruit (A)
Alternaria/ Target Spot	3
Fusarium	See wilt column
Virus Symptoms	no new symptoms discernable

INSECTS:

Loopers	0
Armyworms	old damage to fruit
Fruitworms	0
Pinworms	moderate III-IV in foliage. More common on edge of plots
Whiteflies	1/10 adults/leaf 9/5 immatures/trifoliate
Aphids	few wingless singles
Leafminers	5/5 larvae/trifoliate Stippling 0-1 Adults 0
Thrips	No blooms. None seen on foliage

(A) Appears to be slightly more in #36 & 25.

Wind damage on west side of west rows and on north and south edges of trial. Foliage is desiccated on west side of plants at west edge of trial.

M. de Ruiter

INSECT/DISEASE REPORT

Study# 92-01-33-01
Exp# 92-449-704
Site# 4
Farm 11

48	43	42	38
47	45	41	37
46	44	40	39

N ↑

Date 2/16/93
(B) Stage Bloom-Fruit/2 Tie
Week# 8

Planting Date 12/31/92

Plot#	Plant Loss	Viral Symptoms	
37	0/20	1/20 Plt.# 9	
38	0/20	0/20	
39	0/20	0/20	
40	0/20	0/20	
41	0/20	2/20 Plt.# 8, 19	
42	1/20	1/20 Plt.# 10	
43	0/20	0/20 (A)	
44	0/20	1/20 Plt# 20	
45	0/20	0/20	
46	0/20	0/20	
47	0/20	0/20	
48	0/20	2/20 Plt. # 4, 19	

(A) Plant #7 appears normal now. Was abnormal appearing 2 weeks ago.
(B) Fruit up to ~ 3/4 inch long.

DISEASES:

Bacterial Spot	0
Alternaria/ Target Spot	0
Fusarium	0

INSECTS:

Loopers	0
Armyworms	0
Fruitworms	0
Pinworms	0
Whiteflies	0/10 Adults/leaf 0/5 Immatures/Trifoliate
Aphids	0
Leafminers	0/5 Larvae/Trifoliate Stippling 0-1 Adults- none seen
Thrips	

Will Carlson

Appendix IV. ACCd ELISA Validation Data Summary

Appendix IV. ACCd ELISA Validation Data Summary

Measure	Tissue Type: Red Ripe	Tissue Type: Leaf
I. Precision:		
QC Sample Variability (%CV)	19.9	15.7
Variability of Analysis[1] (%CV)	37.3	21.8
II. Assay Working Limits:		
Limit of Detection (ng/well)	0.150	0.203
Criteria of Detection (OD)	0.177	0.226
III. Accuracy:		
Extraction Efficiency (%)	94.8	87.2
Spike and Recovery (%)	112.6	201.8
IV. Stability of ACCd:		
Red Ripe Fruit Extract[2]	87% remaining after 60 days[3]	
Red Ripe Fruit Tissue[4]	> 7 months	
Leaf Extract	91% remaining after 60 days[5]	
Leaf Tissue	> 7 months	
V. Accept Reject Criteria:		
Quality Control Sample[6]	±2 standard deviations from the established mean	
Mean of Blank[7]	≤0.600 OD at 405nm, 655 nm	
OD of 3 ng standard	between 1.5-2.5 OD	
Replicate Wells Coefficient of Variance	≤10% CV	
Curve Fit Standard Error	≤0.100	

1 derived from analysis of tissue storage stability studies over a period of 8 months
2 red ripe fruit and leaf extracts stored at - 80° C
3 percent remaining decreases 13.5% for each 30 day period of storage
4 red ripe fruit and leaf tissues stored at - 80°C
5 percent remaining decreases 4.4% for each 30 day period of storage
6 Quality Control sample is an ACCd positive line. An extract is made for each of the tissue types aliquoted and stored at - 80°C
7 Blank value is the absorbance at 405 nm in wells containing ELISA buffer spiked with plant extract

Appendix V. NPTII ELISA Validation Data Summary

Appendix V. NPTII ELISA Validation Data Summary

Measure	Tissue Type: Red Ripe	Leaf
I. Precision:		
QC Sample Variability (%CV)	30.7	23.2
Variability of Analysis[1] (%CV)	30.1	34.2
II. Assay Working Limits:		
Limit of Detection (ng/well)	0.075	0.085
Criteria of Detection (O.D.)	0.076	0.062
III. Accuracy:		
Extraction Efficiency (%)	98	91
Spike and Recovery (%)	109	96

IV. Stability of NPTII:
There is no statistical evidence of decay of NPTII in either the extracts or processed tomato tissues over a seven month period when stored at -80°C

V. Accept/Reject Criteria:	
Quality Control Sample[2]	±2 standard deviations from the established mean
Mean of Blank[3]	< 0.350 O.D. at 450nm reference 655 nm
OD of 3 ng standard	> 0.500 O.D. at 450nm reference 655 nm
Replicate Wells Coefficient of Variance	≤10% CV
Curve Fit Standard Error	≤0.100

1 derived from analysis of tissue storage stability studies over a period of 8 months
2 Quality Control sample is an NPTII positive line. An extract is made for each of the tissue types aliquoted and stored at - 80°C
3 Blank value is the absorbance at 450 nm in wells containing ELISA buffer spiked with plant extract

Appendix VI. An assessment of the weediness potential of Delayed Ripening tomato line 8338 compared to control parental line UC82B.

[

CBI DELETED

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Pages 155-183

UNIVERSITY OF CALIFORNIA, DAVIS

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COLLEGE OF AGRICULTURAL AND
ENVIRONMENTAL SCIENCES
AGRICULTURAL EXPERIMENT STATION

DEPARTMENT OF VEGETABLE CROPS
DAVIS, CALIFORNIA 95616

June 5, 1986

Dr. Sheila McCormick
Biological Sciences
Montsanto Company
700 Chesterfield Village Parkway
St. Louis, MO 63198

Dear Dr. McCormick:

Your letter of June 2nd suggests that you did not receive my reply of May 25. Then, I am also confused by the questions in your latest letter because they do not jibe with the ones you posed in our phone conversation. At any rate, here goes:

1. No probability whatever of tomatoes outcrossing with any native or introduced plants in the US. The only intercrossable wild spp. are limited to Latin America.

2. Little or no research has been done on minimum pollination buffer distances; furthermore, data obtained for one region might not be applicable to others, since the pollinating bee spp. differ from one region to another. No evidence whatever for wind transport of pollen under field conditions; the responsible agents there are native bees: bumble bees, and various groups of solitary bee spp. We have some evidence for wind transport of pollen in greenhouse conditions, but the level is extremely low — something like 1 seed produced from such outcrossing per 50-100 flowers.

3. Other researchers in your area who might assist:

Dr. Victor Lambeth
Dept. Horticulture
Univ. Missouri - Columbia

Dr. Stanley Z. Berry
Ohio Agric. Expt. Sta.
Wooster, Ohio

Dr. Wm. George
Dept. Hort., Univ. Illinois
Urbana, IL

It would seem to me that glyphosate resistance would be a great asset in tomatoes. In Calif., for example, bindweed and nightshade are horrific pests in tomato fields, and a system to selectively eliminate them with a general spray would be highly advantageous to growers.

Sincerely yours,

United States Department of Agriculture

Agricultural Research Service

Central Plains Area
Plant Introduction
Research Unit

Plant Introduction Station
Iowa State University
Ames, Iowa
50011

13 June 1986

Shelia McCormick
Mail Zone AA3E
Monsanto
700 Chesterfield Village Parkway
St. Louis, MO 63198

Dear Shelia:

Per our phone conversation today, I have no reason to suspect you would experience any "escaped" pollen from the California-type tomato material you are working with. We regularly increase our stocks of L. esculentum in the field in Ames without protecting our flowers against incoming or outgoing pollen as they are considered to be completely self-pollinating in nature.

Additionally, there are no native species of plants in the midwest with which tomato will cross, even if tomato pollen were artificially transferred to them. We have over 3,000 accessions of tomatoes in our collection here and have never seen a single outcross to wild species.

Sincerely,

Raymond L. Clark

RAYMOND L. CLARK
Research Leader and Coordinator
Regional Project NC-7

UNIVERSITY OF FLORIDA

INSTITUTE OF FOOD AND AGRICULTURAL SCIENCES

GULF COAST RESEARCH AND
DUCATION CENTER, BRADENTON
5007 60TH STREET EAST
TELEPHONE: 813_755-1568
SCOM: 539-1101

BRADENTON, FLORIDA 34203

June 13, 1986

Dr. Shiela McCormick
Monsanto Company
700 Chesterfield Village Parkway
St. Louis, Missouri 63198

Dear Dr. McCormick:

This letter is in response to your letter of June 2, 1986.

1) I doubt that tomatoes would cross with any weed species and am aware of no such report.

2) In the seed industry no buffers are used between varieties being increased for seed production. This cross pollination is negligible where stigmas are not exserted naturally or by emasculation. I've enclosed a list of references I have regarding tomato cross pollination.

3) You might contact a tomato breeder with a seed company such as Fred Angel (now with Asgrow) or Pat Crill. You could also try Hogenboom at Waginengen or Nachum Kedar in Israel.

4) Glyphosate tolerance would be useful in many production areas but since plastic mulch is used in Florida it would probably not be that important. Row middles only have to be sprayed - generally with paraquat. Hopefully, your tolerance would not only allow the tomatoes to survive but also prevent any fertility problems which roundup can cause.

5) There is no problem in your use of this information if it is needed.

Sincerely Yours,

Jay W. Scott
Associate Professor

JWS/st

POLLINATION LIST

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Appendix VII

Tomatoes with a
Delayed Ripening gene

To: Glenn D. Austin, Monsanto

Re: Field evaluation of delayed ripening tomato trial (exp.# 92-449-703)

On March 2, 1993 I evaluated three tomato breeding lines at the BHN Research Facility in Bonita Springs, FL. The three genotypes examined were UC82B, 5673, and 8338. Some general observations of the trial as a whole might be an appropriate starting point.

The varieties appeared to all be processing tomatoes and the size and density of the bush was somewhat sub-standard to what I am used to seeing in a production field. I wrote this off to the fact that they were "grown for research" and researchers tend to be better scientists then farmers. I was very impressed, and am always relieved, to find an extremely high rate of consistency of observable traits across the repetitions. This lack of variation due to reps gave me a high degree of certainty that variation between treatments was due to the genetics and not the environment.

The following is a synopsis of the characteristics I observed for each of the varieties:

UC82B: This variety exhibited a short plant height, with an unusually dense bushy terminal growth habit on some of the plants. The fruit set was heavy, and consisted of a high percentage of red ripe fruit from the top to the bottom of the plant. Fruit size was very uniform in size and firmness. Some uneven ripeness was present, there were essentially no flowers on the plant, and 66% of fruit was trilocular.

5673: This variety had a similar plant height as UC82B, but did not show the bushy terminal growth habit. Fruit were a bit smaller, but had a heavy set, and 66% were bilocular. These fruit characteristics may have been a function of being less mature than UC82B. Only a small percentage had turned red. Fruit tended to have more interlocular air space. There were no flowers present and some of the outer most leaves exhibited a distinct purple cast. This was not due to any frost injury, but may have been a function of phosphorus deficiency.

8338: This variety had by far the tallest bush and the most immature fruit (no red fruit at all). Fruit was 66% trilocular and exhibited a lot of interlocular air space. Flowers were present on many of the plants, and there was a high incidence of dead or dying plants (most likely due to crown rot or a vascular wilt disease).

Please let me know if I can answer any questions or be of further assistance.

[CBI DELETED]

Gainesville, Florida
March 6, 1993

Glen Austin
Mail Zone AA3E
Monsanto Company
700 Chesterfield Village
Chesterfield, MO 63198

Dear Mr. Austin:

Enclosed is the tomato information which we discussed by telephone. This study was supported by the Florida Tomato Exchange and they were provided a report. Since retirement I can't find much of the routine paper work and reports: the original records I do have.

Preliminary study June 1984

Flavor of table-ripe tomatoes harvested mature-green vs "vine-ripe" (turning).

[illegible]
Flora-Dade
Sunny

Immediately after harvest, the mature-green tomatoes were exposed to ethylene gas for 48 hours, at which time only fruits at the "turning" stage were selected as those which were mature-green at time of harvest: all other maturity stages were discarded. To coincide with these selected fruits reaching the turning stage during 48 hours in ehtylene gas, the "vine ripe" fruits were harvested in the field and both lots were then ripened to the table-ripe stage at 20 C.

The taste panel consisted of 45 - 50 judges. The triangle taste method was used. There was no visible difference between samples harvested at the two maturities.

Results - For all three varieties, judges were unable to differentiate between ripe fruits which had been harvested "mature-green" as compared to "vine-ripe".

Expanded study, June 1985

Quality & composition of tomatoes harvested at different stages of maturity.

Cultivars:

1. Flora-Dade
2. Sunny

3. FTE-12
4. Duke

no comparisons were made between cultivars, only harvest maturity.

Harvest maturity:

A. Immature green (breaker/turning at 72 hrs in ethylene)
B. Mature-green (breaker/turning at 24 hrs in ethylene)
C. Vine-ripe (breaker/turning at harvest)
D. Pink (advanced turning to minimum pink at harvest)

As in the preliminary study, only fruits of the 4 harvest maturities were used which reached the table-ripe stage on the same day.

Edible quality was evaluated by the Triangle tests test. Composition was evaluated by standard laboratory methodology.

Taste Test Results:

Comparisons	Statistical significance
Maturity B vs C	ns
Maturity B vs D	ns
Maturity A vs B	*
Maturity A vs C	**
Maturity A vs D	**

There was a flavor difference between fruits harvested immature green and those of the more advanced stage of maturity. In general, the panel rated fruits harvested immature green as being more sour and lacked flavor intensity. However, the panel could not detect flavor differences (could not pair correctly) between fruits harvested mature-green and the two more advanced maturities. All samples ripened to a good red color.

[CBI DELETED]

Table 1. Composition of ripe fruit from the expanded study, June 1985. Each figure is the average of four replications

Cultivar	Harvest Maturity	Firmness A	Acidity B	Sweetness C	Sugar/acid Ratio D	Vitamin C E	Vitamin A F	Ripe color G	Dry Weight H
Flora-Dade	IG	9.3	5.3	3.8	11.4	18.2	0.26	2.8	5.2
	MG	9.1	5.9	4.2	11.6	20.4	0.28	2.8	5.3
	VR	7.4	6.1	4.3	11.2	21.7	0.28	2.8	5.3
	PK	7.8	5.9	4.2	11.0	19.5	0.26	2.8	5.4
Sunny	IG	14.4	6.0	3.8	10.3	16.3	0.26	2.8	5.3
	MG	13.7	5.8	4.2	11.3	20.2	0.27	2.7	5.2
	VR	10.2	6.3	3.8	9.0	18.8	0.32	2.7	5.2
	PK	10.2	6.2	3.6	9.7	19.0	0.32	3.0	5.1
FTE-12	IG	11.8	5.2	[illegible]	12.0	18.3	0.27	2.8	5.0
	MG	11.4	6.3	4.1	10.6	20.6	0.32	2.8	5.4
	VR	8.8	5.9	4.1	10.9	20.8	0.32	2.9	5.3
	PK	[illegible]	5.7	4.3	[illegible]	[illegible]	[illegible]	3.2	5.6
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	MG	10.4	5.9	4.4	11.3	19.4	0.28	2.6	5.2
	VR	9.4	6.0	3.8	12.2	20.5	0.23	2.7	5.2
	PK	9.2	4.8	4.0	13.2	19.8	0.23	2.8	5.2

Harvest maturity:
IG = immature green
MG = mature green
VR = vine ripe (see text)
PK = pink (see text)

A - value shown X 0.254=mm deformation: softer fruit have higher values. In general, fruits harvested immature green were soft when they ripened.

B - citric acid equivalents

C - soluble solids

D - sugar/acid ratio: normally, a high ratio signifies more flavorful fruits. However, the taste panel said that the immature fruits were more sour and lacked "intensity" when they ripened.

E&F - Fruits harvested immature tend to have a lower content of vitamins A and C when they ripened.

G - Hunter Color a/b ratio

H - percent

Appendix VIII. FDA Memorandum of Conference, Sepember 19, 1994

MEMORANDUM OF CONFERENCE
September 19, 1994

Participants:

Monsanto:

Bruce Hammond
Glen Austin
Jim Altemus
Marty Strauss
Andrew Reed
Roy Fuchs
Daryl Thake
Stephen Rogers

FDA:

F. Owen Fields	HFS-207
James Maryanski	HFS-13
Jeanette Glover Glew	HFS-246
Nega Beru	HFS-206
Zofia Olempska-Beer	HFS-247
Carl B. Johnson	HFS-226
Thomas A. Cebula	HFS-237

Subject: Delayed ripening tomato.

Keywords: Tomato; *Lycopersicon esculentum;* delayed ripening; ACC deaminase (ACCd) from *Pseudomonas chloraphis* strain 6G5; *kan*^{r}; APH(3')II; Npt II.

This meeting was intended to bring Monsanto's consultation with FDA on the food and feed safety of this product to closure.

Intended Effect and Food/Feed Use

The intended effect of this genetic modification is to delay the ripening of fruit from tomato *(Lycopersicon esculentum)* plants. Tomato fruits are primarily used for human food; animal feed use is minor and is limited to occasional seasonal use.

Mechanism of Intended Effect

Ethylene serves as an endogenous phytohormone in tomatoes where it initiates and controls the rate of fruit ripening. 1-aminocyclopropane-1-carboxylic acid (ACC) is an intermediate in ethylene's biosynthetic pathway. Monsanto has isolated a gene from the common soil bacterium *Pseudomonas chloraphis* which encodes an ACC deaminase (hereafter referred to as ACCd). Expression of ACCd in tomatoes results in reduced levels of ACC and ethylene and confers a delayed ripening phenotype.

Molecular Alterations and Characterization

A restriction map of the binary vector used for *Agrobacterium*-mediated transformation of tomato plants is shown on page 15 of Monsanto's submission of August 26, 1994. Based on restriction mapping of genomic DNA from the final transgenic lines intended for commercialization, Monsanto has concluded that the inserted DNA spans the region between breakpoints which lie within a few hundred base pairs of the right and left borders of the T-DNA (refer to the Figure on page 15 of the submission). According to Monsanto, the inserted DNA is capable of expressing two proteins; 1) ACCd; and 2) the APH(3')II (Npt II) enzyme. Because APH(3')II has been approved by the agency for this intended use in tomatoes (21 CFR 173.170 and 573.130), this memorandum will not further address aspects specific to APH(3')II. Monsanto stated that sequences outside of the right and left T-DNA borders in the binary vector (specifically, the spectinomycin/streptomycin resistance gene and the pUC *ori)* were not present in tomato lines intended for commercialization as judged by Southern analysis.

Based on genomic restriction mapping and genetic analysis, Monsanto has concluded that the ACCd-expressing transgene is present in one copy, is integrated at a single locus, segregates as a single dominant Mendelian trait, and is molecularly stable over several generations. Monsanto also stated that the trait is phenotypically stable over several generations.

Expressed Protein

As stated above, the only protein expected to be expressed in Monsanto's delayed ripening tomato lines which has not previously been considered by the agency is ACCd.

ACCd catalyzes the deamination of ACC to alpha-ketobutyrate and ammonia, both of which are common metabolic intermediates in plants and other organisms. ACCd was reported by Monsanto to be inactive against amino acids other than ACC and to be heat-labile. Monsanto stated that ACCd activity has been demonstrated to be widespread

in common yeasts and bacteria. Monsanto has inferred similarity in primary sequence among these various ACCd enzymes based on the observed cross-reactivity of many with polyclonal antisera raised against ACCd from *Pseudomonas chloraphis*. According to Monsanto, ACCd is not significantly homologous in primary structure to any known toxins or allergens. Monsanto also stated that ACCd does not fit the profile of the typical allergen because 1) it is not heat stable; 2) it is not a major protein in tomatoes; and 3) it is not resistant to digestion.

In order to produce sufficient material for safety and metabolism studies, Monsanto has produced ACCd in *E. coli*. Based on comparison of molecular weight, N-terminal sequence, specific activity, immunoreactivity, and lack of glycosylation, Monsanto has concluded that *E. coli*-produced ACCd is equivalent to ACCd purified from tomatoes. According to Monsanto, ACCd is rapidly digested in simulated gastric fluid and, as expected, showed no acute toxicity in a mouse gavage study.

Compositional Analysis

Based on the nature of the genetic modification, it was not expected that delayed-ripening tomatoes would differ significantly in composition from other tomato varieties. To confirm this expectation, Monsanto carried out a compositional analysis on whole fruits.

Based on their analysis of whole fruit, Monsanto has concluded that their delayed-ripening tomatoes are not significantly different from other tomato varieties in total solids, ash, fat, total protein, carbohydrates, vitamins A, C, and B6, folic acid, riboflavin, thiamin, niacin, calcium, magnesium, iron, sodium, phosphorus, fructose, glucose, sucrose, citric acid, malic acid, lactic acid, natural tomato soluble solids, pH, titratable acidity, lycopene, and tomatine content.

Wholesomeness Studies

Monsanto described the results of a wholesomeness study they carried out in rats. On the basis of their consideration of the results of this study, Monsanto has concluded that there is no significant difference in the wholesomeness of delayed-ripening and control lines of tomatoes, as expected from their compositional analysis.

Conclusions

Monsanto has concluded, in essence, that the delayed-ripening tomato varieties they have developed are not significantly altered within the meaning of 21 CFR 170.30(f)(2) when

compared to tomato varieties with a history of safe use. At this time, based on Monsanto's description of its data and analysis, the agency considers Monsanto's consultation on this product to be complete.

F. Owen Fields, Ph.D.

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