94-228-01P

DNA PLANT TECHNOLOGY CORPORATION

August 15, 1994

Mr. Michael A. Lidsky
Deputy Director
BBEP, APHIS, USDA
6505 Belcrest Road
Federal Building
Hyattsville, MD 20782

Re: **Petition for Determination of Non-regulated Status**
Delayed-Ripening Tomato Line 1345-4

Dear Mr. Lidsky:

DNA Plant Technology Corporation submits this petition to request that the Animal and Plant Health Inspection Service (APHIS) determine that the Delayed-Ripening Tomato Line 1345-4 described herein is no longer considered a regulated article under 7 CFR Part 340.

In support of this petition, I enclose a full statement explaining the factual grounds why the Delayed-Ripening Tomato Line 1345-4 should not be regulated under 7 CFR Part 340, including information set forth in paragraph (c) of 7 CFR 340.6. This petition does not contain any trade secret or confidential business information (CBI) and is so marked.

Please contact me at 510/450-9310 if you have any questions concerning our petition.

Very truly yours,

J. Scott Thenell
Regulatory Affairs Manager

Enclosures (2)

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Petition for Determination of Non-regulated Status:

Delayed-Ripening Tomato Line 1345-4

Submitted by:

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August 15, 1994

Contains No Confidential Business Information

Summary

DNA Plant Technology Corporation is submitting a Petition for Determination of Non-regulated Status to the USDA Animal and Plant Health Inspection Service (APHIS) for tomatoes that contain a gene that delays fruit ripening. DNAP requests a determination from APHIS that the Delayed-Ripening Tomato line 1345-4, and any progeny derived from hybrid crosses between this line and other non-transformed tomato varieties, no longer be considered a regulated article under 7 CFR Part 340.

Using Transwitch™ gene suppression technology, company scientists introduced a truncated version of an aminocyclopropane carboxylate (ACC) synthase gene (isolated from tomato) into the tomato genome in the "sense" or normal orientation, resulting in tomato plants which exhibit significantly reduced levels of ACC synthase and ethylene biosynthesis. Ethylene is an endogenous plant hormone known to play an important role in fruit ripening in climacteric fruit such as tomato. ACC synthase is the rate limiting enzyme that converts s-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid, the immediate precursor to ethylene. Inhibition of ACC synthase biosynthesis results in reduced levels of ethylene biosynthesis. Fruit of these plants exhibited a delayed ripening phenotype, but ripened normally when external ethylene was applied.

The tomato line for which this determination is requested, line 1345-4, contains a gene which is derived from the tomato ACC synthase gene, but which does not encode a functional ACC synthase enzyme. Tomato plants were produced by inserting the truncated ACC synthase gene into the genome of tomato cultivar 91103-114.

Delayed-Ripening Tomatoes can improve current fresh market tomato production and distribution by: (1) allowing fruit to mature on the vine longer for enhanced flavor without the threat of spoilage; (2) reducing harvest frequency, thereby reducing labor costs; (3) permitting transportation over longer distances; and (4) potentially, opening up international markets for U.S. tomato growers.

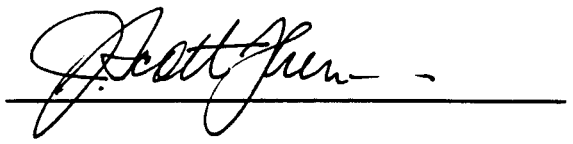
Delayed-Ripening Tomato lines have been field tested since 1992 in the major tomato growing regions of the United States under field release permits granted by APHIS (USDA Permit Nos. 92-035-05, 92-301-01, 93-056-01, 93-252-07, 93-302-01, 93-351-02, 94-060-04) and are currently being tested in additional locations in California (USDA Permit No. 94-126-01, 94-158-01, 94-172-01). Data collected from these trials, laboratory analyses and literature references presented herein demonstrate that the Delayed-Ripening Tomato line 1345-4: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than the non-modified parental variety; 3) is unlikely to increase the weediness

potential of any other cultivated plant or native wild species; 4) does not cause damage to processed agricultural commodities; and 5) is unlikely to harm other organisms that are beneficial to agriculture.

DNA Plant Technology Corporation requests a determination from APHIS that the Delayed-Ripening Tomato line 1345-4, and any progeny derived from hybrid crosses between this line and other non-transformed tomato varieties, no longer be considered a regulated article under 7 CFR Part 340.

Certification

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



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A. Statement of Grounds for Non-regulated Status

Statement of Grounds for Decision

I. Rationale for Development of Delayed-Ripening Tomato

DNA Plant Technology Corporation developed the Delayed-Ripening Tomato as an improvement in the flavor and overall quality of fresh market tomatoes and to make them available to consumers year-round. Research indicates that 85% of U.S. households consume fresh market tomatoes during the year. Despite such widespread use, consumers cite the tomato as the most consistently disappointing vegetable. We believe that a large percentage of tomato consumers drop out of the market at certain times of the year due to dissatisfaction with the taste and texture of available product.

More than 80% of tomatoes (by volume) currently sold in the United States are picked while green. Growers harvest green tomatoes for several reasons: (1) green tomatoes are firmer, enabling them to withstand shipping and handling with less injury; (2) a green tomato harvest is less labor-intensive and less costly than a vine-ripe harvest and (3) green fruit stays in the field for a shorter period of time reducing the risk of loss from weather or pests. After harvest, either the packer or the repacker exposes the green tomatoes to an external source of ethylene gas to cause the tomatoes to develop red color. This practice reddens the fruit, but these tomatoes will not always develop full flavor when picked at the green stage. Fruit that have begun to ripen need to be shipped at a low temperature to delay ripening, however these low temperatures decrease fruit quality. Frequently, a portion of the green fruit is harvested at the immature green stage, which means that it will never achieve full ripeness even with the application of ethylene. Such tomatoes, together with other distribution-damaged tomatoes, do not achieve full flavor potential and, we believe, are a major factor contributing to consumer dissatisfaction with current fresh market tomatoes.

In tomato (and other so called climacteric fruit), fruit ripening is associated with a burst of respiration and a concomitant increase in ethylene production. Once ripening is initiated, the endogenous ethylene production rises autocatalytically. The ethylene produced affects gene transcription in fruit tissue and there is much evidence which indicates that ethylene is the natural ripening hormone.

By controlling ripening, Delayed-Ripening Tomatoes can be left on the vine longer to develop the fruit components which contribute to flavor. At the stage when tomatoes are normally too ripe to survive the rigors of the packing and distribution system, the delayed-ripening tomato has developed more flavor than green-picked tomatoes, yet is still firm enough for distribution. Thus, growers can deliver tomatoes from growing regions far from consumer markets.

To achieve ripening control, company scientists used a gene isolated from tomato that encodes the enzyme ACC synthase, the rate limiting step in ethylene biosynthesis. Reducing ethylene biosynthesis in the fruit slows the ripening process in tomato, resulting in fruit that retains its sensory properties for an extended period. In such fruit, complete ripening does not occur even though fruit are left on the vine or in storage for prolonged periods.

Using Transwitch™ gene suppression technology, company scientists introduced a truncated version of the fruit-specific ACC synthase gene, isolated from tomato, into the tomato genome in the normal or "sense" orientation. Plants which carry this truncated ACC synthase transgene have reduced levels of the ACC synthase enzyme, resulting in tomatoes which retain desirable taste, color, and texture for an extended period of time.

We believe the Delayed-Ripening Tomato offers consumers better fresh market tomato flavor and overall quality year-round. By improving flavor and availability, consumers may be encouraged to increase their consumption of fresh market tomatoes, a beneficial component of a nutritious diet. We also believe that the Delayed-Ripening Tomato will offer considerable cost savings in production and distribution by reducing the number of harvests in a given production field. Further, the ability to hold the Delayed-Ripening Tomato off the vine will allow the tomato packer/repacker some additional flexibility in controlling tomato inventory and will permit shipments over longer distances, thereby opening potential international markets to U.S. farmers.

II. The Tomato Family

A. The Origins and History of Tomato

The tomato is native to South and Central America. The natural distribution of the genus *Lycopersicon* extends from northern Chile to southern Colombia and from the Pacific coast (including the Galapagos islands) to the lower eastern foothills of the Andes (Esquinas-Alcazar, 1981). Many species overlap, but no evidence of natural introgression has been found with the exception of *L. pimpinellifolium* and *L. esculentum.* All the species have well-defined ranges of distribution, except *L. esculentum* var. *cerasiforme* (cherry tomato) which is the only wild *Lycopersicon* found outside South America (Taylor, 1986).

While the original site of domestication is uncertain, most evidence suggests that the tomato was first domesticated in Mexico (Taylor, 1986). Its introduction into Southern Europe can be traced to the 16th century (Davies and Hobson, 1981). Its first recorded mention in North America appeared in the early 18th century (Tigchelaar, 1986). Today, hundreds of tomato cultivars are grown commercially and in home gardens making the tomato one of the most popular fruits world-wide.

B. Taxonomy of the Genus Lycopersicon

The genus *Lycopersicon* is a member of the family Solanaceae, subfamily Solanoideae, and the tribe Solaneae. The genus *Lycopersicon* may be further sub-divided into those species which can be easily crossed with the commercial tomato (the *esculentum* complex) and those which are crossed with considerable difficulty (the *peruvianum* complex).

The subgenus *esculentum* consists of seven *Lycopersicon* species (*L. esculentum, L. cheesmanii, L. chmielewskii, L. hirsutum, L. parviflorum, L. pimpinellifolium,* and *L. pennelli*). The *peruvianum* subgenera consists of only two species, *L. chilense* and *L. peruvianum,* which do not naturally inter-cross with species of the *esculentum* subgenera. All *Lycopersicon* species have the same number of chromosomes (2n = 2x = 24) and chromosome morphology (Rick, 1976).

C. Genetics of Tomato

The genetic structure and crossability of *Lycopersicon* species have been extensively described (Stevens and Rick, 1986). The cultivated tomato is self-fertile and almost exclusively self-pollinating, generally requiring human intervention for cross-pollination. The factors (both pre- and post-fertilization

barriers) that prevent cross-pollination between *Lycopersicon* species are well documented (Rick, 1979; Taylor, 1986). Inter-species hybrids between *L. esculentum* and wild *Lycopersicon* species can only be made by hand-pollination, and only with varying degrees of success. For example, certain pest resistance traits have been successfully introduced into *L. esculentum* through controlled crosses with *L. pimpinellifolium, L. peruvianum* (Rick, 1983), *L. hirsutum, L. chilense, and L. esculentum* var. *cerasiforme* (Esquinas-Alcazar, 1981). Additionally, traits affecting soluble solids, fruit abscission and retention of pedicels have been introduced from *L. chmielewskii* (Rick, 1983). While these reports indicate that crosses between *L. esculentum* and wild *Lycopersicon* species are possible, it appears that natural crosses are quite limited. Wide hybridization between members of the *esculentum* and *peruvianum* subgenera can only be accomplished using specialized laboratory procedures such as embryo culture, and usually lead to early embryo breakdown and nonviable seed (Taylor, 1986).

Attempts to cross *L. esculentum* and close genetic relatives of the genus Solanum have had mixed results. Crosses between *L. esculentum* and *S. ochranthum* failed (Rick, 1979). Crosses between *L. esculentum* and *S. rickii* were successful using a sesquidiploid bridging hybrid (DeVerna et al., 1990). Crosses between *L. esculentum* and *S. lycopersicoides* are possible, but hybrids are usually sterile (Stevens and Rick, 1986).

D. Tomato as a Crop

Essentially all cultivated forms of tomato belong to the species *Lycopersicon esculentum* Mill. Tomato is grown commercially wherever environmental conditions permit an economic yield to be obtained. The edible portion of tomato is botanically a fruit, although it is commonly considered a vegetable. Its popularity and value as a crop have led to wide dissemination around the world.

Gross morphological characteristics of tomato include herbaceous perennial growth, sprawling or prostrate habit, pinnately segmented leaves, stem organization in sequences of 2- or 3-leafed sympodia, cymose inflorescences, yellow corolla and anthers, anthers connate or connivent, and fruit as a soft berry (Rick, 1979). Under optimal temperature and growth conditions, the tomato will complete its reproductive cycle in 95-115 days. The first flowers open 7-8 weeks after seeding, and an additional 6-8 weeks elapse from first flower to ripe fruit (Tigchelaar, 1986).

Within the past century, the tomato has become one of the most popular and widely consumed vegetable crops. Annual world production approaches 50 million metric tons (Tigchelaar, 1986). Per capita consumption in the United States has more than tripled in the past 50 years to approximately 56 lb. per person annually, largely due to increased use of processed tomato products. The tomato's versatility in fresh and processed form has played a major role in its

rapid and widespread adoption as an important food commodity. Today, processing tomatoes are prepared for a variety of uses, canned in various forms or processed into paste, ketchup, tomato juice, specialty sauces and soups. Fresh market tomatoes are eaten whole, sliced or diced in a variety of foods, as well as cooked in many foodservice and home use applications. While the tomato does not rank high in nutritional value, it contributes a significant portion of the dietary intake of vitamins A and C by virtue of the volume consumed (Davies and Hobson, 1981).

Fresh tomatoes are available in the United States in greatest supply from June through September. Florida, California and Mexico provide the bulk of annual fresh market supplies due to their long cropping season and mild winters. California supplies the vast majority of tomatoes for processing in the United States (Tigchelaar, 1986). Of the 500,000 acres of tomatoes grown in the United States, approximately 40% are grown for fresh market consumption; the balance are grown for processing. Fresh market production in the United States is reported as approximately 1.8 million tons in 1992 (USDA, 1993).

E. Weediness Potential

The USDA defines a "weed pest" as "a plant that grows persistently in locations where it is unwanted" (USDA, 1992). The cultivated tomato is not considered a weed pest and is unlikely to become a weed pest.

The cultivated tomato is a perennial which is grown almost exclusively as an annual crop in the United States. Tomato is considered to be a highly inbred, well-characterized crop plant that is not persistent in undisturbed environments without human intervention. Although tomato volunteers are not uncommon, they are easily controlled using herbicides or mechanical means. Tomato also possess few of the characteristics of plants that are notably successful weeds (e.g., it does not produce abundant, long-lived seed; it does not propagate vegetatively; it does not compete well with other plant species in the environment).

Furthermore, the tomato has been grown for centuries throughout the world without any reports that it is a serious weed pest. In the United States, tomato is not listed as a weed in the major weed references (Crockett, 1977; Holm et al., 1977; Muenscher, 1980), nor is it present on the lists of noxious weed species distributed by the State of California or the Federal Government (7 CFR Part 360). The cherry tomato (*L. esculentum* var. *cerasiforme*) is believed to be the direct ancestor of the modern cultivated form of tomato and is capable of hybridization with large-fruited tomato. It is widespread in the Andean region of Central and South America where it grows as a weed in irrigated fields or areas of high rainfall. However, it is not considered a weed pest in the United States (USDA, 1992).

F. Potential for Outcrossing

1. Wild related species

Hybridization with wild species of *Lycopersicon*

The only wild species that can cross with cultivated tomato are members of the genus *Lycopersicon*, subgenus *esculentum*. Interspecific hybrids between *L. esculentum* and wild *Lycopersicon* species can be achieved, but the only opportunities for hybridization would occur in the Andean region of South America where the wild perennial species are endemic (USDA, 1993).

Hybridization with related species

The nightshade family contains many other species which are found as weeds in tomato fields. *Solanum nigrum* is the only major weed pest related to tomato (Lange et al., 1986). Other members include: *S. sarrachoides* (hairy nightshade), the groundcherries (*Physalis heterophylla* Nees., *P. lanceifolia, P. ixocarpa,* and *P. acutifolia), Nicotiana bigelovii* (Indian tobacco), and jimsonweeds (*Datura stramonium L., D. meteloides,* and *D. ferox*), (University of California 1985). Other weedy Solanaceae species are: *Hyoscyamus niger* (black henbane), *Lycium ferocissimum* (African boxthorn), *P. virginiana* var. *sonorae* (smooth groundcherry), *P. viscosa* (grape groundcherry), *S. cardiophyllum* (heartleaf nightshade), *S. carolinense* L. (horsenettle), *S. dimidiatum* (Torrey's nightshade), *S. dulcamara* L. (bitter nightshade) (Lorenzi and Jeffery, 1987), *S. elaeagnifolium* (white horsenettle), *S. lanceolatum* (lanceleaf nightshade), *S. marginatum* (white-margined nightshade), and *S. torvum* (turkeyberry).

L. esculentum is sexually incompatible with all of these weedy species. Two *Solanum* species, *S. lycopersicoides* and *S. rickii*, can be crossed with *L. esculentum* under specific controlled conditions, but they do not naturally cross with *L. esculentum*, nor are they weed pests in the United States.

2. Cultivated tomato

Tomato is a self-compatible species and is predominately self-pollinated when field grown. Flowers are easily manipulated, facilitating hybridization. Rates of natural cross-pollination in temperate zones have been reported to vary from 0.5 to 4% (Rick, 1978). Rick suggests that the change from moderate cross-fertilization to almost exclusive self-fertilization in cultivated tomato coincides with a change in stigma position from outside to within the anther cone. Present cultivated varieties form a tight protective anther cone surrounding the stigma which greatly reduces the possibility for natural outcrossing. Taylor (1986) reports, "All representatives of *L. esculentum* are self-compatible and exclusively

inbreeding." Hybridization among cultivated tomatoes is reported to vary from 0 - 5%, with rates of 0 - 2% most often observed in the United States (USDA, 1993). Insect activity is a factor in outcrossing, but the inserted stigma of cultivated tomato significantly limits occurrence. Seed purity in tomato is maintained using standard breeding practices, including minimum isolation distances.

III. The Transformation System and Plasmid Used

The Delayed-Ripening Tomato line 1345-4 contains a gene (the AccS transgene) which is derived from a tomato fruit-specific ACC synthase gene, but that does not encode a functional ACC synthase enzyme. The AccS transgene is a truncated coding region from the tomato *Acc2* gene which is fused to a 35S promoter from cauliflower mosaic virus and a *nos3'* termination sequence from *Agrobacterium tumefaciens*. The plasmid pWTT2144/AccS, used to transform the parental line 91103-114 to generate line 1345-4, also contains a selectable marker gene (*nptII*) from transposon Tn5 under transcriptional control of a *nos* promoter and *ocs3'* termination sequence. The plasmid was transferred to the parental line using the *Agrobacterium tumefaciens*-mediated transformation technique. Stable insertion of the AccS transgene into the tomato genome in the sense orientation results in down-regulation of expression of the corresponding endogenous *Acc2* gene and a reduction in ethylene biosynthesis in the ripening fruit. The technique is an example of Transwitch™ suppression, a patented gene suppression technology pioneered by DNA Plant Technology Corporation.

The Transwitch™ technology is a method for targeted of gene expression. The technique has been used to suppress the expression of a wide range of genes ranging from enzymes involved in anthocyanin biosynthesis to enzymes involved in fruit ripening (Zhang et al., 1992; Nasrallah et al., 1992; Bird et al., 1993; Fray and Grierson, 1993; Elkind et al., 1991; Gottlob-McHugh et al., 1992; Napoli et al., 1990; van der Krol et al., 1990; Angenent et al., 1993; van der Krol et al., 1993). The Transwitch ™ method employs a transcribable DNA segment, homologous to a target endogenous gene, which is functionally linked to a promoter. This construct is introduced into the plant genome. Among the resulting population of transformants, there are individuals with varying degrees of target gene suppression which can be selected. The trait (level of suppression), once selected through the first sexual generation, behaves in a simple Mendelian fashion.

A key feature of Transwitch™ gene suppression is the reduced accumulation of the targeted gene mRNA (reduced steady state RNA). In several examples which have been analyzed in detail, it is clear that the transcription of the targeted gene is not reduced, but rather a post-transcriptional mechanism is in effect. In the cases studied, Transwitch™ suppression results in an inhibition of translation of both the transgene and the target gene (Dunsmuir, unpublished).

A. Agrobacterium-mediated transformation system

Introduction of DNA into plant tissue by *Agrobacterium*-mediated transformation has been previously described (Klee and Rogers, 1989; Zambryski, 1988). The vector system used to transfer the AccS transgene into tomato is based on the Ti plasmid from *Agrobacterium tumefaciens*. The vector system is "disarmed" or

non-pathogenic by deletion of all genes normally found in the T-DNA which are responsible for crown gall disease. This system is also "binary," with the genes to be transferred on one plasmid and the genes encoding necessary functions for transfer, the *vir* genes, on a second plasmid, pAL4404. Genes on the second plasmid are not transferred to the engineered plant.

Following the use of *Agrobacterium* for plant transformation, the *Agrobacterium* are killed with carbenicillin or cefotaxime (Fillatti et al., 1987) so no subsequent infection or transformation can occur. The transformed plants are grown to flowering and seed is collected and used for future generations of plants before the final generation is selected for field production. Because of these procedures, the original plant transformation vector (Ti plasmid) does not remain associated with the plants and any further transfer of genes from such plasmids to humans, animals or the environment could not occur.

B. Construction of the Plasmid Used for Transformation

The T-DNA plasmid, pWTT2144, used in this cloning is composed of: (1) the replication of origin from pACYC184 that ensures replication in *Escherichia coli;* (2) the pVS1 replicon (derived from *Pseudomonas aeruginosa* DNA) that ensures replication in *A. tumefaciens;* (3) the tetracycline resistance marker from plasmid RP1 that allows for selection of the binary plasmid in *A. tumefaciens* and *E. coli,* and (4) the left and right border regions of T-DNA from an octopine strain of *A. tumefaciens* which surround the DNA insertion in the plant genome.

Within the T-DNA are the *nptII* gene from transposon Tn5 that encodes the enzyme neomycin phosphotransferase II and serves as a selectable marker for transformed plant cells, fused to a nopaline synthase (*nos*) promoter sequence and octopine synthase (*ocs3'*) termination sequence from *A. tumefaciens*, and the LacZ' polylinker region with multiple restriction sites for cloning of genes to be transferred. The T-DNA has an insertion of a truncated *Acc2* gene coding region fused to the 35S promoter from cauliflower mosaic virus and the *nos3'* termination sequence in the LacZ' polylinker region of pWTT2144. Plasmid pWTT2144 was transferred from *E. coli* to *A. tumefaciens* LBA4404, which carries the pAL4404 *vir* plasmid, by a triparental mating procedure (Figurski et al., 1979).

As will be demonstrated in detail in the following sections, only a portion of the DNA sequences of pWTT2144/AccS were inserted into the genomic DNA of the tomato line 1345-4. Prior to transfer of the T-DNA region from *Agrobacterium* to the plant cell, single strand nicks are introduced adjacent to the left and right border regions and it is only the sequences between these nicks which are transferred. Hence, of the DNA which is transferred to the tomato plants, the regions derived from *Agrobacterium* or CaMV include the *nos* promoter, *ocs3'*, *nos3'*, 35S promoter and the T-DNA left and right border fragments.

The complete sequence of the T-DNA region is shown in Appendix 1. A map of vector pWTT2144/AccS is shown in Figure III.1. The description of the DNA elements in pWTT2144/AccS is shown in Table III.1.

C. Open Reading Frames Contained in pWTT2144/AccS

1. AccS. The AccS gene is a truncated version of a fruit-specific ACC synthase gene (*Acc2*) isolated from *L. esculentum* (Rottman et al., 1991). The AccS gene is derived from the *Acc2* gene, but is missing 149 bases at the 5' end of the coding region and 220 bases at the 3' end. The gene was prepared using PCR synthesis and two oligonucleotide primers which correspond to the zucchini ACC synthase gene sequence. The *Acc2* gene is induced in tomato fruit at the onset of ripening, and it reaches maximum expression at the pink fruit stage. The AccS gene does not encode a functional ACC synthase enzyme.

2. *nptII* The neomycin phosphotransferase (*nptII*) gene was isolated from the Tn5 transposon, and is found throughout nature, often in soil microorganisms (Leff et al., 1983). The *nptII* gene encodes a protein, neomycin phosphotransferase II, which confers resistance to certain antibiotics used in the selection of transformed plant cells.

Figure III.1 Plasmid Map of pWTT2144/AccS

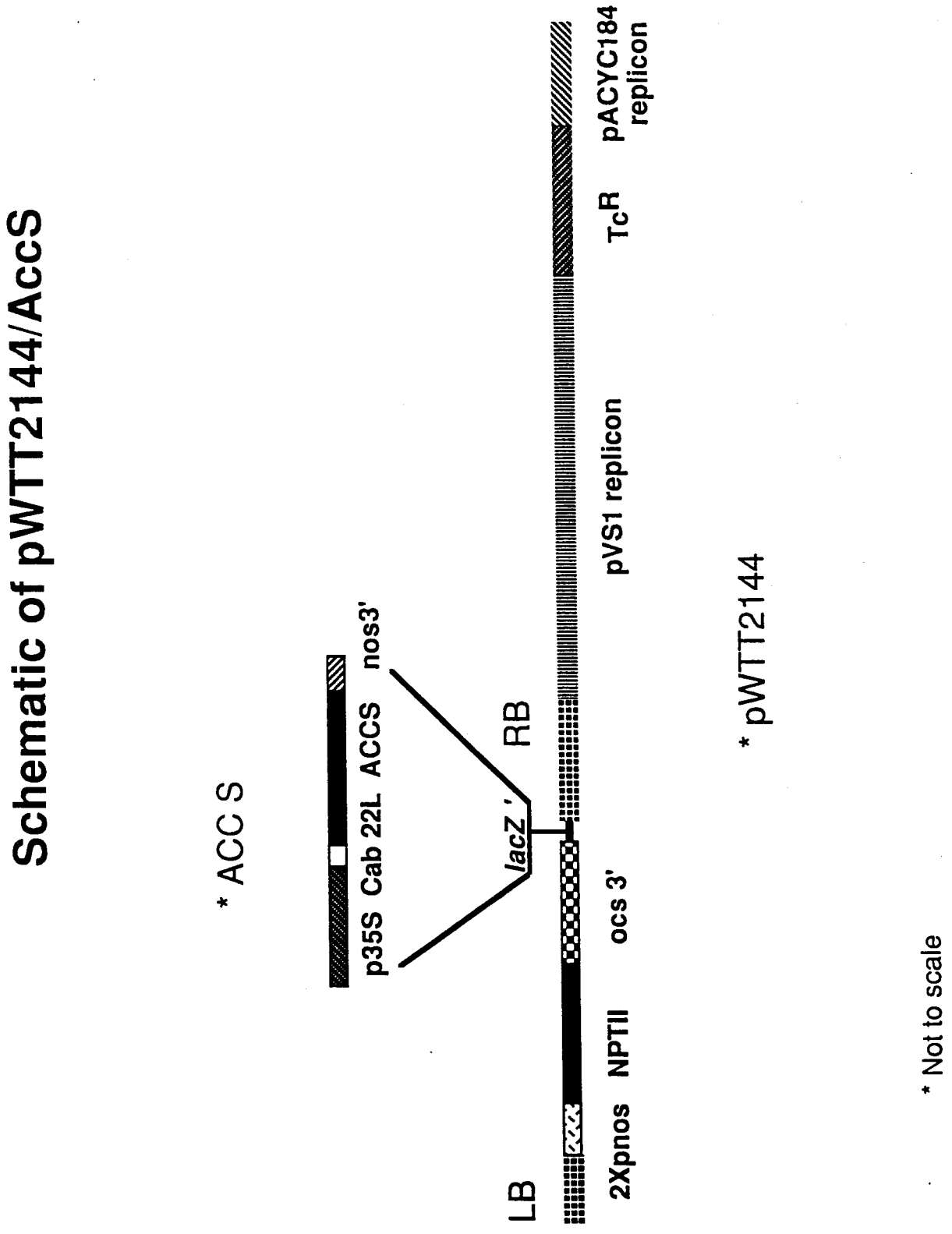


Table III. 1 Summary of Sequences of pWTT2144/AccS

Genetic Element	Size, Kb	Function
LB	0.880	The left and right border regions of T-DNA from Agrobacterium tumefaciens (Barker et al., 1983; van den Elzen et al., 1985)
2Xpnos	0.6	The tandem duplicate untranslated promoter region of the nopaline synthase gene from Agrobacterium tumefaciens (Depicker et al., 1983) controls expression of the *nptII* gene.
nptII	1.02	The neomycin phosphotransferase II gene from Tn5 (Beck et al., 1982) encodes a protein which renders transformed cells resistant to kanamycin.
ocs3'	0.56	The untranslated 3' region of the octopine synthase gene from Agrobacterium tumefaciens (DeGreve et al., 1983) controls expression of the *nptII* gene.
lacZ'	0.466	The untranslated LacZ polylinker sequence (Yanish-Perron et al., 1985).
p35S	0.96	The 35S gene promoter from cauliflower mosaic virus (Odell et al., 1985) controls expression of the AccS transgene.
Cab22L	0.069	The gene leader sequence corresponding to the 5' untranslated region of the Cab22R gene from petunia (Dunsmuir, 1985) fused to the 35S promoter.
AccS	1.09	The truncated coding region from the tomato *Acc2* gene which corresponds to a 1088 bp region from base 149 to base 1237 (Rottman et al., 1991).
nos3'	0.27	The untranslated 3' region of the nopaline synthase gene from Agrobacterium tumefaciens (Depicker et al., 1983) controls expression of the AccS transgene.,
RB	1.900	The left and right border regions of T-DNA from Agrobacterium tumefaciens (Barker et al., 1983; van den Elzen et al., 1985)
pVS1	8	The pVS1 replicon derived from Pseudomonas aeruginosa DNA which ensures replication in Agrobacterium tumefaciens (Itoh et al., 1984).
Tc^R	2.5	The tetracycline resistance marker gene from plasmid RP1 allows for selection of the binary plasmid in Agrobacterium tumefaciens and E. coli (Waters et al., 1983).
pACYC184	1.535	The origin of replication from plasmid pACYC184 which ensures replication in E. coli (Chang et al., 1979).

IV. The Donor Gene in Delayed-Ripening Tomato Line 1345-4 to be Considered for Non-regulated Status

The Delayed-Ripening Tomato line 1345-4 has been considered a regulated article because it contains DNA sequences from *Agrobacterium tumefaciens* and cauliflower mosaic virus, organisms which are considered to be plant pests. This section contains a description of the genetic material in line 1345-4 to be considered for non-regulated status.

CaMV35S The 35S promoter region is derived from cauliflower mosaic virus (Gardner et al., 1981) and controls expression of the AccS gene. Cauliflower mosaic virus is a doublestranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. The region of the CaMV genome which we used corresponds to nucleotides 6500 to 7460. The 35S promoter directs high level constitutive expression and is widely used as a promoter for high expression of genes (Harpster et al., 1988). This sequence, as used in producing the Delayed-Ripening Tomato, does not cause these tomatoes to become a plant pest risk.

Cab22R leader The Cab22R leader sequence (Cab22L) is a 69 bp fragment of *Petunia hybrida* genomic DNA which was derived from the Cab22R gene and corresponds to the 5' untranslated region for that gene. This sequence is not derived from an organism considered to be a plant pest and does not cause line 1345-4 to become a plant pest risk.

AccS The AccS gene is a truncated coding region derived from an ACC synthase gene (*Acc2*) isolated from tomato (*L. esculentum*). The AccS gene corresponds to a 1088 bp region of the *Acc2* gene from base 149 to base 1237. The AccS gene does not encode a functional ACC synthase enzyme. As shown in Section V, insertion of the AccS gene into the tomato genome in the sense orientation interferes with normal expression of the endogenous *Acc2* gene by significantly reducing accumulation of the levels of the endogenous *Acc2* mRNA. This results in a reduction of active ACC synthase enzyme and ethylene in the fruit, the intended technical effect. The down-regulation of ethylene biosynthesis in the fruit results in the arrest of fruit ripening, but produces no other significant changes in fruit composition or agronomic characteristics.

Termination sequences The nopaline synthase (*nos3'*) and octopine synthase (*ocs3'*) gene termination sequences from *A. tumefaciens* function in the expression of the AccS and nptII genes, respectively. No *A. tumefaciens* disease symptoms were observed in any plants in any of the field trials (Appendix 2). These sequences, as used in producing the Delayed-Ripening Tomato, no longer function as regulated sequences since they are not associated with the nopaline synthase or octopine synthase coding regions which function in *A. tumefaciens* and thus do not make the Delayed-Ripening Tomato a plant pest risk.

2Xpnos Promoter The *nos* promoter is present in line 1345-4 as a duplicate tandem repeat of the untranslated 5' region of the nopaline synthase gene from *A. tumefaciens*. It functions in line 1345-4 in the expression of the *nptII* selectable marker gene. This sequence, as used in line 1345-4, no longer functions as a regulated article since it is not associated with the nopaline synthase coding region which functions in *A. tumefaciens*.

NptII The *nptII* gene is a coding region originally isolated from transposon Tn5. It encodes a protein, neomycin phosphotransferase II, which catalyzes the phosphorylation of certain aminoglycoside antibiotics, rendering transformed cells resistant to kanamycin. It functions in line 1345-4 as a selectable marker gene to aid in the laboratory selection of transformed tomato cells. The *nptII* gene is not derived from an organism considered to be a plant pest and does not make line 1345-4 a plant pest risk. The environmental consequences of using the *nptII* marker gene in tomato and other crops has been previously evaluated (USDA, 1992).

LacZ' polylinker sequence The untranslated LacZ' polylinker sequence functions in line 1345-4 as a site for cloning the AccS transgene into the binary vector pWTT2144. It is not derived from an organism considered to be a plant pest and does not make line 1345-4 a plant pest risk.

Borders The left and right border regions of T-DNA from *A. tumefaciens* function in the transfer of gene sequences into the tomato genome. The border regions (Barker et al., 1983) are the only necessary *cis*-acting elements in T-DNA (Klee and Rogers, 1989) for T-DNA insertion. The use of a binary vector system allows for other necessary transfer elements to act in *trans* so that only the border regions are required to be integrated into the plant host genome (Zambryski, 1988). The T-DNA borders are only partially transferred to the tomato genome; during the transformation process, the left border is cut between nucleotides 293 and 294 (left border nick) while the right border is cut between nucleotides 7603 and 7604 (right border nick). This cleavage reduces the length of the right border fragment of pWTT2144/AccS from 1900 bp to 303 bp in the T-DNA and the left border fragment of pWTT2144/AccS from 880 bp to 589 bp in the T-DNA (Figure V.1.) These border regions functioned as predicted in facilitating integration of the specific gene sequences into the tomato genome. No *A. tumefaciens* disease symptoms were observed in any plants in any of the field trials (Appendix 2). In *A. tumefaciens*, the T-DNA border regions flank the agropine biosynthesis genes and consequently (upon plant infection) integrate these genes in the plant genome where they become an important component of the tumorous tissue. However, the T-DNA borders as used herein do not flank genes which could contribute to the tomato becoming a plant pest risk.

V. Genetic Analysis, Agronomic Performance, and Compositional Analysis of Delayed-Ripening Tomato Line 1345-4

A. Description of Non-transformed Tomato Cultivar 91103-114

DNAP tomato line 91103-114 is a somaclone derived from the breeding line FL7181 developed by Dr. Jay Scott at the University of Florida. Line FL7181 is characterized as a determinate large-fruited variety with an average fruit weight of 8 oz. Fruit are globate to slightly elliptical in shape and are substantially firmer than fruit of comparable varieties (i.e., Floradade). Fruit shoulders are smooth and exhibit a darker green shade than the rest of the fruit surface before ripening. Fruit ripen to a deep crimson red interior color due to the presence of the og^c allele. The fruit stem (pedicel) lacks a joint. This line is known to be resistant to Verticillium wilt race 1 and Fusarium wilt races 1 and 2.

The DNAP line 91103-114 exhibits all of the traits described above, but differs from FL7181 principally by reduced blossom end scar size, strong main stem and increased foliage cover for fruit. Line 91103-114 also differs from FL7181 in its adaptation to diverse growth environments; while FL7181 is specifically adapted to Florida growing regions, 91103-114 has proven to grow well in other regions (e.g., California).

Seed of the DNAP tomato line 91103-114 is not publicly available. A notice of patent allowance has been issued to DNA Plant Technology Corporation for line 91103-114.

B. Description, History and Mendelian Inheritance of Delayed-Ripening Tomato Line 1345-4

The line 1345-4 is a homozygous T_1 selection from an original T_0 transformant 1345, obtained after *Agrobacterium tumefaciens* transformation of the DNAP line 91103-114 with binary vector pWTT2144/AccS. Line 1345-4 is not intended to be a finished commercial variety, but will be used in a breeding program for the development of fresh market tomatoes with the delayed-ripening phenotype.

The primary transformant 1345 was selected in a 1992 field screen of several hundred primary transformants (USDA# 92-035-05) to have fruit which did not ripen when left on the vine. Subsequently, 20 of the 1345 T_1 seed which were pre-screened for kanamycin resistance were planted in the field in 1993 and observations on the plant and fruit phenotype were made. Plant 1345-4 was selected as having fruit which did not ripen on the vine. Seed from the self-fertilization of 1345-4 have been subsequently analyzed in multiple field trials in Ruskin, Florida; Indio, California; and Brentwood, California during 1994. In

addition, seed from 1345-4 were planted in Molokai, Hawaii (USDA# 93-302-03) and served as the female or male parent in the production of hybrid seed.

We have not observed any instability in the delayed-ripening phenotype in any of our field trials through three sexual generations. There are two independent ways in which we have monitored the stability of line 1345-4. First, it is possible to establish that the T-DNA insertion is stable and intact by measuring kanamycin resistance in large populations of seedlings in the greenhouse. We initially established that the 1345 plant was carrying a single T-DNA insertion by evaluating the segregation of kanamycin resistance in the primary transformant. Subsequently, we selected multiple kanamycin-resistant T2 plants and then evaluated the segregation of the kanamycin-resistant phenotype in progeny plants arising from self-fertilization of the selected T2 plants. We found that the T3 plants from 1345-4 were all kanamycin resistant, hence we deduced that the 1345-4 plant was homozygous. We have since collected seed and evaluated kanamycin resistance in an additional three generations derived from 1345-4 and we have not seen any kanamycin-sensitive plants in any of these seed lots which have been collected from field-grown fruit.

The presence of an active *nptII* gene in the genetically engineered 1345 line was assayed by a kanamycin germination assay and by quantitation of the gene product (neomycin phosphotransferase II) using an immunological assay. In the germination assay, the seeds arising from the self-fertilization of the primary transformant 1345 were germinated on media containing the antibiotic. The *nptII* -containing seedlings form long healthy roots in the medium, while sensitive seedlings have short stunted roots. Using this assay we found that the *nptII* locus in 1345 seed segregated as a single locus (Table V. 1). When we tested the seed arising from the self-fertilization of the 1345-4 plant, we found that over 98% of the seed was kanamycin resistant which indicated that the *nptII* locus was homozygous in this selection. (We have found that the germination assay is not absolutely reliable, in that occasionally a seed may appear to be kanamycin sensitive, but would not germinate even in the absence of selection. This may be the explanation of the two 1345-4 progeny seed which appear to be sensitive. In most seed lots there is a low frequency of seed which do not germinate, and this is true for the parental and the transgenic material. In a control experiment where both 1345-4 and 91103-114 seed were germinated on non-selective medium, the frequency of non-germination was similar at 1-2%.) In a progeny plant from 1345-4, namely 1345-4-S, we found that the seed did not segregate any sensitive progeny indicating that it was homozygous, as expected.

The second approach to evaluating the stability of line 1345-4 is through observation of the delayed-ripening phenotype in the field. To date, we have performed eight separate field trials for the evaluation of the homozygous 1345-4 line and progeny derived from it. These trials have involved the evaluation of fruit on at least 2000 separate plants of 1345-4. During these evaluations, which involved observations of the individual plants at multiple times during the

ripening process and the harvest of fruit from all of the plants, we have not observed exceptional plants in which the fruit ripen at the normal rate. On the rare occasion where we have seen a single fruit ripening normally on the plant, we have always found that the fruit is damaged by cracking or infection. In this case, we infer that the fruit is ripening as a consequence of the induction of wound ethylene.

Table V. 1 Kanamycin Germination Data for Delayed-Ripening Tomato Lines

Line	Resistant	Sensitive	X^2
1345	25	8	0.01*
1345-4	112	2	na
1345-4-S	128	0	na

* Chi-square goodness-of-fit test for hypothesis of 3:1 segregation. X^2 =3.84, for a 3 to 1 ratio at p=0.05, df=1

These data, describing the inheritance of the kanamycin-resistance phenotype and the direct observation of the delayed-ripening phenotype, support the position that the T-DNA insertion in the initial 1345 transformant has been stably maintained and expressed through four generations for which we have collected data.

C. DNA Analysis of Delayed-Ripening Tomato Line 1345-4

To determine the nature and number of insertions which have occurred in line 1345, we used Southern hybridizations to characterize the structure of the T-DNA inserts in the genomic DNA (Southern, 1975) in conjunction with the *nptII* segregation data described above which indicates the T-DNA locus number. The T-DNA is defined as the region between the left and right borders of the binary vector pWTT2144/AccS that is transferred into the plant (see Figure V. 1). This region includes the *nptII* selectable marker and the truncated ACC synthase gene (AccS), together with the left and right border sequences. Figure V. 2 shows the restriction enzymes used to cleave the DNA and the location of the four probes used to determine the structure of T-DNA insert, the left border (LB), *nptII*, *Acc2*, and right border (RB) probes.

1. Copy number

The number of additional AccS genes in 1345 was determined by digesting genomic DNA from transgenic plants with HindIII and EcoRI, then after electrophoresis and transfer to nylon membranes, hybridizing to a ^{32}P-labeled

Acc2 probe. This probe hybridizes to a 0.9 kb EcoRI-HindIII fragment from the transgene (AccS) and a 1.2 kb HindIII fragment from the endogenous *Acc2* gene which includes an additional 300 bp of intron sequence (Figure V. 3). By comparing the intensity of the transgene and endogenous bands, a determination of the number of copies of the transgene can be made in either the hemizygous primary transformant or homozygous S1 progeny. Figure V. 4 shows the hybridization pattern of genomic DNA from an untransformed tomato plant, Baxter's Early Bush Cherry (BEB) and 1345-4 digested with HindIII and EcoRI and hybridized to the *Acc2* probe. In 1345-4, the homozygous S1 progeny of 1345, the endogenous gene to transgene ratio is greater than 2 suggesting that there are at least 2 copies of AccS in the transgenic DNA. Since the *nptII* gene segregates as a single locus, it is most probable that the 2-3 copies of the T-DNA are present at a single locus. The differences in hybridization intensity between the BEB untransformed control and 1345-4 in Figure V. 4 is due to different amounts of total DNA in the separate lanes on the gel which was transferred.

2. T-DNA Structure

It is known that a single intact copy or multiple T-DNA copies can be inserted at a single locus as direct or inverted repeats around either the left or right border, and it is known that deletions of the T-DNA or insertions of genomic DNA may be present between the T-DNA copies (Jorgenson et al., 1987). To determine the organization of the T-DNAs in the 1345 genome, we hybridized several different probes to the 1345-4 genome digested with several restriction enzymes. Figure V.2 shows the relative map position of the probes in the T-DNA. Figure V.5 shows the hybridization pattern to 1345-4 DNA after digestion with the indicated restriction enzymes and hybridization to four different probes. The enzymes used are EcoRI (E), NcoI (N), HindIII (H), and XbaI (X).

Left and Right Borders: EcoRI digestions were done to determine the number of intact left and right border fragments. EcoRI sites in the T-DNA are located approximately 500 bp in from the LB and 2.1 kb in from the RB (within the AccS transgene). The fragments hybridizing to the specific border probe will be at least this size. If there is an inverted repeat at the LB and the borders are intact and flush, we would expect to see a 1 kb EcoRI fragment hybridizing only to the LB. For the RB, an intact inverted repeat would give a 4.2 kb fragment that hybridizes to the RB fragment as well as to the AccS probe. A direct LB-RB repeat would result in a fragment of 2.6 kb that hybridizes to both border probes and AccS.

In 1345-4 DNA cut with EcoRI, a 2.9 kb fragment hybridizes to the LB probe only and a 3.5 kb fragment hybridizes to the RB probe only, indicating that there is one intact left and right border. Likewise, the NcoI digestions show single hybridizing bands with both probes and the fragments are of appropriate sizes (LB fragment, greater than 1.6 kb and RB fragment greater than 2.3 kb). The HindIII and XbaI digests also show single bands of appropriate sizes which

suggests that there is a single complete T-DNA insertion. However, we know from the copy number blots (see above) that there are at least two copies of the AccS transgene. Together these results suggest that a deleted form of T-DNA, containing an intact AccS gene but missing one or both of the borders, is also present. There are no direct repeat structures since the LB and RB probes do not hybridize to the same fragment. There could, however, be inverted or indirect repeats around either border. Hybridizations with the *nptII* and AccS probes were done to further characterize the T-DNA insertions.

nptII: If the T-DNA is intact, the *nptII* probe will hybridize to a single 2.4 kb EcoRI fragment containing the entire 2Xpnos-*nptII*-ocs3' fusion (see Figure V.2). In 1345-4 genomic DNA cut with EcoRI, the expected 2.4 kb fragment is present in addition to two other hybridizing fragments of 5.2 kb and 2.9 kb. The 2.9 kb fragment also hybridizes to the left border probe which indicates that there is probably one complete internal copy and 2 incomplete copies of T-DNA with deletions occurring at the LB. Since the 2.9 kb EcoRI fragment hybridizes to both *nptII* and LB probes, this junction is likely an inverted repeat with a deletion of one of the borders that includes the EcoRI site. Hybridization of an EcoRI fragment to both the LB probe and the *nptII* probe can only occur if a deletion eliminates one of the EcoRI sites.

Since NcoI cleaves within the *nptII* coding sequence, digestion of the T-DNA with this enzyme will give two *nptII* fragments, one of 2.1 kb which spans the *nptII* 3' coding region up to the NcoI site located at the ATG of the AccS transgene, and one of 1.2 kb in length which includes the 5' *nptII* coding region and LB to the next NcoI site in either genomic DNA or adjacent T-DNA insertion. If there is a perfect inverted repeat at the LB, we expect a 3.3 kb NcoI fragment that hybridizes to both the *nptII* and LB probes. Based on the results from the EcoRI digestion, we expect the 2.1 kb fragment to be present, as well as two fragments greater than 1.2 kb. One of these will also hybridize to the LB. As predicted, the 2.1 kb fragment is present as well as a 6.6 kb fragment and a 2.9 kb fragment which also hybridizes to the LB probe. This is consistent with the presence of three T-DNA copies, one which is complete and intact, one which is an inverted repeat with a deletion extending to the LB, and a second inverted repeat with a deletion extending to the right border.

AccS: Hybridization with the AccS probe will give fragments greater than 2.3 kb and 2.1 kb for NcoI and EcoRI digests respectively. These fragments will also hybridize to the RB probe if the border is intact. The results show two hybridizing fragments for NcoI, 15 kb and 3.8 kb, and two for EcoRI, 4 kb and 3.5 kb. The 3.8 kb NcoI fragment and the 3.5 kb EcoRI fragment also hybridize to the RB. These results confirm that there are at least 2 copies of AccS, and that one copy is present on a T-DNA with a deleted RB. To demonstrate that AccS itself has not been deleted or rearranged, NcoI/XbaI double digests were done to drop out the intact 1.1 kb fragment containing the complete truncated gene (Figure V.6).

Linkage of AccS and nptII : XbaI and HindIII cleave the T-DNA once approximately 1 kb in from the right border. Digestion with either of these enzymes will generate fragments containing both the *nptII* and AccS genes. After XbaI digestion, a 9.2 kb and a 6.2 kb fragment hybridized with both probes. After HindIII digestion, a 9.2 kb and 6.4 kb fragment hybridized to both probes confirming that each copy of AccS is linked to a copy of *nptII*. The 9.2 kb and 9.3 kb fragments also hybridize to the LB. The size of the fragments are consistent with the presence of inverted T-DNA repeats at the LB and RB.

The structure for 1345-4 T-DNA insertion is shown in Figure V. 7. It consists of three T-DNAs assembled in inverted repeats at the LB and RB. At the LB-LB and RB-RB junction, one border is deleted such that there is only one complete border at each junction. The LB and RB at either end of the T-DNA structure are also deleted. The endpoint rightward is internal to the *nptII* gene and deletes the internal NcoI site, and the endpoint leftward lies between the *Acc2* gene and the right border.

To confirm that the T-DNA remains intact in subsequent generations, we examined the hybridization pattern of 12 individual progeny plants from the S2 generation and found that the pattern remained the same with all four probes. No gross deletions or rearrangements were observed. An example of one of these digests is shown in Figure V. 8.

3. Acc2 Expression Levels

To determine the level of the *Acc2* gene expression in the ripening delayed 1345-4 fruit, we measured the steady state RNA levels corresponding to this gene. Total RNA was extracted from fruit samples at different stages of development and then the RNAse protection technique was used to quantitate the expression of *Acc2* and the AccS transgene. The data of Figure V. 9 illustrate the level of *Acc2* RNA which occurs in normal control fruit (lane 5). The *Acc2* gene transcript is significantly reduced in the 1345-4 fruit tissue (lanes 1-4). The RNAse protection assay also detects the expression of the truncated *Acc2* transgene which occurs as a smaller band present in the 1345-4 tissue, but is absent from the control fruit.

In addition to *Acc2* RNA measurements, we have performed extensive measurements of ethylene production in the 1345-4 transgenic fruit and we find that the levels are reduced by at least 50 fold compared to wild-type fruit.

4. NptII Expression Levels

For the detection and quantitation of neomycin phosphotransferase II protein (NptII) in tomato fruit extracts, we used the NptII Elisa Kit from 5'-3' Inc. This NptII Elisa is a sandwich immunoassay. Rabbit polyclonal antibody specific to the NptII protein encoded by Tn5 is coated onto polystyrene wells. Then

transgenic fruit extracts, non-transgenic fruit extracts, and purified NptII protein standards are added to individual wells. Unbound proteins are removed by aspiration and washing of the wells, and then a biotinylated secondary antibody is bound to the immobilized primary antibody/NptII complex. The bound biotinylated antibody is then quantitated colorimetrically by incubation with streptavidin-conjugated alkaline phosphatase and substrate. The resultant color development is proportional to the concentration of NptII protein in each microwell.

Samples from several different transgenic lines were evaluated using the NptII Elisa kit: 1345-4 fruit which was harvested at breaker and then exposed to ethylene for 7-10 days to achieve full ripeness (sample #1), 1345-4 fruit which was harvested at breaker and then stored for 7 days (sample #2), *cel*1-4 fruit which expresses a CaMV35S/*nptII* gene, and commercially available Flavr Savr fruit which expresses a Mas/*nptII* selectable marker. Tissue from multiple fruit were pooled and then 1 gram of pooled tissue was extracted with 3ml extraction buffer and this sample material was loaded directly onto the microtitre wells. The results of analysis are shown in Table V. 2. These data indicate that there are relatively low levels of NptII protein present in the 1345-4 line, and furthermore these levels do not dramatically change with post-harvest storage.

Table V. 2 Quantities of NptII Protein in Line 1345-4 Fruit and Other Transgenic Fruit

Transformant	Protein conc. ug/ml	pg NptII/ ug total protein	% NptII of total protein	pg NptII/ ml total extract	ng NptII per gm fresh wt.
1345-4#1	296	2.6	0.00026	787	2.36
1345-4#1	219	1.5	0.00015	325	0.98
1345-4#1	276	2.4	0.00024	667	2.0
1345-4#2	128	1.88	0.00019	240	0.72
cel1-4	528	117	0.0117	6198	18.6
cel1-4	380	138	0.0138	5282	15.8
cel1-4	366	63.9	0.0064	2339	7.01
Flavr Savr	560	4.3	0.00043	2412	7.24

D. Field Tests of Delayed-Ripening Tomato

DNAP has conducted ten field trials of Delayed-Ripening Tomatoes (both fresh market and cherry cultivars) in the principal tomato producing states in the U.S. since 1992 (Table V.3). These trials have been conducted to generate plant material for molecular expression analysis, fruit quality analysis, and agronomic performance analysis of delayed-ripening tomato lines including line 1345-4. Line 1345-4 has been field tested in five trial sites in Florida, California and

Virginia from 1992 through spring 1994 and is being grown in three additional trial sites in California during the summer, 1994. Line 1345-4 was also planted in a winter nursery (1994) in Hawaii to produce quantities of hybrid seed. Appendix 2 contains reports of each environmental release completed or harvested to date in which line 1345-4 has been evaluated.

Table V. 3 DNAP Field Trials of Delayed-Ripening Tomatoes

Permit #	Site	Date Issued	Trial Status
92-035-05	Brentwood, CA	05/04/92	Completed
92-301-01	Brentwood, CA	02/12/93	Completed
93-056-01	Brentwood, CA	04/30/93	Completed
93-252-07	Naples, FL	09/30/93	Completed
93-302-01	Ruskin, FL	11/16/93	Completed
93-302-03	Molokai, HI	11/16/93	Completed
93-351-02	Indio, CA	12/28/93	Completed
94-060-04	Painter, VA	03/15/94	Harvested
94-126-01	Brentwood, CA	05/20/94	In Progress
94-158-01	Brentwood, CA	07/07/94	In Progress
94-172-01	Firebaugh, CA	07/07/94	In Progress

E. Horticultural Characteristics

Company researchers and tomato breeders made visual observations of plant stature, vigor, flower set, fruit set and firmness during field trials of Delayed-Ripening Tomato lines. Except for the expected delayed-ripening phenotype, no differences in these plant horticultural characteristics were detected between line 1345-4 and the control line 91103-114 (see Appendix 2, field trial reports). Further, company tomato breeders observed no obvious differences in flower morphology or pollen production of line 1345-4 during hybrid test crosses with line 1345-4 and non-transgenic lines.

Tomato growers make a concerted effort to eliminate volunteer tomato plants from production fields since tomato plants can attract and harbor the specific pests which damage the desired crop. Applications of pre-emergent and foliar herbicides or cultivation and hand-rouging, provide effective control of volunteer tomatoes in production fields. To date, we have completed field trials of line 1345-4 in five different geographic locations. In each of the trials, we have used field cleanup procedures comparable to those used in commercial tomato production. This involved spraying the vines with herbicide, followed by burning and disking the vines and fruit into the soil, or allowing the vines to

desiccate, followed by disking into the soil for natural decay. We have observed no volunteer tomato plants in any of the test sites following these trials.

We have not directly tested the ability of line 1345-4 to overwinter and germinate under field conditions. However, we have observed plots ranging in size from 0.1 to 0.3 acres during and after the 1992/93 and 1993/94 winter months at the Brentwood, CA site (USDA# 92-35-05, 92-301-01) and found no tomato volunteers the following spring. Additionally, we have measured germination rates (percent germination) in line 1345-4 and parental line 91103-114, and find them to be comparable and within a range normally seen for commercially available cultivars (Table V.4). Thus, we have no reason to believe that line 1345-4 has any ability to survive in the environment which differs from the parental line.

Table V. 4 Germination Rates for Line 1345-4 and Parental Line*

Line	Seed lot 1	Seed lot 2	Seed lot 3	Seed lot 4	Seed lot 5
1345-4	93% (119/128)	83% (66/80)	94% (75/80)	90% (115/128)	96% (2640/2750)
91103-114	84% (107/128)	89% (71/80)	93% (74/80)	89% (114/128)	88% (112/128)

* Percent germination for 5 independent seed lots; lots 1, 2, and 3 were S3 generation seed, lots 4 and 5 were S4 generation seed. Values in parentheses are number plants germinated/ number seeded. Measurements made 2 weeks after seeding into potting trays in greenhouse. Lots 4 and 5 were transplanted into the field and survival rates (stand counts) were between 97 - 100%.

F. Yield Characteristics

Yield comparisons were made to determine the feasibility of reduced harvest frequency for the delayed ripening tomato lines. In Indio, CA (USDA# 93-351-02), we performed a single harvest on delayed ripening and control tomato lines when approximately 10-20% of the fruit on the vines of control lines were at the breaker stage. In Ruskin, FL (USDA# 93-302-03), we performed a single harvest on all lines at the 50% breaker stage. Fruit was collected and sorted into color groups: mature green/breaker, pink/turning, light red, and red. Fruit in the mature green/breaker to light red categories was considered commercially usable.

In the Indio, CA trial, the delayed-ripening lines (including 1345-4) exhibited a lower total yield than the non-transgenic parental line. However, the yields of marketable fruit from delayed-ripening lines were comparable to the non-transgenic control lines, and the percentage of marketable fruit from delayed-

ripening lines was significantly higher than control lines. The delayed ripening lines had fewer culls due to overmature fruit and the damage associated with over maturity. Two of three delayed-ripening hybrid lines exhibited lower yield of marketable fruit as compared to the 91103-114 control, but were not significantly different than their delayed-ripening parent.

In the Ruskin, FL trial there was no significant difference between total yield between delayed-ripening inbred lines including line 1345-4, F1 hybrid 93397 and any of the non-transgenic control lines. When the fraction of marketable fruit arising from the single point harvest was measured, there was a significant difference (P=0.05) between the delayed-ripening lines (inbreds including 1345-4 and hybrid). There were higher quantities of marketable fruit harvested from the delayed-ripening lines compared to the control lines; approximately 95% of fruit was considered marketable from these lines compared to 54 to 73% marketable fruit from the control lines.

We believe the difference in total yield observed in the Indio, CA trial is likely related to the trial size and not related to either the transformation event or ethylene suppression. The standard practice in tomato breeding involves evaluating progenies from the original crosses over several years before selecting commercial lines. This practice is likely to remove any yield reduction (if any actually exists) associated with line 1345-4. We believe these data support the hypothesis that delayed ripening tomatoes can be harvested less frequently than current commercial practices without sacrificing marketable fruit yield. Additional field tests to evaluate harvest yield (via single point and cumulative harvest strategies) and pack-out through commercial handling and distribution are planned in 1994.

G. Disease and Pest Characteristics

Company agronomists and plant pathologists monitored each of the field trials for disease and pest susceptibility of line 1345-4 and control lines. Plots were evaluated for overall plant vigor, stature, and evidence of fruit or plant tissue damage due to bacterial speck and leaf spot, Fusarium crown rot, target spot, early and late fungal blight, and insects such as whiteflies, aphids and thrips which vector plant viruses, tomato pinworms, and vegetable leafminers. Detailed insect counts or disease incidence data were not collected on experimental and control plants from these field trials. However, company researchers made observations of the appearance of disease symptoms such as stunted growth, wilting, crown rot, spotted leaves or fruit, leaf necrosis, or yellowing, and assigned ratings of the disease status of the transgenic and non-transgenic lines (see Appendix 2, field trial reports). No significant differences in disease or pest susceptibilities between line 1345-4 and control line 91103-114 were observed in this manner.

Field trial sites were managed in the same manner as commercial tomato production with respect to use of crop protection chemicals and pest management techniques. Due to the proximity of the test plots to commercial production fields, the same disease and insect pressures in production fields were generally found in the test plots. Crop specialists routinely scout the fields during production to monitor the presence of disease symptoms and insects, and recommend specific control measures to mitigate pest damage. Company researchers observed no differences between 1345-4 and control lines with respect to pesticide treatments used.

Line 1345-4 and parental line 91103-114 were evaluated for resistance to *Fusarium oxysporum f.sp. radicis lycopersici* (Crown rot), *Fusarium oxysporum f.sp. lycopersici* Race 2 and Race 3, and *Verticillium dalhae* Race 1. Seven to 10 day old tomato seedlings were inoculated with standardized spore suspensions and grown in the greenhouse, then removed and assayed for disease. Measurements of shoot height and disease severity were made. The data were analyzed as a completely randomized design using ANOVA protocols of Statistical Analysis Systems. Means comparisons were made using the Duncan multiple range test. The results are shown in Table V.5 below.

Table V. 5 Fungal Disease Screen for Line 1345-4 and Parental Line*

Line	Crown rot		Fusarium wilt Race 2		Fusarium wilt Race 3		Verticillium wilt Race 1	
	height (cm)	disease rating	height (cm)	disease rating	height (cm)	disease rating	height (cm)	disease rating
1345-4	4.1a	4.5b	3.0a	1.8b	3.3a	1.8a	4.6a	1.1a
91103-114	4.2a	4.4b	2.5b	2.4b	3.1a	2.5b	4.7a	1.9b

* Disease severity ratings were made at the crown area for infection incited by the Crown rot pathogen or, for the other pathogens, the root and hypocotyl was cut longitudinally and rated as follows: 1=healthy plant, 2=1 to 25%, 3=25 to 75%, 4=75 to 100% of the area exhibiting necrosis, 5=dead plant. Means compared using Duncan multiple range test, where values followed by similar letter are not significantly different (P=0.05). Mean values are based on 16 observations per treatment. Height measurements (cm) without pathogen inoculation was not significantly different (P=0.05) between line 1345-4 (3.3 cm) and 91103-114 (3.4 cm).

Both line 1345-4 and 91103-114 exhibited susceptibility to Crown rot; there was no significant difference in disease severity between these lines. Lines 1345-4 and 91103-114 exhibited only minor symptoms of Fusarium wilt Race 2 and 3, with line 1345-4 exhibiting significantly less infection against Race 3 than did parental line 91103-114. We noted a small, but statistically significant difference in plant height between line 1345-4 and the parental line inoculated with Fusarium wilt Race 2; experiments will be repeated to confirm this result. Resistance to

Fusarium wilt Race 2 is tightly linked to resistance to Fusarium wilt Race 1. Both lines showed resistance to infection from Verticillium wilt Race 1.

H. Compositional Analysis

There is considerable variability in the composition of tomato fruit, influenced predominately by genetics, environmental growing conditions and post-harvest handling practices. The composition of tomato fruit has been extensively described (see for example, Davies and Hobson, 1981 and references cited therein). Fresh fruit quality is determined mainly by appearance, firmness and flavor, whereas the quality of processing tomatoes is largely determined by total solids content, color, pH and firmness (Stevens, 1986). According to Stevens, the solids content of tomato fruits has received more attention than any other component. Solids contribute significantly to fresh fruit flavor (especially higher sugar levels and suitable sugar:acid ratios) and to the flavor and yield of processed tomato products. The most common means of estimating solids content is by refractive index. The value obtained is often called total soluble solids reported as degrees brix and is highly correlated to total solids. Total solids in ripe fruit of tomato cultivars range from 4.5 to 6.5%.

Reducing sugars (primarily, fructose and glucose) make up about 50% of the dry matter of tomatoes. Based on figures in Stevens (1986), the reducing sugars in ripe fruit of commercial tomato cultivars range from 2 to 3.7%. Cantwell (1990) reports percent reducing sugars in ripe fruit of 3 common cultivars in the range of 1.5 - 2.3%.

Organic acids contribute to the sourness of tomato fruit and are also a major factor in flavor intensity. Acid concentration varies significantly among genotypes, and its impact on flavor make it the most important component in flavor variation in fresh fruits. Additionally, acid concentration is a factor in the safety and quality of thermally processed tomato products. Stevens (1986) reports that a study of 250 tomato accessions demonstrated a wide variation in titratable acidity ranging from 0.40 to 0.91% (expressed as a percentage citric acid).

We compared the soluble solids, percent reducing sugars and titratable acidity of fruit from line 1345-4, 91103-114 parental line, a transgenic delayed-ripening hybrid 93397 and a non-transgenic control hybrid 92030 grown in a winter 1994 trial in Indio, CA (USDA# 93-351-02) and an early spring 1994 trial in Ruskin, FL (USDA# 93-302-01). Fruit were hand harvested from the field at comparable stages of maturity, shipped to DNAP laboratories, ethylene treated to complete ripening and analyzed by staff scientists. Purees were made from red ripe fruit and all measurements made on the same sample. Soluble solids were measured by refractometer, titratable acidity by titration with 0.1N NaOH and percent

reducing sugars by colorimetric assay (Somogyi method). The results of analysis are shown in Table V. 6.

The titratable acidity of Delayed-Ripening Tomatoes (line 1345-4 and hybrids) was slightly higher than controls in both the Indio, CA and Ruskin, FL trials. These results confirm the findings of in-house sensory panelists which consistently rate the flavor of Delayed-Ripening Tomatoes as slightly more acidic than the parental line. The sugars and solids of Delayed-Ripening Tomatoes (line 1345-4 and hybrids) were comparable to controls in both field tests. Generally, the levels of solids, sugars and acids were well within the range typically seen in other ripe tomato varieties. We conclude there are no significant changes in the composition of line 1345-4 fruit compared to its parental variety.

Table V. 6 Compositional Analysis of Delayed-Ripening Tomato and Control Fruit*

Location	Line	Brix, °	Reducing Sugars, %	Titratable Acidity, %
Indio, CA	1345-4	4.5	2.2	0.6
	93397 transgenic hybrid	5.0	2.9	0.75
	91103-114 control	4.2	2.1	0.5
	92030 control hybrid	5.2	2.8	0.6
Ruskin, FL	1345-4	4.7	2.0	0.5
	93397 transgenic hybrid	5.6	2.6	0.4
	91103-114 control	5.3	2.5	0.4
	92030 control hybrid	5.6	3.0	0.3
Typical Range in Tomato		4.5 - 6.5	1.5 - 3.7	0.4 - 0.9

* Each value is the mean of 3 replicate assays on duplicate samples. Each sample consisted of a composite of 5-10 fruit.

We are collecting and evaluating additional fruit quality data on line 1345-4, control line 91103-114, and selected hybrids, including the vitamin A and vitamin C content of fruit grown in representative field trials. These data will be reviewed in consultation with the FDA in support of the food safety of Delayed-Ripening Tomato.

I. Tomatine Content

Tomatine is a steroidal glycoalkaloid which occurs naturally in certain Solanaceous plants (Roddick, 1974). It is found in the leaves, stems and immature fruit of cultivated tomato plants and, in unusually high doses, can be

toxic (Nishie et al., 1975). Tomatine levels are highest in young developing fruits and plant structures, but levels rapidly decline to barely detectable levels as fruit ripens. Tomatine levels of 87, 45 and 36 mg/100 gm of fresh weight have been recorded in green, yellow and red ripe fruit of cultivated varieties respectively, but are almost completely absent from ripe fruit held on the plant for an additional 2-3 days (Roddick, 1974). Eltayeb and Roddick (1984) report levels of tomatine in red ripe fruit in the range of 0.3-0.6 mg/100 gm fresh weight for normal ripening cultivars and 0.9-1.2 mg/100 gm in the non-ripening *nor* and *rin* mutants. Friedman et al. (1994) reports levels of tomatine ranging from 4-17 mg/100 gm fresh weight in unripe green tomatoes and from 0.03-0.6 mg/100 gm in red ripe tomatoes.

Studies in mice reveal the oral LD_{50} value for tomatine to be 500 mg per kg body weight; thus in ripe tomato, tomatine normally doe not pose a food safety hazard (Grierson and Kader, 1986).

The tomatine content of green and ripe fruit of line 1345-4, parental line 91103-114 and a commercial fresh market variety, Sun J, grown in Indio, CA was measured by HPLC (Friedman et al., 1994). The results of analysis are shown in Table V. 7. As expected, the tomatine content in all varieties decreased with fruit ripening. The tomatine levels in line 1345-4 were comparable to the parental line and the other commercial control line grown under the same conditions. Additionally, all tomatine levels were within the ranges typically seen in tomato fruits at comparable stages of maturity.

Table V. 7 Tomatine Content of Fresh Tomatoes (mg/100 g fresh weight)*

Fruit Stage	1345-4	91130-114 Control	Sun J Control
Immature	1.15, 1.15, 1.37, 1.13	3.37, 3.59	4.04, 4.04
Mature Green	1.56, 1.50	0.68, 0.68	2.57, 2.48
Breaker	0.60, 0.63, 0.62, 0.63	0.36, 0.33	0.67, 0.82, 0.73, 0.70
Red Ripe	0.11, 0.11	0.07, 0.07	0.12, 0.12

* Each value is the average of 3 HPLC injections

Figure V.1. Schematic of the T-DNA region of pWTT2144/AccS

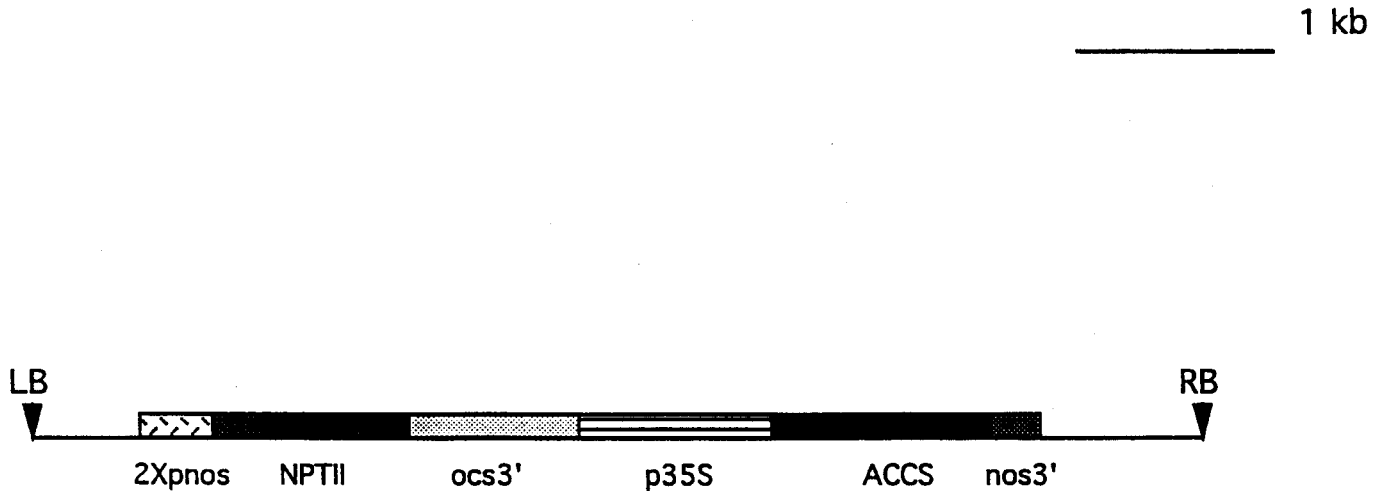


Figure V.2. Restriction map of the T-DNA region of pWTT2144/AccS showing the location of the hybridization probes used in the genomic mapping

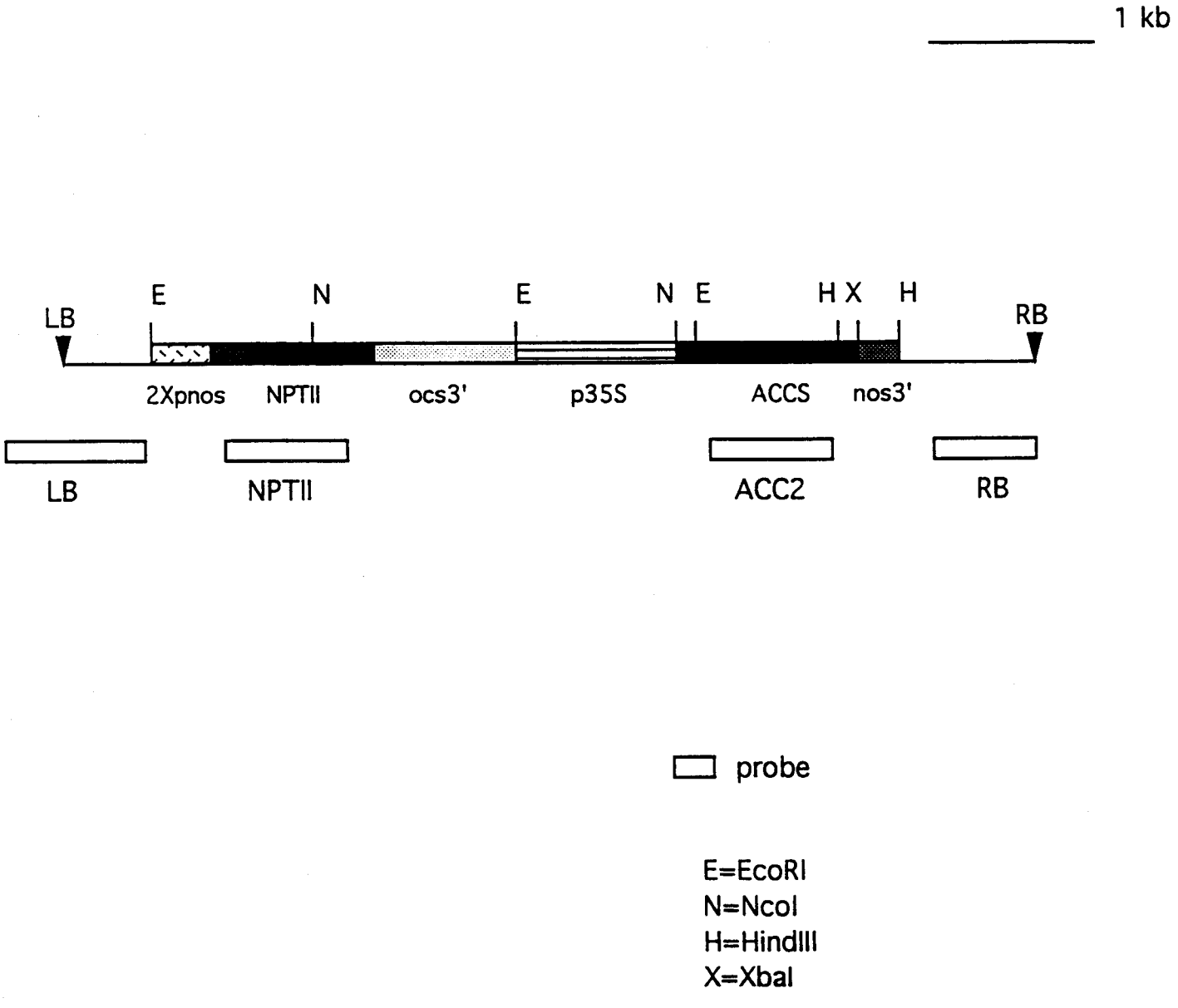


Figure V.3. Schematic showing the *Acc2* genomic region and *Acc2* cDNA and the fragments used to determine transgene structure in the 1345-4 transformant

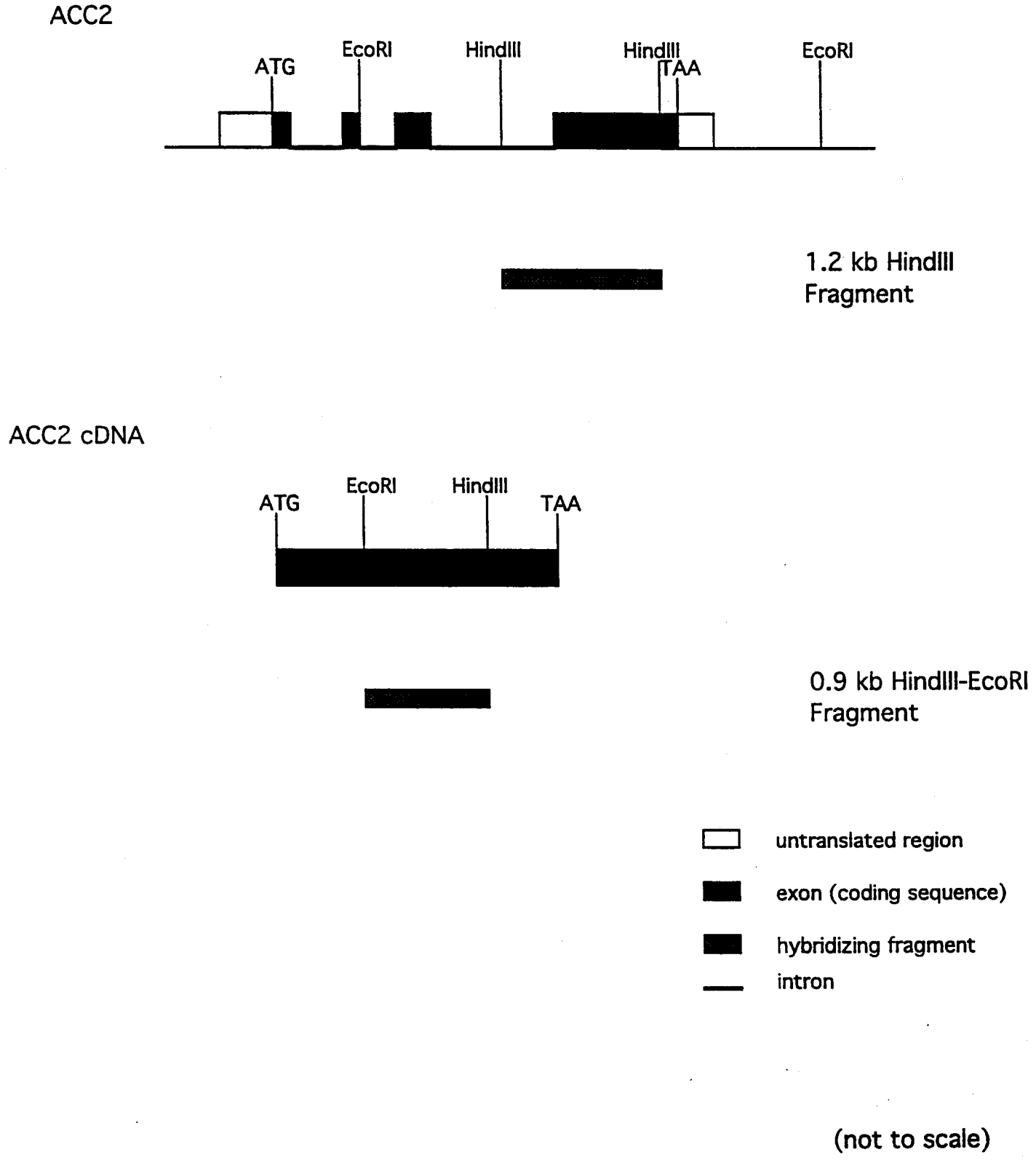


Figure V.4. Genomic Southern hybridization with *Acc2* probe showing copy number. 2 ug genomic DNA from untransformed Baxter's Early Bush Cherry (BEB) and 10 ug 1345-4 DNA was digested with EcoRI and HindIII and separated on an agarose gel. The digested DNA was transferred to a nylon filter and hybridized to a ^{32}P-labeled *Acc2* probe.

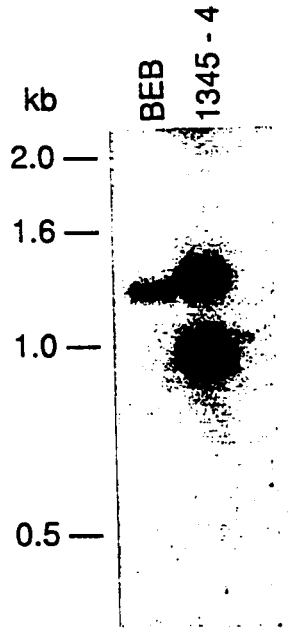


Figure V.5. Genomic Southern hybridizations with LB (left border), *nptII*, *Acc2* and RB (right border) probes. Digestions were done as indicated above the lane. E=EcoRI, N=NcoI, H=HindIII, X=XbaI.

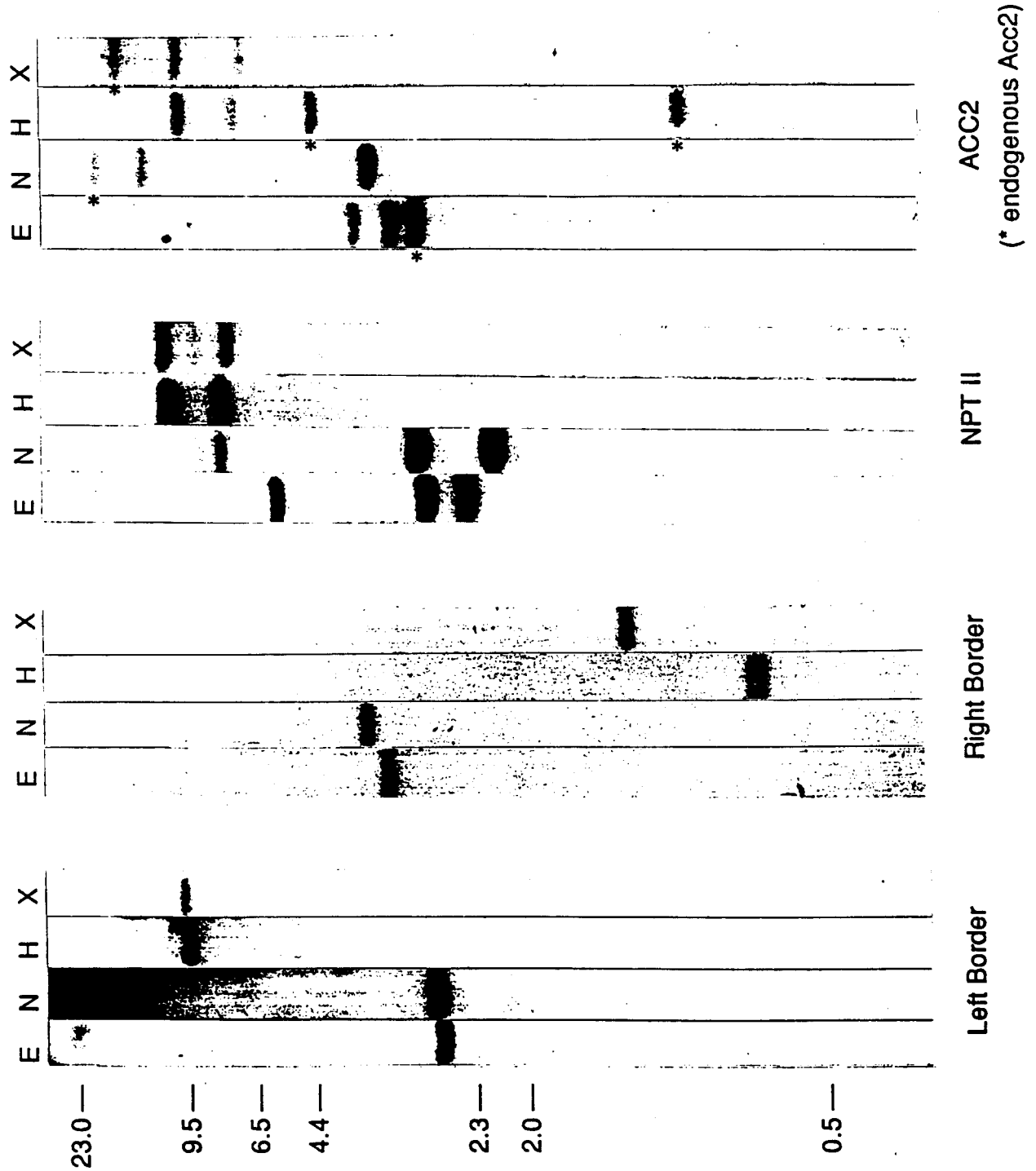
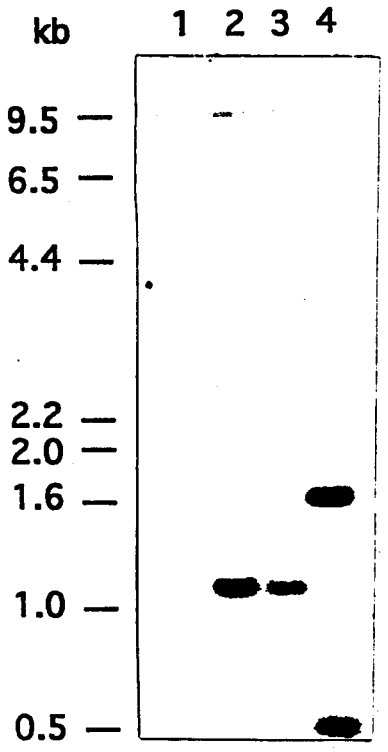


Figure V.6. Genomic Southern blot. 10 ug of genomic DNA was digested with NcoI and XbaII, separated on 1% agarose gel and transferred to nylon membrane. The membrane was hybridized to a ^{32}P-labeled *Acc2* SacI-HindIII riboprobe. B324-6 is a cherry tomato line that has been transformed with pWTT2144/AccS.



1. untransformed control
2. 1345-4
3. B324-6
4. size marker

Figure V.7. Structure of 1345-4 T-DNA insertion.

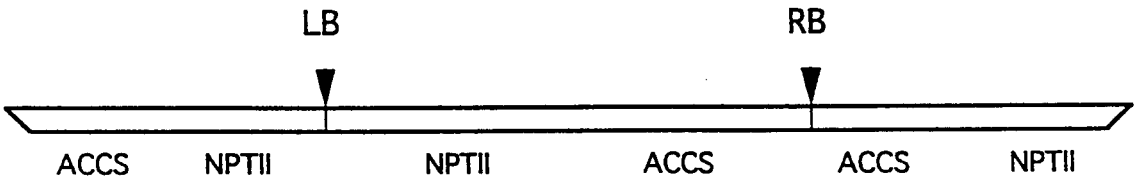


Figure V.8. Genomic Southern hybridization with *nptII* probe. Genomic DNA from 1345-4 (lane 1) and 12 independent S2 progeny (lanes 2-13) were digested with EcoRI and hybridized to ^{32}P-labeled *nptII* probe.

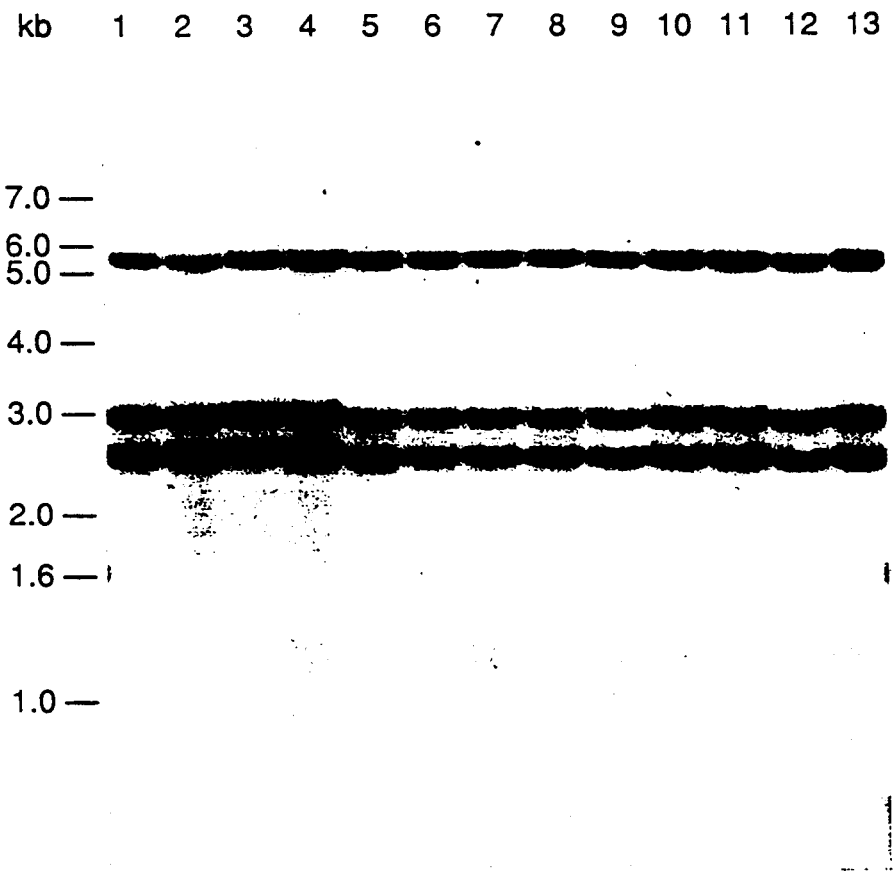
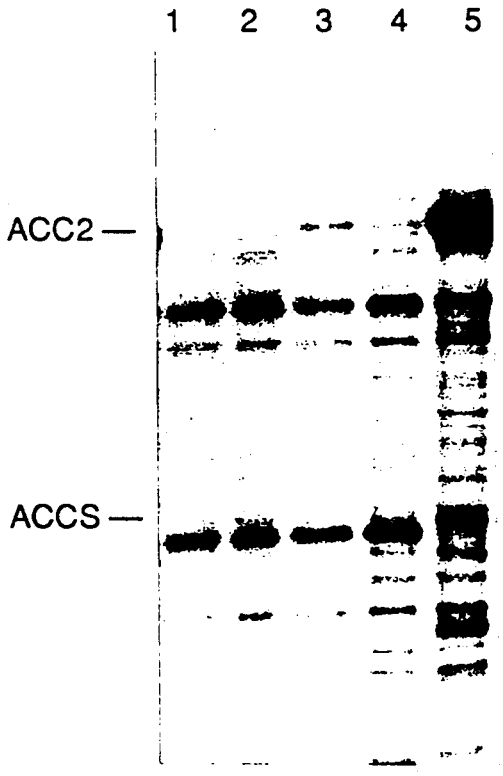


Figure V.9. RNAse Protection analysis on RNA from 1345-4 fruit and untransformed control fruit. 10 ug of total fruit RNA was hybridized to a labeled probe fragment which corresponds to the 3' end of the *Acc2* gene and transgene. Lane 1, 1345-4 fruit harvested at breaker; lane 2, 1345-4 fruit at 7 days post harvest; lane 3, 1345-4 fruit harvested 14 days after breaker; lane 4, 1345-4 fruit harvested at 14 days post breaker and stored for additional 7 days; lane 5, untransformed control fruit harvested at breaker.



VI. Environmental Consequences of Introducing Delayed-Ripening Tomato Lines

A. Ripening-Inhibited Tomatoes

Delayed-Ripening Tomato line 1345-4 contains a gene which inhibits expression of ACC synthase, an enzyme which catalyzes the conversion of s-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid, the immediate precursor to ethylene. By suppressing the expression of ACC synthase, fruit of line 1345-4 produce significantly less ethylene and do not fully ripen until an external source of ethylene is applied, thereby improving the ability to hold fruit on the vine for an extended period of time prior to harvest. Fruit ripen normally when treated with ethylene for 7-10 days at 21°C. Additionally, fruit of line 1345-4 may be held off the vine (prior to ethylene treatment) for an extended time with no appreciable loss of sensory properties. The use of ethylene regulation in tomato can enhance flavor by allowing fruit to remain on the vine longer without the threat of spoilage, thereby delivering better tasting tomatoes to consumers.

SAM plays at least four important roles in plants. First, it is the predominant methyl donor in all methyl transfer reactions. The second role is as the donor of the a-aminopropionyl group in sperminine and spermine synthesis. The third is in the regulation of the synthesis of methionine and other amino acids of the aspartate family. Fourth, it functions as an intermediate in the conversion of methionine to ACC and ethylene. The diversion of SAM into ACC, the ethylene precursor, is minor compared to the other cellular reactions involving SAM, and as such it does not deplete steady state levels of SAM (Abeles et al., 1992). We have not measured the steady state levels of SAM in delayed-ripening tomato lines, however it is likely that these would not be significantly different from levels in control lines.

The AccS gene (a truncated *Acc2* gene prepared from tomato) responsible for suppression of ACC synthase does not encode a functional protein, producing only a truncated messenger RNA. The AccS gene affects only the biosynthesis of ethylene in the fruit and has no significant effect on the agronomic or compositional characteristics of the plant, except for delayed ripening. The AccS gene does not alter seed germination or confer any other apparent selective advantage which would enhance survival of the Delayed-Ripening Tomato in the field or result in any significant adverse environmental consequence.

The other introduced gene sequences (*nptII* and specific regulatory sequences) in line 1345-4 do not confer any selective advantage which would enhance survival in the field or result in any significant adverse environmental consequences. The human and environmental safety of the use of the *nptII* marker gene in tomato has been described previously and evaluated as safe (USDA, 1992; FDA, 1994).

Fruit of line 1345-4 contain approximately 5-fold less neomycin phosphotransferase II than the amounts evaluated as safe for human consumption in the Flavr Savr Tomato.

Because the genetic modification used to produce the Delayed-Ripening Tomato involves an ACC synthase gene from *L. esculentum* which is re-inserted into the same species, it is generally considered a class of modification that is inherently safe (Keeler, 1988). This becomes an even stronger argument when the nature and function of the ACC synthase gene are considered, since the gene inhibits ethylene production, an effect that has no selective advantage for persistence in the environment. Greenhouse and field observations to date show that there are no deleterious effects on humans involved in cultivation, post-harvest production, or consumption of Delayed-Ripening Tomatoes.

B. Effects on Agricultural and Cultivation Practices

Delayed-Ripening Tomatoes can improve agricultural practices by: 1) reducing the number of harvests required in a production field; 2) reducing the amount of labor and other inputs in fruit harvest; 3) reducing the losses in shipping and distribution due to uneven ripening, over-ripening and immature fruit; 4) reducing the variability in market supply by extending the holding capacity of the fruit; and 5) expanding the geographic range for delivery of better-tasting tomatoes which could open up new markets for U.S. tomato producers. The principle effect of Delayed-Ripening Tomatoes is expected to be availability of better tasting fruit for human consumption.

Delayed-Ripening Tomatoes will be ripened with ethylene prior to retail distribution. By inhibiting ACC synthase, we have reversibly arrested the fruit ripening process and extended the holding capacity of the fruit both on and off the vine. Thus, fruit may be harvested later in the ripening process than current practice for fresh market tomatoes, or harvested and held unripened. We believe this trait allows flexibility in the timing and frequency of harvest, with significant potential for labor and production cost savings. We are in the process of assessing the specific impacts on pre- and post-harvest handling practices via tests which measure the yield of marketable fruit from field harvest through commercial packing/repacking and distribution.

Standard cultivation practices for tomatoes were used in each field test of Delayed-Ripening Tomato lines. Company researchers and agronomists observed no significant differences in horticultural traits or disease and pest characteristics of line 1345-4 compared to control line 91103-114 (Section V.). Based on observations of six field trials of various Delayed-Ripening Tomato lines, no adverse effects on tomato cultivation practices are predicted.

C. Human and Animal Exposure

There are two major markets for tomatoes: fresh market and processed tomato products. U.S. tomato production in 1992 was 1,806,400 tons for fresh market and 8,776,500 tons for processing (USDA, 1993). Delayed-Ripening Tomato line 1345-4 is a fresh market variety intended to be consumed fresh. Delayed-Ripening Tomatoes could eventually be used for a significant portion of the fresh market product in the U.S. Fresh market tomatoes are most commonly processed commercially by the following procedure: 1) harvesting; 2) washing/grading; 3) packing; 4) ripening; 5) shipping; 6) repacking; and 7) distributing to wholesaler/retailers. We do not anticipate that Delayed-Ripening Tomatoes will be used differently than current fresh market varieties.

Tomatoes are also processed into a variety of forms including canned whole and cut, paste, sauces, ketchup and specialty items. Thermal processing is commonly used to preserve tomatoes and tomato products. While the procedures used for processing tomatoes are different from fresh market tomatoes, we know of no reason why Delayed-Ripening Tomatoes would adversely affect processed tomato products.

The exposure of animals to Delayed-Ripening Tomatoes is expected to be no different than current exposure to tomatoes. We do not anticipate any increase in the use of tomato as animal feed as a result of commercialization of the Delayed-Ripening Tomato.

D. Effects on Non-Target Organisms

Line 1345-4 has been field tested at sites in the major growing regions in the United States and has shown no toxicity towards beneficial insects, birds or other species that frequent tomato fields (see field trial reports, Appendix 2). This result is to be expected since line 1345-4 contains a gene from tomato (AccS transgene) which regulates ethylene biosynthesis, but does not encode a protein. Line 1345-4 also contains a gene which codes for the NptII protein. The *nptII* gene and its gene product has been extensively evaluated in tomato and other feeding studies and has not been found to be toxic (USDA, 1992; FDA; 1994).

E. Weediness Potential

A general consensus upon 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, pollinated by

unspecialized visitors or wind pollinated; 7) high seed output in favorable environments and some seed production in a wide range of environments; 8) adaptation for short- and long-distance dispersal; 9) if perennial, vegetative production or regeneration from fragments and brittleness; and 10) ability to compete by special means (rosette formation and presence of allelochemicals).

In general, tomato possesses few of the characteristics of plants that are notably successful weeds (e.g., it does not produce abundant, long-lived seed; it does not propagate vegetatively; it does not compete well with other plant species in the environment.) Tomato is considered to be a highly domesticated, well-characterized crop plant that does not persist in undisturbed environments without significant human intervention (USDA, 1992).

The parental tomato line 91103-114 which has been genetically modified is not considered to be a weed pest and the introduction of the delayed-ripening trait into this cultivar has not imparted any new "weedy" characteristics. No changes in seed germination, seed production, disease or insect susceptibility, or plant growth characteristics were noted in any of the greenhouse or field trials of line 1345-4 compared to the control line 91103-114 (Section V.).

F. Potential for Outcrossing

1. Wild species

As discussed in section I., hybridization between *L. esculentum* and wild *Lycopersicon* species can be achieved, but only with significant human intervention. Tomato has no wild relatives in the United States with which it can naturally cross. Accordingly, there is little probability of an unaided cross between line 1345-4 and wild *Lycopersicon* species.

2. Domesticated species

As discussed in section I., cultivated tomato is self-fertile and almost exclusively self-pollinating, due in part to the presence of an inserted stigma developed through more than 50 years of breeding. Cultivated tomato is not wind-pollinated and insect pollination is limited. Accordingly, there is little probability of an unaided cross between line 1345-4 and other cultivated tomato species. Cultivated tomato varieties are routinely improved through hybridization with other domesticated tomato cultivars, and line 1345-4 will be used in a breeding program directed toward developing such hybrids.

3. Other organisms

Movement of transgenes from the engineered crop plant to microorganisms has been suggested as a risk if such crops are released into the environment. As

stated in the USDA's Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status (FR 57, No. 202, pp. 47608-47616, October 19, 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 suggest 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 occur, transfer of the AccS or *nptII* genes to microbes would not pose any plant pest risk. Neither the AccS or *nptII* gene are derived from a plant pest organism and there is no evidence to suggest that the transfer of these genes to a microorganism would result in the microbe becoming a plant pest risk.

VI. Statement of Unfavorable Grounds

No negative aspects have been determined for the Delayed-Ripening Tomato.

VII. References

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VIII. Appendices

Appendix 1. – T-DNA Sequence
Appendix 2. – Field Trial Reports

Permit No. 92-035-05, Brentwood, CA
Permit No. 92-301-01, Brentwood, CA
Permit No. 93-252-07, Naples, FL
Permit No. 93-302-01, Ruskin, FL
Permit No. 93-302-03, Molokai, HI
Permit No. 93-351-02, Indio, CA
Permit No. 94-060-04, Painter, VA

Appendix 1. – T-DNA Sequence

WTT2144/ACCS

left border->

1 GGCAGGATATATTCAATTGTAAATGGCTTCATGTCCGGGAAATCTACATG

51 GATCAGCAATGAGTATGATGGTCAATATGGAGAAAAAGAAAGAGTAATTA

101 CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAAA

151 ATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATA

201 GGCGAAAGCAATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGACG

251 TGTCTACATTCACGTCCAAATGGGGGCTTAGATGAGAAACTTCACGATCG

301 ATGCCTTGATTTCGCCATTCCCAGATACCCATTTCATCTTCAGATTGGTC

351 TGAGATTATGCGAAAATATACACTCATATACATAAATACTGACAGTTTGA

401 GCTACCAATTCAGTGTAGCCCATTACCTCACATAATTCACTCAAATGCTA

451 GGCAGTCTGTCAACTCGGCGTCAATTTGTCGGCCACTATACGATAGTTGC

501 GCAAATTTTCAAAGTCCTGGCCTAACATCACACCTCTGTCGGCGGCGGGG

<-left border/pUC8->

551 CCCATTTGTGATAAATCCACCCATCGGATCTGAATTCTCACTCATTAGGC

601 ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATTG

<-pUC8/pnos->

651 TGAGCGGATAACAATTTCACACAGGAAACAGGATCATGAGCGGAGAATTA

701 AGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTAC

751 GTTTGGAACTGACAGAACCGCAACGATTGAAGGAGCCACTCAGCCGCGGG

801 TTTCTGGAGTTTAATGAGCTAAGCACATACGTCAGAAACCATTATTGCGC

851 GTTCAAAAGTCGCCTAAGGTCACTATCAGCTAGCAAATATTTCTTGTCAA

901 AAATGCTCCACTGACGTTCCATAAATTCCCCTCGGTATCCAATTAGAGTC

pnos->

951 TCATATTCACTCTCAATCCAAATAATCTGGATCATGAGCGGAGAATTAAG

1001 GGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGT

1051 TTGGAACTGACAGAACCGCAACGATTGAAGGAGCCACTCAGCCGCGGGTT

1101 TCTGGAGTTTAATGAGCTAAGCACATACGTCAGAAACCATTATTGCGCGT

1151 TCAAAAGTCGCCTAAGGTCACTATCAGCTAGCAAATATTTCTTGTCAAAA

1201 ATGCTCCACTGACGTTCCATAAATTCCCCTCGGTATCCAATTAGAGTCTC

<-pnos/NPTII->

1251 ATATTCACTCTCAATCCAAATAATCTGGATCTGATCAAGAGACAGGATGA

1301 GGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGC

1351 CGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCG

1401 GCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTT

1451 CTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGA

1501 GGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTG

1551 TGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAA

1601 GTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGT

1651 ATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTA

1701 CCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACT

1751 CGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCA

1801 GGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCG

1851 ACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATC

1901 ATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGG

1951 TGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTG

2001 AAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATC

2051 GCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTT

2101 CTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAA

2151 CCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGG

2201 GCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGG

<-NPTII/ocs3'->

2251 GATCTCATGCTGGAGTTCTTCGCCCACCCCCTGCTTTAATGAGATATGCG

2301 AGACGCCTATGATCGCATGATATTTGCTTTCAATTCTGTTGTGCACGTTG

2351 TAAAAAACCTGAGCATGTGTAGCTCAGATCCTTACCGCCGGTTTCGGTTC

2401 ATTCTAATGAATATATCACCCGTTACTATCGTATTTTTATGAATAATATT

2451 CTCCGTTCAATTTACTGATTGTACCCTACTACTTATATGTACAATATTAA

2501 AATGAAAACAATATATTGTGCTGAATAGGTTTATAGCGACATCTATGATA

2551 GAGCGCCACAATAACAAACAATTGCGTTTTATTATTACAAATCCAATTTT

2601 AAAAAAAGCGGCAGAACCGGTCAAACCTAAAAGACTGATTACATAAATCT

2651 TATTCAAATTTCAAAAGGCCCCAGGGGCTAGTATCTACGACACACCGAGC

2701 GGCGAACTAATAACGTTCACTGAAGGGAACTCCGGTTCCCCGCCGGCGCG

2751 CATGGGTGAGATTCCTTGAAGTTGAGTATTGGCCGTCCGCTCTACCGAAA

<-ocs3'/

2801 GTTACGGGCACCATTCAACCCGGTCCAGCACGGCGGCCGGGTAACAGCGG

lacZ->

2851 CCGCTGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATT

2901 AATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC

2951 AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACA

3001 CTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAAT

3051 TTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACC

<-lacZ/p35S->

3101 CGGGATCTCCTTTGCCCCGGAGATTACAATGGACGATTTCCTCTATCTTT

3151 ACGATCTAGGAAGGAAGTTCGAAGGTGAAGGTGACGACACTATGTTCACC

3201 ACTGATAATGAGAAGGTTAGCCTCTTCAATTTCAGAAAGAATGCTGACCC

3251 ACAGATGGTTAGAGAGGCCTACGCAGCAGGTCTCATCAAGACGATCTACC

3301 CGAGTAACAATCTCCAGGAGATCAAATACCTTCCCAAGAAGGTTAAAGAT

3351 GCAGTCAAAAGATTCAGGACTAATTGCATCAAGAACACAGAGAAAGACAT

3401 ATTTCTCAAGATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGC

3451 TTCATAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTT

3501 CCTACTGAATCTAAGGCCATGCATGGAGTCTAAGATTCAAATCGAGGATC

3551 TAACAGAACTCGCCGTGAAGACTGGCGAACAGTTCATACAGAGTCTTTTA

3601 CGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGAGCACGACAC

3651 TCTGGTCTACTCCAAAAATGTCAAAGATACAGTCTCAGAAGACCAAAGGG

3701 CTATTGAGACTTTTCAACAAAGGATAATTTCGGGAAACCTCCTCGGATTC

3751 CATTGCCCAGCTATCTGTCACTTCATCGAAAGGACAGTAGAAAAGGAAGG

3801 TGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCATTCAAG

3851 ATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGC

3901 ATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTG

3951 ATGTGACATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTT

4001 CGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACA

4051 CGCTCGAGCTCATTTCTCTATTACTTCAGCCATAACAAAAGAACTCTTTT

<-p35S/ACCS->

4101 CTCTTCTTATTAAACCATGGGTTTAGCAGAAAATCAGCTTTGTTTAGACT

4151 TGATAGAAGATTGGATTAAGAGAAACCCAAAAGGTTCAATTTGTTCTGAA

4201 GGAATCAAATCATTCAAGGCCATTGCCAACTTTCAAGATTATCATGGCTT

4251 GCCTGAATTCAGAAAAGCGATTGCGAAATTTATGGAGAAAACAAGAGGAG

4301 GAAGAGTTAGATTTGATCCAGAAAGAGTTGTTATGGCTGGTGGTGCCACT

4351 GGAGCTAATGAGACAATTATATTTTGTTTGGCTGATCCTGGCGATGCATT

4401 TTTAGTACCTTCACCATACTACCCAGCATTTAACAGAGATTTAAGATGGA

4451 GAACTGGAGTACAACTTATTCCAATTCACTGTGAGAGCTCCAATAATTTC

4501 AAAATTACTTCAAAAGCAGTAAAAGAAGCATATGAAAATGCACAAAAATC

4551 AAACATCAAAGTAAAAGGTTTGATTTTGACCAATCCATCAAATCCATTGG

4601 GCACCACTTTGGACAAAGACACACTGAAAAGTGTCTTGAGTTTCACCAAC

4651 CAACACAACATCCACCTTGTTTGTGACGAAATCTACGCAGCCACTGTCTT

4701 TGACACGCCTCAATTCGTCAGTATAGCTGAAATCCTCGATGAACAGGAAA

4751 TGACTTACTGCAACAAAGATTTAGTTCACATCGTCTACAGTCTTTCAAAA

4801 GACATGGGGTTACCAGGATTTAGAGTCGGAATCATATATTCTTTTAACGA

4851 CGATGTCGTTAATTGTGCTAGAAAAATGTCGAGTTTCGGTTTAGTATCTA

4901 CACAAACGCAATATTTTTTAGCGGCAATGCCATCGGACGAAAAATTCGTC

4951 GATAATTTTCTAAGAGAAAGCGCGATGAGGTTAGGTAAAAGGCACAAACA

5001 TTTTACTAATGGACTTGAAGTAGTGGGAATTAAATGCTTGAAAAATAATG

5051 CGGGGCTTTTTTGTTGGATGGATTTGCGTCCACTTTTAAGGGAATCGACT

5101 TTCGATAGCGAAATGTCGTTATGGAGAGTTATTATAAACGATGTTAAGCT

5151 TAACGTCTCGCTTGGATCTTCGTTTGAATGTCAAGAGCCAGGTTGGTTTC

<-ACCS/nos3'->

5201 GAGTTTGTTTCTAGAGTCAAGGGGCAGATCGTTCAAACATTTGGCAATAA

5251 AGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATA

5301 ATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGA

5351 CGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATT

5401 TAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATC

<-nos3'/lacZ->

5451 GCGCGCGGTGTCATCTATGTTACTAGATCGACCTGCAGGCATGCAAGCTT

5501 GGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTA

5551 CCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAAT

5601 AGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAA

<-lacZ/right border->

5651 TGGCGAATGGCGCAGATCCCTGAAAGCGACGTTGGATGTTAACATCTACA

5701 AATTGCCTTTTCTTATCGACCATGTACGTAAGCGCTTACGTTTTTGGTGG

5751 ACCCTTGAGGAAACTGGTAGCTGTTGTGGGCCTGTGGTCTCAAGATGGAT

5801 CATTAATTTCCACCTTCACCTACGATGGGGGGCATCGCACCGGTGAGTAA

5851 TATTGTACGGCTAAGAGCGAATTTGGCCTGTAGACCTCAATTGCGAGCTT

5901 TCTAATTTCAAACTATTCGGGCCTAACTTTTGGTGTGATGATGCTGACTG

5951 GCAGGATATATACCGTTGTAATT

Appendix 2. -- Field Trial Reports

Permit No. 92-035-05
June 30, 1994

ENVIRONMENTAL RELEASE REPORT

USDA Permit No. 92-035-05

DNA Plant Technology Corporation
6701 San Pablo Avenue
Oakland, California 94608-1239
Tel: 510/547-2395
Fax: 510/547-2817

Permittee: Dr. Pamela Dunsmuir, DNAP, 510/547-2395

Dates of Release: June, 1992; April, 1993

Site of Release: DNAP, Brentwood, California

Purpose of Release:
This experiment consisted of a field evaluation of transgenic tomatoes (large-fruited and cherry) expressing a sense or antisense transcript of an ACC synthase gene to determine whether suppression of the fruit specific ACC synthase activity can be a useful way to control the process of fruit ripening and consequently improve the fruit quality for the consumer.

The test was divided into two parts. Part 1 consisted of i) an evaluation of a genetically engineered tomato line containing an antisense construct and ii) development of hybrids of this line with non-transgenic tomato lines. These field trials were designed to evaluate the inheritance and phenotypic stability of the delayed-ripening phenotype and to evaluate the agronomic performance of the lines. In Part 2 of the trial, additional genetically engineered ripening impaired lines were evaluated. The lines which were evaluated included both large-fruited and cherry tomato lines containing either a sense (designated 300 series) or an antisense (designated 100 series) construct of the fruit specific ACC synthase gene.

Part 1 of the experiment included the following genotypes and treatments:

- 1 transgenic line of tomato cultivar VF36 transformed with pPO35 (designated line A11.1)
- non-transgenic VF36 control
- three cultivars (Cal Ace, Floradade and VF36) of non-transgenic pollen-parent lines

In order to evaluate tomato ripening, 40-45 day old transplants of transgenic (A11.1) and non-transgenic tomato (VF36) were planted in single rows spaced 18 inches within the row on 5 ft beds. A total of 16 transgenic plants and 16 non-transgenic controls were transplanted to the field. The two treatments were completely randomized within two rows, each row was 24 ft long. Border rows of Cal Ace and Floradade were planted on each side.

Development of hybrids lines consisted of utilizing six transgenic tomato plants both as pollen donors and recepients of pollen from non-transgenic tomato lines, VF36, Cal Ace and Floradade. All crossing experiments were conducted in the greenhouse. The plants were allowed to set fruit and develop seed. The hybrid seed was collected and held for subsequent analysis and experimentation. When the female parent was the non-transgenic partner , the confirmation of the hybrid nature of the seed could be obtained by germination of the seed on agar plates containing kanamycin at 100 ug/ml. and evaluating root formation.

Part 2 of the experiment included the following genotypes and treatments:

- *Antisense lines (designated 100 series)-transformed with pWTT2144/ACCA*

  A single transgenic line of each cultivar Floradade [F146], 91103-114 [1112] and Gold Nugget [G116] was evaluated. Ten to 18 plants per line were planted in blocks and rated for agronomic characteristics and ripening phenotype of the fruit. The A11.1 line was used as a positive control.

- *Sense lines (designated 300 series)-transformed with pWTT2144/ACCS*

  Four independent transgenic lines of cultivar Floradade [F347, F349, F359, F388], one of 91103-114 [1345], four of NC8288 [N351, N354, N363, N388] and two of Gold Nugget [G306, G352] were evaluated. Ten to 18 plants per line were planted in blocks and rated for agronomic and ripening phenotype.

- Nine independent selections of Baxter's Early Bush Cherry cultivar (BEBC) derived from six primary transformants carrying the T-DNA from pWTT2144/ACCS (a sense construct) were evaluated. Ten to 18 plants per line were planted and rated for agronomic and ripening.

- Non-transgenic controls of BEBC, Gold Nugget, Floradade and NC8288 were used in blocks of 15-20 plants and also rated for agronomic properties and fruit ripening phenotype.

- Transgenic control lines which carry the T-DNA from the pWTT2144 vector (Golden Nugget, G001; NC8288, N001; Floradade, F001; BEBC, B003-8) were used in blocks of 15-20 plants and rated for agronomic performance and fruit ripening properties.

This part of the experiment consisted of planting the S1 selection of primary tomato transformants (15 to 20 plants per line) to the field in single rows and allowing them to set fruit. F1 transplants of antisense hybrid lines were also included. Plants were screened for the ripening impaired phenotype on the vine and off the vine. Further analysis consisted of removing fruit from the vine, storing it at 20°C and 85% relative humidity, and over time making colorometric and ethylene measurements. A total of 750 transgenic plants were transplanted to the field during the course of this two-year experiment.

Schedule of Major Operations - 1992 Growing Season

May, 1992	Seed sown in greenhouse at the DNAP research facility in Oakland, California.
June, 1992	Plants transported to field site in Brentwood, California and transplanted to field.
July-September, 1992	Fruit and leaf samples collected for analysis at DNAP, Oakland facility. Seed produced for further experimentation.
November, 1992	Release terminated; plants disked into the field. The plot was monitored over the winter months (approx. 90 days). The field was observed visually for volunteer tomato plants. No volunteer plants were observed during the monitoring period.

Schedule of Major Operations - 1993 Growing Season

March, 1993	Seed sown in greenhouse at the DNAP research facility in Oakland, California.
April-June, 1993	Plants transported to field site in Brentwood, California and transplanted to field.
July-November, 1993	Fruit and leaf samples collected for analysis at DNAP, Oakland facility. Seed produced for further experimentation.
November, 1993	Release terminated; plants were disked into the field. Plot fumigated with methyl bromide. The plot was visually monitored over the winter months (approx. 90

days) for volunteer tomato plants. No volunteer plants were observed during the monitoring period.

Plant Growth and General Observations

Both transgenic and non-transgenic plants grew normally during the course of the experiment. Experimental plots were evaluated by company researchers and tomato breeders for color, fruit quality, plant vigor and disease characteristics. Ratings were assigned according to rating scales developed by company tomato breeders, and are based upon visual observations made in the same general manner in which a tomato breeder would examine other new varieties to determine acceptability for commercial release.

There were no major differences between the growth habit, flowering time, fruit set, general plant vigor, and disease susceptability between the control and ripening impaired lines (Table 1). The only difference observed was in the ripening phenotype of the fruit in the control and ripening impaired lines. Amongst the various transgenic lines which were selected for field trialing, there was considerable variation in the extent of ripening inhibition. However, generally the ripening impaired lines showed less fruit color development when the fruit was left on the vine and also in fruit which was removed from the vine and stored.

The ripening inhibited phenotype was found to be heritable and dominant since the F1 hybrids between the homozygous A11.1 line and the VF36, Floradade, and Cal Ace cultivars exhibited similar ripening properties to those of the transgenic ripening impaired parent.

The plots were regularly monitored for *Agrobacterium* infection symptoms. No symptoms of infection were found in any transgenic plant. No significant fruit damage was observed from birds or rodents.

Means and Effectiveness of Containment Measures

The experiment was planted more than 30 ft. from other tomato experiments conducted at this site. The predominance of self pollination in tomato minimizes the opportunity for pollen dispersal.

The plants and fruit remaining at the end of the experiments were destroyed by disking into the field. The experimental plot site was monitored for 3 months after the termination of the experiments for evidence of any remaining growth of test plants or volunteers. No evidence of volunteers were observed in the test site.

Table 1. Agronomic traits of transgenic antisense and sense tomato lines that are impaired in fruit ripening.

Lines	Transgene	Vigor	Color on vine	Col. off vine a/b ratio	Disease
VF36	not transgenic	5	6	1.5	4
A11.1	ACC-A	5	2	0.1	4
F001	none	4	6	1.5	3.5
F146	ACC-A	4	2	0.05	4
F388	ACC-S	4	6	1.2	4
F349	.. ..	4	5	1.0	4
F347	.. ..	4	6	1.0	4
F359	.. ..	4	6	1.45	4
91103-114	not transgenic	4	6	1.2	4
1112	ACC-A	4	2	0.01	4
1345	ACC-S	4	2	0.08	4
NC8288	not transgenic	4	6	1.2	4
N001	none	4	6	1.2	4
N363	ACC-S	4	5	1.14	4
N354	.. ..	4	5	1.3	4
N388	.. ..	4	4	1.2	4
N351	.. ..	4	5	1.2	4
Gold Nugget	not transgenic	4	6	na	4
G001	none	3	6	na	3.5
G116	ACC-A	4	5	na	4
G306	ACC-S	4	5	na	4
G352	.. ..	4	6	na	4

Scale: Vigor; 1 to 5: 1=stunted and small plant to 5 =very vigorous plant.

Color on vine; 1 to 6: 1=fruit at mature green stage to 6=fruit at red ripe.

Color off vine: a/b ratio determined with a Minolta CR300 colorimeter.

Disease; 1 to 5: 1 to 5 1=very diseased plant to 5 =very healthy plant.

Table 1 (cont). Agronomic traits of transgenic antisense and sense tomato lines that are impaired in fruit ripening.

Lines	Trans gene	Vigor	Color on vine	Col. off vine a/b ratio	Disease
B003-8	none	3.5	6	1.52	3.5
B368	ACC-S	4	5	1.0	3.5
B309-5	.. ..	4	5	1.07	4
B309-6	.. ..	4	5	1.3	4
B320-2	.. ..	4	4.5	0.9	4
B316-1	.. ..	4	5	0.7	4
B316-2	.. ..	4	6	1.3	4
B316-3	.. ..	4	2	0.2	3.5
B316-4	.. ..	4	3	0.6	4
B324-6	.. ..	4	2.5	0.4	4
B324-4	.. ..	4	2.5	0.42	4
B324	.. ..	4	2	0.4	4
B391	.. ..	4	3	0.4	4
Hybrids					
VF36xA11-2	ACC-A	4	2	0.35	4
VF36xA11-4	.. ..	4	2	0.07	4
VF36xA11-7	.. ..	4	2	0.16	4
Floradade x A11-1	.. ..	4	2.5	0.1	4
Floradade x A11-2	.. ..	4	2	0.07	4
Floradade x A11-3	.. ..	4	2	-0.1	4
CalAce x A11-1	.. ..	4	2	0.1	4
Floradade x VF36	.. ..	4	6	1.1	3.5
CalAce x VF36	.. ..	4	6	1.26	4

Scale:

Vigor;	1 to 5:	1=stunted and small plant to 5 =very vigorous plant.
Color on vine;	1 to 6:	1=fruit at mature green stage to 6=fruit at red ripe.
Color off vine:	a/b ratio	determined with a Minolta CR300 colorimeter.
Disease; 1 to 5:	1 to 5	1=very diseased plant to 5 =very healthy plant.

Permit No. 93-252-07
June 30, 1994

ENVIRONMENTAL RELEASE REPORT

USDA Permit No. 93-252-07

DNA Plant Technology Corporation
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Permittee: Scott Thenell, DNAP, 510/547-2395

Date of Release: December, 1993

Site of Release: Six-L's Farms, Naples, Florida

Site Contact: Wesley Roan, Six-L's Farms

Purpose of Release:
This experiment consisted of a field evaluation of transgenic cherry tomato lines expressing a sense transcript of a truncated form of the fruit specific ACC synthase gene. The purpose of this trial was to determine whether inhibition of fruit ripening, through the control of ethylene biosynthesis in the fruit, was an approach which could be used to improve fruit quality and fruit production parameters.

This experiment included the following genotypes and treatments:

- Two independent transgenic lines of Baxter's Early Bush Cherry (BEBC) transformed with the construct pWTT2144/p35S:ACCS (designated lines B316-3 and B324-6)

- One non-transgenic control of Baxter's Early Bush Cherry transformed with pWTT2144 (the empty binary vector)

The experiment consisted of planting a randomized complete block design with three replications. Each plot consisted of a single row of 50 plants spaced 18 inches within the row with 5 ft. spacings between rows. To determine the potential harvest cost savings and yield increases associated with the ripening impaired (RI) phenotype, the yields were compared for each of the lines after the normal practice of multiple harvests (cumulative yield) or as a single harvest after holding of the fruit on the vine. The multiple harvests were taken every 4 to 6 days over a two week period as would occur for a normal commercial pick, and the single point harvest was taken at the end of the same two week period. At each harvest, the fruit was sorted into color groups: mature green/breaker,

pink/turning, light red and red. A total of 600 transgenic plants and 300 non-transgenic controls were transplanted to the field, for a total of approximately 0.2 acres.

Schedule of Major Operations

October, 1993	Seed sown in transplant facility in Naples, Florida at Six-L's Farm.
December, 1993	Plants transplanted at Six-L's Farm site in Naples, Florida.
March, 1994	Plots harvested and ship fruit shipped to DNAP, Oakland, California for evaluation of fruit quality and composition.
April, 1994	Release terminated. The plants and remaining fruit were sprayed with paraquat and allowed to dessicate prior to burning. After burning, the stakes and plastic row cover was pulled and the plot disked. Six-L's Farm staff routinely use this procedure in production fields as a highly effective means of preventing volunteer plants. To date, there is no evidence of volunteer plants in the experimental plot.

Plant Growth and General Observations

Both transgenic and non-transgenic plants grew normally during the course of the experiment (Table 1) based on ratings for disease, vigor, foliage cover, % crown and top fruit set. The line B316-3 did exhibit a slightly higher incidence of crown rot symptoms than either B324-6 or the control line. In all likelihood this increased susceptability is unrelated to the lowering of ACC synthase levels since we have not observed this effect in any other of the ripening impaired lines; possibly the process of transformation and regeneration has given rise to a somaclone. The ratings for disease, vigor and cover were made at two evaluation times: 3 wks before the first harvest and 2 wks after the the first. Ratings were assigned by company researchers and tomato breeders according to rating scales developed by company tomato breeders.

The plots were regularly monitored for *Agrobacterium* infection symptoms. No symptoms of infection were found in any transgenic plant. No significant fruit damage from birds or rodents was observed. The plants and remaining fruit were destroyed according to protocol.

Table 1. Agronomic traits of ripening impaired lines and BEBC: Naples trial

Evaluation time	Lines	Agronomic traits				
		Disease	Vigor	Cover	%Crown set	%Top set
3 weeks before first harvest	B316-3	2.6	2.6	2.7	50	na
	B324-6	2	2.7	3	50	na
	BEBC	2	2.7	2.7	50	na
2 weeks after first harvest	B316-3	2.1	3.6	2.7	75	30
	B324-6	3	4	4	73	37
	BEBC	3	4	3.8	78	35

Disease rating (1=high amount of disease to 5=very healthy plant)
Vigor rating (1=stunted small plant to 5=vigorous plant growth)
Cover (1=fruit very exposed to 5=fruit covered by leaf canopy)
Crown and top set (average percent fruit set on each racime)

Fruit samples were collected for analysis of fruit quality, composition and sensory characteristics. A discussion of these analyses will be presented in a petition for non-regulated status.

Means and Effectiveness of Containment Measures

The experiment was planted more than 30 ft. from other tomato experiments conducted at this site. The predominance of self pollination in tomato minimizes the likelihood of pollen dispersal.

Following termination and clean up, the plot was monitored for 30 days and visually observed for volunteer plants. No volunteer plants were observed during the monitoring period.

Permit No. 92-301-01
June 30, 1994

ENVIRONMENTAL RELEASE REPORT

USDA Permit No. 92-301-01

DNA Plant Technology Corporation
6701 San Pablo Avenue
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Tel: 510/547-2395

Permittee: Scott Thenell, DNAP, 510/547-2395

Date of Release: June, 1993

Site of Release: DNAP, Brentwood, California

Purpose of Release:
This experiment consisted of a field evaluation of transgenic tomatoes (large-fruited and cherry tomatoes) expressing a sense transcript of an ACC synthase gene to determine its efficacy for control of fruit ripening and quality. This experiment included the following genotypes and treatments:

Group A
Plants transformed with a truncated ACC synthase gene in the sense orientation; construct in pWTT2144/p35S-ACCS [300 series plants]

- 8 transgenic selections of cultivar Baxter's Early Bush Cherry, derived from four independent primary transformants (B316, B324, B329, B391)
- 1 transgenic line of cultivar Floradade (F308)
- 3 selections from one primary transformant of cultivar 91103-114 (1345)

GroupB
Plants transformed with the full length ACC synthase gene sense construct in pJJ2964/p35S-ACCS were included in an observational trial [400 series plants].

- 10 independent transgenic lines of Baxter's Early Bush Cherry (416, 419, 453, 461, 466, 469, 476, 481, 482, 4110).

Group C
Non-transgenic controls of cultivar Baxter's Early Bush Cherry, Floradade and 91103-114.

The experiment was conducted in two parts. Part one was as a randomized complete block design with three replications per line [300 series (truncated ACCS) plants plus controls]. Each plot consisted of a single row of 20 plants

spaced 18 inches within the row and with 5 ft. spacing between the rows. In the second part, the ten lines expressing the full length ACC-S construct [400 series plants] were not replicated, but rather evaluated in single block observational trial. A total of approximately 1400 transgenic plants and 200 non-transgenic controls were transplanted to the field in an area approximately 0.3 acres.

Schedule of Major Operations

May to June, 1993	Seed sown in greenhouse at the DNAP research facility in Oakland, California.
June to early July, 1993	Plants transported to field site in Brentwood, California and transplanted to field.
July through September	Fruit and leaf samples collected for analysis at DNAP, Oakland. Seed produced for further experimentation.
November, 1993	Release terminated; plants disked into the field. The plot was monitored over the winter months (90 days) and visually observed for evidence of volunteer plants. No volunteer tomato plants were observed during the monitoring period.

Plant Growth and General Observations

Baxter's Early Bush Cherry

The agronomic characteristics of the vines and the fruit from BEBC and derivative ripening impaired lines were evaluated. Experimental plots were evaluated by company researchers and tomato breeders for plant growth habit, plant vigor, fruit quality and disease characteristics. Ratings were assigned according to rating scales developed by company tomato breeders, and are based on visual and other observations made in the same manner that a tomato breeder would examine other new varieties for commercial acceptability. Measurements of disease, vigor, vine cover, fruit set, fruit firmness, and fruit diameter were made at several times throughout the growing season (Table 1). There were no significant differences between the transgenic lines and the parental BEBC line for any of these parameters. As expected, fruit of the ripening-inhibited lines developed less color than the parental lines.

Seeds of ripening impaired (RI) lines germinated as well as BEBC and there were no differences between these lines and the parental cultivar with respect to survival after transplant.

The plots were regularly monitored for *Agrobacterium* infection symptoms. No symptoms of infection were found in any transgenic plant. No significant fruit damage from birds or rodents was observed. The plants and fruit remaining in the field at the end of the experiment were disked into the field.

Fruit samples were collected for evaluation of the ripening characteristics, fruit quality and sensory analysis. These analyses were restricted to the selections B316-3 and B324-6 rather than for the complete set of BEBC ripening impaired lines since a) they derived from independent transformants, b) they each exhibited the most pronounced phenotype with respect to ripening inhibition and c) we had accumulated the most data on the 300 series selections. A discussion of these fruit evaluations will be presented in connection with a petition for non-regulated status.

91103-114 and Floradade lines

The agronomic characteristics of the vines from the 91103-114, Floradade and derivative ripening impaired lines were evaluated in the field where the plants were grown in a standard replicated plot. Measurements of disease, vigor, cover, fruit set, fruit firmness, and fruit diameter were made several times throughout the growing season. There were no differences between either of the transgenic lines and the parental 91103-114 line for disease occurrence, plant vigor and fruit set (Table 2). Fruit from all lines, including the control, showed signs of cracking around the stem end, but since development occurred during the hot periods of August and early September, it could have been attributed to excess heat. Evaluations of the one Floradade ripening impaired line was discontinued since the fruit size of the F308 ripening impaired line were consistently too small, less than the 2 inch small grading standard. The small fruit size was most probably attributed to somaclonal variation.

Seeds of the ripening inhibited lines germinated as well as 91103-114 and Floradade, and there were no differences between the transgenic lines and the parental cultivar with respect to survival after transplant.

Color development for the RI lines (1345-1, -4, -7) and the parental line (91103-114) was measured at 14 days post breaker picked fruit, and the transgenic fruit were significantly different. The RI lines had La/b ratios ranging from 0.35 to 0.56, whereas the control line had La/b ratios ranging from 1.3 to 1.5. However, the color development was comparable when the fruit were ripened with ethylene- the fruit from the RI lines and the control had a/b readings of 1.5 to 1.7. The levels of ethylene produced in the fruit of the transgenic lines was measured as less than 1% (0.65 %) of the level produced in 91103-114 control fruit.

Reducing sugars, titratable acidity and BRIX measurements were made on fruit harvested at breaker, 7 and 14 days post breaker, before and after ripening with ethylene (Table 3). In all lines, the acid levels decreased as the fruit was left on the vine post the breaker stage. This correlated with the reduction in titratable acidity which occurred if the fruit were ripened with ethylene. Reducing sugar values were generally similar for RI lines 1345-1, 1345-7, and 91103-114, but levels were reproducibly higher for the 1345-4 line. This same pattern was observed after ripening. Generally, the reducing sugars level decreased the longer fruit was left on the vine for all lines.

The BRIX readings were also significantly higher for line 1345-4 compared to the control and the other RI lines (Table 3). The BRIX readings (like the reducing sugars measurements) decreased the longer the fruit was left on the vine, and generally were a little lower after ripening compared to the before ripening values.

A taste panel ranked ethylene ripened fruit from these lines for aroma, sweetness, sourness, green flavor, tomato flavor, overall impact, juiciness, firmness, mealiness and skin residue. The fruit of 91103-114 and RI lines was harvested at the breaker stage and a) ripened for tasting or, b) the RI fruit was held for an additional 2 weeks and then ripened and used in a taste panel. The control 91103-114 fruit was harvested for the second taste panel at the breaker stage, and then ripened alongside the older RI fruit which had been held post harvest for the two week period. The RI lines consistently were rated with higher aroma, sourness, tomato flavor and overall flavor as compared to the control line. This was true for comparisons between RI fruit picked at the breaker stage and the control line, and also for fruit stored for two weeks after harvest. The RI fruit that had been stored for two weeks had overall lower ratings as compared to the fruit which had been picked at breaker and ripened immediately.

Means and Effectiveness of Containment Measures

The experiment was planted more than 30 ft. from other tomato experiments conducted at this site. The predominance of self pollination in tomato minimizes the likelihood of pollen dispersal.

The experimental plot site was monitored for 3 months after the termination of the experiments for evidence of any remaining growth of test plants or volunteers. No evidence of volunteers were observed in the test site.

Table 1. Agronomic characteristics of Baxter's Early Bush Cherry and ripening impaired lines

Line	Disease[a]	Vigor[b]	Cover[c]	Fruit Firmness[d]	Fruit set[e]	Fruit diameter (cm)[f]
B316-1	4	4	4	3	3	2.7
B316-3	4	4	4	4	3	na
B324-4	4	4	4	3.5	3	na
B324-6	4	4	4	3.5	3	2.8
B329-6a	4	4	4	3	3	na
B391-1/8	4	4	3.5	3	3	na
B391-7	4	4	4	3	3	na
B329-1/8	4	4	3.5	3	3	na
B416	4	4	3.5	3	3	na
B453	4	3	3.5	3	3	na
B461	3	3	4	3	3	na
B466	4	4	3	3	3	na
B469	4	3	3	3	3	na
B476	4	4	3	3	3	na
B481	4	4	3	3	3	na
B4110	3	4	3	3	3	na
B419	4	4	4	3	3	na
B482	4	4	4	3	3	na
BEBC	4	4	3.5	3.5	3	2.6

a Disease rating (1=high amount of disease to 5=very healthy plant)
b Vigor rating (1=stunted small plant to 5=vigorous plant growth)
c Cover (1=fruit very exposed to 5=fruit covered by leaf canopy)
d Firmness (1=very soft fruit to 5=very firm fruit)
e Fruit set (1=poor set to 5 =very good fruit set of each racime)
f Fruit diameter: measurements made of fruit harvested at breaker.

Table 2. Agronomic characteristics of RG103-114 and ripening impaired lines

Line	Fruit Disease[a]	Vigor[b]	Cover[c]	Fruit Firmness[d]	Fruit set[e]	Color Crown fruit[f]
RG103-114	3	3	4	2	4	6
1345-1	3	3	4	4	4	3
1345-4	3	3	4	3.5	4	2.5
1345-7	3	3.5	4	3.5	4	3

a Disease rating (1=high amount of disease to 5=very healthy plant)
b Vigor rating (1=stunted small plant to 5=vigorous plant growth)
c Cover (1=fruit very exposed to 5=fruit covered by leaf canopy)
d Firmness (1=very soft fruit to 5=very firm fruit)
e Fruit set (1=poor set to 5 =very good fruit set of each racime)
f Color crown fruit (rating based on USDA color standards 1=mature green to 6=red ripe)

Table 6. Comparison of titratable acidity, reducing sugar and BRIX measurements for RG103-114 and ripening impaired lines

Days post breaker	Lines	Titratable Acidity (before C2H4)	Titratable Acidity (after C2H4)	Reducing sugar (before C2H4)	Reducing sugar (after C2H4)	BRIX (before C2H4)	BRIX (after C2H4)
At breaker	1345-1	0.58	0.42	2.7	2.7	4.7	4.7
	1345-4	0.79	0.22	3.6	3.2	6.2	4.2
	1345-7	0.68	0.38	2.1	2.6	4.2	4.3
	RG103-114	0.46	0.17	2.7	2.5	4.8	4.4
7 days	1345-1	0.58	nd	2.1	nd	4.1	nd
	1345-4	0.79	nd	2.7	nd	5.2	nd
	1345-7	0.68	nd	2.4	nd	4.5	nd
	RG103-114	0.46	nd	2.4	nd	4.3	nd
14 days	1345-1	0.46	0.28	1.7	2.7	4.1	4.1
	1345-4	0.48	0.25	2.6	3.3	5	4.9
	1345-7	0.54	0.3	1.7	2.3	4.5	4.2
	RG103-114	0.24	0.1	2.2	2.8	4	4.2

Permit No. 93-302-01
August 10, 1994

ENVIRONMENTAL RELEASE REPORT

USDA Permit No. 93-302-01

DNA Plant Technology Corporation
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Permittee: Scott Thenell, DNAP, 510/450-9310

Date of Release: February, 1994

Site of Release: Six-L's Farms, Ruskin, Florida

Site Contact: Wesley Roan, Six-L's Farms

Purpose of Release:
Field evaluation of transgenic delayed-ripening tomatoes (large-fruited and cherry) to determine efficacy of fruit ripening control and quality.

This experiment included the following genotypes and treatments:

Large-fruited tomatoes

- 3 selections derived from primary transformant 1345 of cultivar 91103-114 carrying pWTT2144/p35S:AccS (lines 1345-1, 1345-4 and 1345-7);
- 5 proprietary hybrid lines of 91103-114 with the 1345 derivatives carrying pWTT2144/p35S:AccS (designated lines 93396, 93397, 93398, 93400 and 94401).
- non-transgenic controls of 91103-114, 91155, 91347-RN1 and 92030;

Cherry tomatoes

- 4 independent transgenic lines of cultivar Baxter's Early Bush Cherry (BEBC) carrying pWTT2144/p35S:AccS (designated lines B316-3, B324-6, B329-6a, and B391-7s)
- 8 proprietary hybrid cherry tomato lines of BEBC carrying pWTT2144/p35S:AccS (designated lines 93593, 93594, 93599, 93600, 93602, 93603, 93605, and 93606)
- non-transgenic control Baxter's Early Bush Cherry

Experimental Design

The experiment was conducted in two parts:

i) as a randomized complete block design with four replications per treatment of large-fruited tomatoes, and;
ii) as an observational trial of F1 hybrids of cherry tomatoes.

Each replicated plot consisted of a single row of 20 plants spaced 15 inches within the row with 5 ft. spacing between the row. Each treatment used in the observational trial consisted of a single row of 15 to 20 plants. A total of approximately 800 transgenic and 480 non-transgenic controls were transplanted to the field in approximately 0.25 acres.

Schedule of Major Operations

January, 1994	Seed sown in Six-L's Farms greenhouse facility in Naples, Florida on January 4, 1994.
February, 1994	Plants transported to field site in Ruskin, Florida and transplanted to field on February 24, 1994.
June, 1994	Fruit was harvested on June 6, 1994. Fruit was hand harvested, graded and weighed for yield comparisons. Approximately 50 lbs. of fruit was shipped to DNAP, Oakland under Biotechnology Permit No. 93-302-01 for analysis and sensory evaluations. The remaining fruit was returned to the field for disposal by disking. The plants were sprayed with paraquat on June 13, 1994 and allowed to desiccate prior to planting a crop of cucurbits for fall 1994 harvest.

Plant Growth and General Observations

The data reported here describe the large fruited tomatoes. The data pertaining to the cherry tomatoes will be submitted separately.

The agronomic characteristics of the vines and the fruit from 91103-114 and derivative ripening impaired lines were evaluated. Experimental plots were evaluated by company researchers and tomato breeders for plant growth habit, plant vigor, fruit quality and disease characteristics. Ratings were assigned according to rating scales developed by company tomato breeders and are based on observations made in the same manner that a tomato breeder would examine new varieties for commercial acceptability.

Both transgenic and non-transgenic plants grew normally during the course of the experiment. Measurements of disease incidence, plant vigor, vine cover, fruit set, and fruit color development were made at several times throughout the growing season and these data are collated and summarized in Table 1. There were no significant differences between the transgenic lines and the parental lines for any of these parameters.

The test site and adjacent production fields experienced low incidence of bacterial leaf spot, target spot, and *Fusarium* crown rot, along with moderate tomato pinworm and vegetable leaf miner pressure and relatively high whitefly pressure. Control measures included regular applications of copper compounds (Kocide) with fungicides (Manzate, Bravo) and insecticides (Admire, Assana, Ambush, Monitor, Lannate, Vidate and Thiodan) as recommended by Six-L's Farms' crop specialists. At the time of harvest there was a significant amount of early blight, *Alternaria alternata*, on foliage of all lines whether delayed-ripening or parental lines. The fruit and foliage of a nearby commercial planting of the tomato cultivar Agroset also exhibited a similar amount of early blight infection. Likewise, post harvest decay incited *by Alternaria* and *Rhizopus* was also observed on all lines. Early in the growing season, during March and April, there was a low incidence of bacterial speck and spot observed on all lines in the field. DNAP plant pathologists observed no differences between transgenic and non-transgenic lines with respect to disease or insect susceptibility or response to treatments.

Germination rates and survival after transplanting did not differ between the delayed ripening and parental lines.

Yield

Yield comparisons were made to determine the total pounds per acre, as well as the actual percentage of marketable fruit which results from harvesting 2 weeks post-breaker for the three delayed-ripening inbreds (1345-1, 1345-4, 1345-7) and one hybrid (93397) together with two control inbreds (91103-114, 91347-RN1) and one hybrid (91155). The 2 week post-breaker harvest point was determined based on fruit color development of the 91103-114 parental line when at least 50% of the fruit was at the breaker stage or beyond. These data, which are summarized in Table 2, illustrate that there were no significant differences in total lbs/acre yield between delayed-ripening inbreds, F1 hybrid 99397 and all of the control lines. However, when the fraction of marketable fruit arising from this single point harvest was measured, there were significant (P=0.05) differences between the delayed-ripening lines (inbreds and hybrid) and the normal ripening controls (inbreds and hybrid). There were higher quantities of marketable fruit harvested from the delayed-ripening lines compared to the control lines (Table 2); approximately 95% of fruit was considered marketable from these lines (both inbreds and hybrid) compared to 54 to 73% marketable fruit from the control lines. Furthermore, 29% to 44% of the fruit from the control

lines was at or beyond the light red stage of development, whereas the delayed-ripening inbred lines and three of the F1 hybrids had no fruit at red ripe stage and less than 17% of fruit at the light red stage of development. Two delayed-ripening F1 hybrids showed a higher percentage of fruit at the red ripe stage which may reflect differences in the penetrance of the delayed-ripening phenotype in different backgrounds, or it may reflect differences in the 'earliness' or 'lateness' of the other parent in the hybrids (Table 3).

Titratable Acidity, Reducing Sugars and Brix Measurements

Purees were made from red ripe fruit of 1345-4, selected delayed-ripening F1 hybrids and control lines, and titratable acidity, % reducing sugars and Brix measurements made (Table 4). The fruit from 1345-4 had the highest readings for titratable acidity followed by the 93397 and 93398 and then the control lines (Table 4). The reducing sugars ranged between approximately 2 - 3% and the Brix measurements were 4.7 - 5.5. The delayed-ripening hybrids were consistently intermediate in there levels for titratable acidity and reducing sugar measurements as compared to the delayed-ripening inbred and the hybrid, 92030. Measurements for Brix were comparable between 92030 and the delayed-ripening hybrids.

Sensory Evaluations

A trained in-house taste panel compared fruit from 1345-4, selected delayed-ripening hybrids and control lines (Table 5). Line 1345-4 ranged higher in aroma, sweetness, sourness, tomato, overall and juiciness as compared to its parental line, 91103-114. The delayed-ripening hybrid lines were rated higher in sourness, tomato and overall compared to the control hybrid 92030. Aroma and sweetness ratings were slightly less for the hybrids as compared to 92030.

Means and Effectiveness of Containment Measures

The experiment was planted at least 60 ft. away from any commercial tomato plantings at this site. The predominance of self pollination in tomato makes the loss of containment through pollen dispersal remote. Fruit were hand harvested to prevent intermixing with other non-transgenic tomatoes not part of this experiment. The test site was sprayed with paraquat and allowed to desiccate before planting to cucurbits for fall 1994 harvest. Six-L's Farms routinely uses this method to destroy tomato vines in preparation for double-cropping following tomato production. In Six-L's experience, herbicide treatment is very effective at controlling volunteer tomato growth. As of 53 days following paraquat treatment (August 4, 1994), Six-L's Farms management had observed no tomato volunteers in the test site. In addition, this site will be fumigated with methyl bromide when new plastic mulch is laid in spring 1995.

Table 1. Agronomic traits of ripening impaired and large fruited tomato lines: Ruskin trial

	Agronomic traits						
Lines	**Disease**	**Vigor**	**Cover**	**flower set**	**fruit color crown**	**fruit color upper**	**fruit set**
1345-1	3.5	3	3	3	2	1	3
1345-4	3	3	3	3	2	1	3
1345-7	3	3	3	3	2	1	3
RG103-114	3	3	3	3	6	5	3
91155	3	3	3	3	6	3	3
91347-Rn1	3	3	3	3	6	4	3
93396	3	3	3	3	5	4	3
93397	3	3	3	3	4	2	3
93398	3	3	3	3	4	2	3
92030	3	3	3	3	6	3	3

Disease rating (1=high amount of disease to 5=very healthy plant)
Vigor rating (1=stunted small plant to 5=vigorous plant growth)
Cover (1=fruit very exposed to 5=fruit covered by leaf canopy)
Flower set rating (1=very low flower set to 5 =100% set)
Crown and upper fruit color (USDA fruit color scale used 1=immature green to 6= red ripe)
Fruit set rating (1=very low set to 5 =100% set)

Table 2. Yield comparison of transgenic lines and large fruited lines: Ruskin trial

Lines	lbs/ac	Marketable fruit lbs/ac	Total wt % Marketable
1345-1	52,568 a	50,518 a	96
1345-4	49,296 a	45,849 a	93
1345-7	45,846 a	45,352 a	99
RG103-114	30,855 a	21,041 b	68
91155	44,307 a	32,178 ab	73
91347-Rn1	35,229 a	19,197 b	54
93397	45,846 a	44,030 a	96

lbs/ac were determined from a randomized complete block trial: 20 plants/line replicated in 4 blocks
Six plants were harvested out of the center of each replication.
Mean comparison were made using Duncan's mutiple range comparison (P=0.05)

Table 3. Percentage of fruit at different stages for single harvest of parental and impaired large fruited lines

Lines	Red	lt red	pink	breaker	mature green
1345-1	0	4	14	19	63
1345-4	0	7	11	20	62
1345-7	0	0	80	20	71
RG103-114	14	15	12	12	47
91155	23	8	12	9	48
91347-Rn1	35	9	12	12	33
93396	0	0	34	8	58
93397	0	4	10	18	68
93398	0	17	17	14	52
93401	25	18	14	5	39
93400	12	18	15	20	35

fruit color stages were based on USDA tomato color standards

Table 4. Reducing sugars, titratable acidity and BRIX determination from large fruited ripening impaired and parental lines after ripening

Lines	Titratable Acidity	% Reducing sugar	BRIX
1345-4	0.50	1.95	4.7
RG103-114	0.39	2.40	5.2
91347-Rn1	0.34	2.40	4.9
93397	0.43	2.70	5.5
93398	0.47	2.65	5.4
92030	0.30	3.00	5.5

Puree's were made from 2-150 g samples and measurements made from these samples.

Titrable acidity is based on malic acid equivalence

% reducing sugar is based on glucose equivalence

Table 5 . Sensory analysis for large fruited ripening impaired and parental lines

	Sensory parameters						
Line	**Aroma**	**Sweet**	**Sour**	**Tomato**	**Overall**	**Juicy**	**Firmness**
1345-4	8.1	1.6	5.3	5.6	5.5	10	4.3
RG103-114	6.1	1.3	3.6	4.6	2.9	7.5	8
93397	6.4	2.5	4	5.3	4.7	7.3	5.8
93398	4.9	2.1	4.8	5.9	5.3	7.3	10.6
92030	6.6	2.4	3.4	5.1	4.1	9.3	7.4

Lines were picked at breaker, ripened with ethylene and taste without holding
Each value is the mean of 7 taste panelist.
Ratings are based on a scale from 1 to 15: 1=perceptible, 5=slight, 10=moderate and and 15 =extreme.

Permit No. 93-302-03
August 3, 1994

ENVIRONMENTAL RELEASE REPORT

USDA Permit No. 93-302-03

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Permittee: Scott Thenell, DNAP, 510/450-9310

Date of Release: December, 1993

Site of Release: Hawaiian Research Ltd., Kaunakakai, Molokai, Hawaii

Site Contact: Danny Iaea, Hawaiian Research Ltd.

Purpose of Release:
This release consisted of producing hybrid crosses of transgenic tomatoes (large-fruited) expressing a sense transcript of a truncated fruit specific ACC synthase gene. The release included the following genotype:

- line 1345-4 carrying pWTT2144/p35S:ACCS; this line was utilized as either the pollen or the seed parent in hybrid crosses.
- non-transgenic lines of proprietary tomato breeding lines.

Schedule of Major Operations

November, 1993	Seed transported to Hawaiian Research facility in Kaunakakai, Molokai, Hawaii. Seed sown in isolation cages established as temporary containment facility.
December, 1993	Plants transplanted to field site on December 28-30, 1994. Approximately 700 transgenic 1345-4 and 540 non-transgenic plants were established in a 0.25 acre plot.
February - March, 1994	Hand emasculations and pollinations performed in the field to produce hybrid crosses.
April, 1994	Plot harvested; hybrid seed collected, cleaned and shipped to DNAP, Oakland, California under Biotechnology Permit

	93-302-02. Plants remaining after harvest were mowed and the entire test site was disked.
July, 1994	The field remained fallow for 90 days during which the site was irrigated and monitored for volunteer tomato plants. No volunteer tomato plants were observed. Release terminated July 28, 1994.

Plant Growth and General Observations

Small quantities of hybrid seed were produced from crosses between line 1345-4 and non-transgenic tomato breeding lines.

An off-type plant was observed at 7-8% frequency in line 1345-4. The off-type appeared upright in habit, leaves tightly curled downward, with branching at acute angles to the main stem of the plant. The off-type plants were removed by hand and not used for hybrid crosses.

The non-transformed parent of line 1345-4 was not planted in this release, so no direct comparison between line 1345-4 and non-transformed parent was possible. However except as noted above, researchers observed no dramatic differences between line 1345-4 and other non-transgenic lines with regard to plant height, vigor, cover, or growth habit.

Three pesticide treatments were applied to control insects (white flies, aphids and thrips) and fungal infections; two separate applications of Evade and Diatect were made, and one application of Evade and Bravo 720 was made. Hawaiian Research staff observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to these treatments.

Means and Effectiveness of Containment Measures

There were no other tomatoes planted at this site, so outcrossing or seed mixture with other tomato plantings not part of this release was impossible. The predominance of self pollination in tomato minimizes pollen dispersal.

The plot was visually monitored for the presence of volunteer tomato plants for 90 days after harvest. Four overhead irrigations and 3 diskings were made during that period. No volunteer tomato plants were observed in the test site during the monitoring period. There was no evidence of any unplanned release or loss of transgenic plants or plant parts during any portion of this release.

Permit No. 93-351-02
July 14, 1994

ENVIRONMENTAL RELEASE REPORT

USDA Permit No. 93-351-02

DNA Plant Technology Corporation
6701 San Pablo Avenue
Oakland, California 94608-1239
Tel: 510/547-2395
Fax: 510/547-2817

Permittee: Scott Thenell, DNAP, 510/547-2395

Date of Release: February, 1994

Site of Release: M. Draper Farms, Indio, California

Site Contact: Trevor Suslow, DNAP, 510/547-2395

Purpose of Release:
This experiment consisted of a field evaluation of transgenic tomatoes (large-fruited and cherry) expressing a sense transcript of a truncated form of a fruit specific ACC synthase gene. The purpose of this trial was to evaluate whether suppression of ACC synthase in the fruit affords control of fruit ripening and allows for improvements in fruit quality for the consumer, and lowering of production costs to the producer.

The experiment included the following genotypes and treatments:

- 3 selections from 1 primary transformant (named 1345) of cultivar 91103-114 carrying pWTT2144/p35S:ACCS (designated homozygous selections of the primary transformant 1345 are 1345-1, 1345-4 and 1345-7)

- 4 selections derived from 4 independent primary transformants of cultivar Baxter's Early Bush Cherry (BEBC) carrying pWTT2144/p35S:ACCS (designated lines B316-3, B324-6, B329-6a, and B391-7 which derive from the primary transformants B316, B324, B329, and B391)

- 6 proprietary hybrid lines of 91103-114 carrying pWTT2144/ p35S:ACCS (designated lines 93396, 93398, 93400 which derive from pollination of the 91103-114 flowers with material from the transgenic 1345 derived lines)

- 7 proprietary cherry tomato hybrid lines carrying pWTT2144/ p35S:ACCS (designated lines 93593, 93594, 93599, 93600, 93602, 93605 and 93606 which derived from crosses between the ripening impaired transgenic BEBC lines with DNAP proprietary cherry tomato lines)

- non-transgenic controls of 91103-114 and BEBC

The experiment was a randomized complete block design with four replications. Each plot consisted of a single row of 20 plants spaced 15 inches within the row, with 5 ft. spacing between the rows. A total of approximately 800 transgenic plants, and approximately 480 non-transgenic controls were transplanted to the field in an area of approximately 0.25 acres.

Schedule of Major Operations

December, 1993	Seed sown at DNAP greenhouse facility in Brentwood, California.
February, 1994	Plants transported to field site in Indio, California and transplanted to field.
May, 1994	Plots harvested and fruit samples returned to DNAP, Oakland for analysis.
June, 1994	Release terminated; plants disked into the field.

Plant Growth and General Observations

Both transgenic and non-transgenic plants grew normally during the course of the experiment (Table 1 and 2). There was very little disease on any of the plants within the experiments. Observations by experienced DNAP breeders and plant pathologists indicated there were no differences between transgenic and non-transgenic lines with regard to plant growth or disease susceptibility. Ratings for plant vigor, cover, flower set and fruit set were comparable for ripening impaired lines, impaired hybrids and control lines.

The plots were regularly monitored for *Agrobacterium* infection symptoms. No symptoms of infection were found in any transgenic plant. No significant fruit damage from birds or rodents was observed. The plants and remaining fruit were destroyed according to protocol.

Fruit Evaluations

The ripening impaired parental lines exhibited less color development in fruit on the crown, or fruit in the upper part of the canopy as compared to control lines and to the hybrid lines (Table 1-cherry tomatoes and Table 2-large fruited tomatoes). This inhibition of fruit color development was also observed for both the parental ripening inhibited lines and derivative hybrid lines after harvesting the fruit and holding at 15°C and 85% relative humidity (Table 3 and 4). For the

cherry tomatoes, ethylene biosynthesis was suppressed by more than 99% in ripening impaired parent lines and 90% in hybrid lines (Table 3 and 4) compared to control lines. In the large fruited tomatoes, the color development and ethylene measurements of hybrid impaired lines were intermediate to the ripening impaired parental lines and control lines.

Yield Observations

In order to determine the feasibility of reduced harvesting frequency for the ripening impaired fruit, we performed a single harvest on all of the lines at a time when 10-20% of the fruit on the vines of the control lines were at the breaker stage; the fruit were sorted into color stages and the % marketable fruit determined. Pounds per acre of marketable cherry tomato fruit from 4 out of 5 impaired lines were significantly higher than the BEBC control (Table 5). The percentage of marketable fruit of the impaired lines were also significantly higher for all of the ripening impaired lines as compared to the BEBC control (Table 5). The hybrid lines had an intermediate amount of color development as compared to the control line. On average, approximately 40% of the harvested fruit was at red ripe and 50% was sorted at pink to light red for the hybrid lines, whereas the BEBC control was approximately 65% and 27% respectively for fruit at the red and pink to light red stage pink (Table 7). The inbred impaired lines generally had a higher percentage of fruit at the breaker/turning stage.

Pounds per acre of marketable large fruited tomatoes were comparable between the impaired the control lines (Table 6). However, the percentage of marketable fruit of the impaired lines were significantly higher for all of the 1345 selections as compared to the control lines (Table 6). Two out of three ripening impaired hybrid lines exhibited significantly lower yield of marketable fruit as compared to the 91103-114 control, but were not significantly different than their ripening impaired parent. The ripening impaired hybrid lines exhibited an intermediate amount of color development as compared to the control line (Table 8). On average, approximately 30% of the harvested fruit was at red ripe and 30% was sorted at pink to light red for the hybrid lines, whereas the control was approximately 45% and 35% respectively for fruit at the red and pink to light red stage pink. The inbred impaired 1345 selections generally had a higher percentage of fruit at the breaker/turning stage .

Means and Effectiveness of Containment Measures

The experiment was planted more than 30 ft. away from other tomato experiments conducted at this site. The predominance of self pollination in tomato minimizes the likelihood of pollen dispersal.

The experimental plot site will be visually monitored after the termination of the experiments for evidence of any remaining growth of test plants or volunteers.

Table 1. Agronomic traits of ripening impaired and parental cherry tomato lines: Indio trial

	Agronomic traits						
Lines	Disease	Vigor	Cover	Flower set	Fruit color crown	Fruit color upper	fruit set
B316-3	5	5	4	4	4	2.5	4
B324-6	5	5	4	4	4	2	4
B329-6a	5	4	4	5	4	2	4
B391-7	5	5	5	4	4	2	4
BEBC	5	4	5	4	6	6	4
Br124	5	4	3	4	6	4	4
93594	5	5	4	4	6	5	4
93593	5	5	4	4	6	4	4
93599	5	4	4	4	6	4.5	4
93600	5	5	4	4	6	4	4
93602	4	4	4	4	6	4	4
93605	5	5	4	4	6	5	4
93606	5	5	4	4	6	4	4

Disease rating (1=high amount of disease to 5=very healthy plant)
Vigor rating (1=stunted small plant to 5=vigorous plant growth)
Cover (1=fruit very exposed to 5=fruit covered by leaf canopy)
Flower set rating (1=very low flower set to 5 =100% set)
Crown and upper fruit color (USDA fruit color scale used 1=immature green to 6= red ripe)
Fruit set rating (1=very low set to 5 =100% set)

Table 2. Agronomic traits of large fruited ripening impaired and parental tomato lines: Indio trial

	Agronomic traits						
Lines	Disease	Vigor	Cover	flower set	fruit color crown	fruit color upper	fruit set
1345-1	4	4	4	4	3	2	4
1345-4	4	4	3.5	4	3	2	4
1345-7	4	4	4	4	3	1.5	4
RG103-114	4	4	4	4	6	5	4
91155	4	4	3	4	6	4	4
91347-Rn1	5	4	3	4	6	4	4
93396	5	4	3.5	4	3	2	4
93398	5	4	4	4	4	2	4
93397	5	4	4	4	5	2.5	4
93400	4	4	4	4	5	2	4
93401	5	4	4	4	6	5	4
92030	4	4	4	4	6	4.5	4

Disease rating (1=high amount of disease to 5=very healthy plant)
Vigor rating (1=stunted small plant to 5=vigorous plant growth)
Cover (1=fruit very exposed to 5=fruit covered by leaf canopy)
Flower set rating (1=very low flower set to 5 =100% set)
Crown and upper fruit color (USDA fruit color scale used 1=immature green to 6= red ripe)
Fruit set rating (1=very low set to 5 =100% set)

Table 3. Color development and C2H4 measurements of ripening impaired and parental cherry tomato lines

Lines	a/b ratio		C2H4
	5 days after harvest	12 days after harvest	nl/g-hr
B316-3	0.36	0.52	0.2
B324-6	0.22	0.25	0.1
B329-6a	0.55	0.71	0.3
B391-7	0.12	0.23	0
BEBC	0.95	1.3	51
Br124	0.6	1	7.25
93594	0.68	0.8	0.6
93593	0.57	0.7	1
93599	0.68	0.84	0.65
93600	0.5	0.89	1.5
93602	0.7	1.18	2.15
93605	0.7	0.8	0.3
93606	0.37	0.52	5.25

a/b ratio's were calculated from L. a. b. measurements made with Minolta CR300 colorimeter
C2H4 measurements were made using gas chromotography

Table 4. Color development and C2H4 measurements of ripening impaired and parental large fruited lines

Lines	a/b ratio		C2H4
	5 days after harvest	12 days after harvest	nl/g-hr
1345-1	-0.4	0.19	0.2
1345-4	-0.4	0.28	0.4
1345-7	-0.25	0.28	0.7
RG103-114	0.6	1.22	43.4
91155	0.61	1.27	65.1
91347-Rn1	0.7	1.44	30.2
93396	0.5	1.1	2.3
93398	0.1	0.7	1.4
93397	0.3	0.68	1.4
93400	0.3	0.97	2.2
93401	0.24	0.87	7.3
92030	0.5	1.4	20.5

a/b ratio's were calculated from L. a. b. measurements made with Minolta CR300 colorimeter
C2H4 measurements were made using gas chromotography

Table 5. Yield comparison of transgenic lines and Baxter's Early Bush: Indio trial

Lines	lbs/ac	Marketable fruit lbs/ac	Total wt % Marketable
B316-3	17,236 e	13,327 cd	78 ab
B324-6	37,898 a	34,217 a	90 a
B329-6a	20,793 de	16,021 c	77 ab
B391-7	31070 b	24,471 b	79 ab
BEBC	27,071 bc	9,484 d	35 c
Br124	23,917 bc	15,422 c	64 b

lbs/ac were determined from a randomized complete block trial: 20 plants/line replicated in 4 blocks

Table 6. Yield comparison of transgenic lines and large fruited lines: Indio trial

Lines	lbs/ac	Marketable fruit lbs/ac	Total wt % Marketable
1345-1	14,245 b	13,981 abc	98 a
1345-4	14,927 b	14,324 abc	95 a
1345-7	14,438 b	12,990 abc	89 ab
RG103-114	33,899 a	18,304 ab	54 c
91155	33,100 a	20,304 a	59 c
91347-Rn1	11,842 b	7,121 c	63 c
93396	10,269 b	6,933 c	69 bc
93398	10,153 b	7,149 c	70 bc
93400	14,779 b	10,790 bc	72 bc

lbs/ac were determined from a randomized complete block trial: 20 plants/line replicated in 4 blocks

Table 7. Percentage of fruit at different stages for single harvest of parental and impaired cherry tomato lines

Lines	Mature green	Breaker/ Turning	Pink/lt red	red
B316-3	0	57	21	22
B324-6	0	44	46	10
B329-6a	0	9	69	22
B391-7	1	42	36	21
BEBC	0	8	27	65
Br124	6	19	40	35
93594	0	10	42	48
93593	0	12	55	33
93600	0	13	54	33
93602	0	12	47	41
93605	0	10	50	40
93606	0	18	38	44

fruit color stages were based on USDA tomato color standards

Table 8. Percentage of fruit at different stages for single harvest of parental and impaired large fruited lines

Lines	Mature green	Breaker/ Turning	Pink/lt red	red
1345-1	35	36	27	2
1345-4	17	33	45	5
1345-7	19	43	27	11
RG103-114	0	19	35	46
91155	11	19	29	41
91347-Rn1	7	30	26	38
93396	10	28	31	31
93398	8	30	32	29
93400	8	17	47	28

fruit color stages were based on USDA tomato color standards

1994 Fresh Market Tomato Experiment
USDA Permit No. 94-060-04N

INTERIM REPORT

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This experiment consisted of a field evaluation of transgenic tomatoes expressing a sense transcript of a truncated ACC synthase gene. The plant lines being evaluated have reduced levels of ACC synthase RNA, and ACC synthase activity as a consequence of the introduction and expression of the additional, truncated ACC synthase gene [this phenomenon has been termed 'sense suppression']. We want to determine whether suppression of ACC synthase activity is a practical and commercially useful approach to the control of tomato fruit ripening and improvement in fruit quality.

The transgenic lines which we are evaluating were generated by *Agrobacterium* transformation of tomato explants with the binary plasmid pWTT2144/ACCS. The T-DNA region which has been transferred to the transgenic plants carries a neomycin phosphotransferase (NPTII) coding region fused to the *nos* promoter and *ocs* terminator, which allows for the initial selection of transformed material and also allows for the determination of the segregation of the introduced T-DNA in subsequent generations. In addition the T-DNA includes a 1.2 kb region from the *Acc2* coding region, fused in-frame to the CaMV 35S promoter at the 5' end, and the *nos* terminator. Sequences which encompass the T-DNA left and right border regions flank these transferred genes. A summary of all of the sequences which are included in the transferred T-DNA is provided in Table 4.

The field test was conducted under provisions of the USDA notification procedures described in the Final Rule (7 CFR Part 340, 58 FR 17044, March 31, 1993). USDA Submission No. 94-060-04N was acknowledged on March 15, 1994.

The test was conducted in Painter, Virginia.

This experiment included the following genotypes and treatments:

- two homozygous selections from 1 independent transgenic line of cultivar RG103-114 transformed with the binary plasmid pWTT2144/ACCS (lines designated 1345-1 and 1345-4)

- 2 hybrid lines between 1345-4 (RG103-114 transformed with the binary plasmid pWTT2144/ACCS) and DNAP proprietary non-transgenic lines (designated lines 94655, 93397)

- 4 independent transgenic lines of cultivar Baxter's Early Bush Cherry (BEBC) transformed with the binary plasmid pWTT2144/ACCS (lines designated B316-3, B324-6, B329-6a, and B391-7)

- non-transgenic control lines (RG103-114, 91101-33, 91347, 92030, 91389 and BEBC)

Experimental Design:
The experiment was designed as an observational trial with one replication. Each plot consisted of a single row of 20 plants spaced 15 inches within the row, with 5 ft. spacing between the rows. Approximately 160 transgenic plants and 120 non-transgenic controls were transplanted to the field, for a total of (less than 0.1 acres). The experiment was planted more than 30 ft. away from other tomato experiments conducted at this site.

Schedule of Major Operations

February 28, 1998	Seed sown in greenhouse.
April 16, 1994	Plants transported to field site and transplanted to field.
July 20, 1994	Harvest fruit from plots.
Mid August, 1994	Conclude experiment, clean up experimental plots.
Spring, 1995	Check area for volunteer plants

Plant Growth and General Observations

Cherry tomato lines:

Agronomic Properties

Both transgenic and non-transgenic plants grew normally during the course of the experiment. We did not observe any differences in the growth habit between the transgenic lines and the parental BEBC line. The seedling survival rates for the transgenic and parental lines were similar, ranging from 94 to 100 %. There was very little to no insect damage on any of the lines. We did observe a very low incidence of bacterial speck on lower leaves of both transgenic and parental lines, but no differences in susceptibilities were observed. Likewise, agronomic ratings for disease, vigor, cover, flower and fruit set were comparable between all lines (Table 1).

Fruit Evaluations:

We measured the levels of ACC2 RNA in fruit from the genetically engineered lines and compared these to the normal levels in fruit. We found that the steady state mRNA levels of the ACC2 transcript are significantly reduced in fruit of the selected transgenic lines (ranging from 10-25%) compared to the control lines. We have compared the ripening properties of the control and transgenic fruit. There was more color development on both crown fruit and fruit in the upper part of the canopy in the control line BEBC compared to the four ripening impaired lines (Table 1). At the single harvest point (the point at which approximately 80% of the BEBC fruit was at the red stage) the percentage of marketable fruit ranged from 28 to 87 for the ripening impaired lines but was only 17 % for the control line, BEBC (Table 2). No sensory evaluations were made on the cherry tomato lines.

Large fruited lines

Agronomic Evaluations:

There were no observable differences between the growth properties of the transgenic and non-transgenic plants during the course of the experiment. The survival rates for the transgenic and parental lines ranged from 95 to 99 %. There was very little to no insect damage on any of the lines. There was also a very low incidence of bacterial speck on lower leaves of both transgenic and parental lines, but no differences in susceptibilities were observed. The agronomic ratings for disease, vigor, cover, flower and fruit set were comparable between all lines (Table 1).

Fruit Evaluations:

We have measured the levels of ACC2 RNA in fruit of transgenic and control lines at different stages of development and we have observed that 1345-1 and 1345-4 fruit have levels of ACC2 RNA which are identical to the levels measured for the parental 1345 fruit, and are reduced to less than 10% of the level present in the parental line.

The fruit on the transgenic lines, both inbreds and hybrids appeared to ripen more slowly than the fruit on control plants. There was more color development on both crown fruit and fruit in the upper part of the canopy in the control lines (RG103-114, 911101-33, 91347, 92030 and 91389) compared to the ripening impaired lines (Table 1). Lines 1345-1 and 1345-4 exhibited the greatest amount of ripening suppression in the crown fruit, but ratings for color development in the upper canopy fruit were more comparable between all of the ripening impaired lines (inbreds and hybrids). Fruit was collected and returned to the Oakland laboratory where color measurements were made after holding fruit for 4 wks at 15°C. Fruit from line 1345-4 had an a/b ratio of 0.2 followed by fruit from lines 93397 and 94655 (0.63 and 0.70, respectively). The control lines (RG103-114, 92030 and 91389) had a/b ratios between 1.1 and 1.3. After ripening in ethylene the color measurements (a/b values) for all ripening impaired and control lines were comparable (between 1.15 and 1.3.)

At a single harvest the percentage of marketable fruit ranged from 89 to 100 % for the ripening impaired lines and for 67 to 71% for the control lines (Table 2). The control lines averaged approximately 25 % fruit at the red ripe stage at harvest whereas, the ripening impaired lines were between 0 to 11 % at red ripe. Sensory evaluations have been made on fruit from selected lines after ripening with ethylene (20°C, 120-150 ppm C_2H_4) (Table 3). Sensory parameters for sweetness, sourness, tomato, overall, juicy and firmness were made. In general, any difference amongst lines for a given parameter could be accounted within the mean standard deviation, indicating that the transgenic and parental lines were comparable in their ranking for a trait.

The plots were regularly monitored for *Agrobacterium* infection symptoms. No symptoms of infection were found in any transgenic plant. No fruit damage was observed from birds or rodents. The plants and remaining fruit will be destroyed according to protocol.

The experimental plot site will be monitored for 6 months after the termination of the experiments for evidence of any remaining growth of test plants or volunteers.

Table 1: Agronomic evaluations of ripening impaired and parental lines: Virginia field trial

Lines	disease	vigor	cover	flower set	color crown	color upper	fruit set
Large fruited lines							
RG103-114	4	4	3.5	3	5	2.5	3.5
91101-33	3	3.5	3	3	5	3.5	3
91347	3	3.5	3	3	5	4	3.5
1345-1	4	4	4	3.5	2	1	3.5
1345-4	4	4	3.5	3	3	2	3.5
94655	4	4	3	3.5	5	2	4
93397	5	4	4	3.5	4	1	3.5
92030	3	3.5	3.5	3.5	5.5	4	4
91389	3	3.5	3.5	3.5	5	3.5	3
Cherry tomatoes lines							
B316-3	4	4	4	4	4.5	3	4
B324-6	3	4	3.5	4	5.5	3.5	4
B329-6a	4	4	3.5	4	5.5	4	4
B391-7	4	4	3.5	4	5	3.5	4
BBEC	3	4	3.5	4	6	4	4

scale :

disease	1 to 5 : 1 very diseased to 5 very healthy
vigor	1 to 5: 1 shows little vigor to 5 very vigorous
cover	1 to 5: 1 shows little cover to 5 a lot of cover
flower set	1 to 5: 1 shows little flower set to 5 all flowers setting fruit
crown color	based on USDA color standards: 1=mtgrn, 2= breaker, 3=turn, 4=pink, 5=ltred and 6=red ripe
upper color	based on USDA color standards: 1=mtgrn, 2= breaker, 3=turn, 4=pink, 5=ltred and 6=red ripe
fruit set	1 to 5: 1 shows little fruit set to very good fruit set

Table 2. Percentage of fruit at different color stages for a single harvest: Virginia field trial

Lines	Red	Pink/lt red	Breaker/ turning	Mature green	% Marketable
Large fruited lines					
RG103-114	29	18	14	39	71
91101-33	23	18	18	42	77
91347 Rn1	11	20	25	44	89
1345-1	0	1	9	89	99
1345-4	0	7	9	84	100
94655	11	8	14	67	89
93397	6	3	9	82	94
92030	33	14	25	29	67
91389	23	31	24	21	77
Cherry tomato lines					
B316-3	13	29	28	30	87
B324-6	72	18	7	3	28
B329-6a	68	12	6	14	32
B391-7	43	27	8	23	57
BEBC	83	3	3	11	17

fruit color stages were based on USDA tomato color standards
% Marketable is defined as fruit that could be packed and sold.

Table 3. Sensory evaluation of ripening impaired and parental lines: Virginia field trial

	Sensory parameters					
Line	Sweet	Sour	Tomato	Overall	Juicy	Firmness
91389	4.4	3.8	8.3	8.5	10.0	8.8
92030	4.2	5.4	8.5	8.7	8.9	10.7
RG103-114	3.6	4.0	7.6	7.6	9.2	9.2
93397	3.1	2.4	7.2	6.8	9.5	8.1
1345-4	2.3	2.1	5.4	5.2	8.1	7.0
94655	3.6	4.6	7.6	7.2	9.5	9.5
mean standard deviation	2.4	1.9	2.6	2.6	2.0	2.4

Lines were picked at breaker, shipped overnight to the Oakland laboratory, ripened with ethylene and tasted.
Each value is the mean of 7 taste panelist.
Ratings are based on a scale from 1 to 15: 1=perceptible, 5=slight, 10=moderate and and 15 =extreme.

Table 4 1 Summary of Sequences of pWTT2144/AccS

Genetic Element	Size, Kb	Function
p35S	0.96	The cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985; Harpster et al., 1988)
Cab22	0.069	The plant gene leader sequence from the Cab22L chlorophyll a/b binding protein of petunia corresponding to the 5' untranslated region of the Cab22R gene (Dunsmuir, 1985; Harpster et al., 1988)
AccS	1.09	The truncated coding region from the tomato *Acc2* gene which corresponds to a 1088 bp region from base 149 to base 1237 (Rottman et al., 1991)
nos3'	0.27	The untranslated 3' region of the nopaline synthase gene from Agrobacterium tumefaciens (Depicker et al., 1983)
pnos	0.6	The untranslated promoter region of the nopaline synthase gene from Agrobacterium tumefaciens (Depicker et al., 1983)
nptII	1.02	The neomycin phosphotransferase II gene from Tn5 (Beck et al., 1982)
ocs3'	0.56	The untranslated 3' region of the octopine synthase gene from Agrobacterium tumefaciens (DeGreve et al., 1983)
LacZ'	0.466	The untranslated LacZ polylinker sequence (Yanish-Perron et al., 1985)
pACYC184	1.535	The origin of replication from plasmid pACYC184 which ensures replication in E. coli (Chang et al., 1979)
pVS1	8	The pVS1 replicon derived from Pseudomonas aeruginosa DNA which ensures replication in Agrobacterium tumefaciens (Itoh et al., 1984)
Tc	2.5	The tetracycline resistance marker from Tn803 which allows for selection of the binary plasmid in Agrobacterium tumefaciens and E. coli (Waters et al., 1983)
LB	0.589	The left and right border regions of T-DNA from Agrobacterium tumefaciens (Barker et al., 1983; van den Elzen et al., 1985)
RB	0.303