

96-248-010



CALGENE

August 30, 1996

Dr. Arnold Foudin
Deputy Director, Biotechnology Permits
USDA, APHIS, BBEP
4700 River Road, Unit 147
Riverdale, MD 20737-1237
phone 301-734-7612

Subject: Request to amend the Determination of Non-regulated Status to add one FLAVR SAVR™ tomato line

Dear Dr. Foudin,

Enclosed is a request to add one additional FLAVR SAVR tomato line, 532A 4109a 5166, to the existing Determination of Non-regulated Status for this product.

Calgene asks that the review be expedited in a similar manner to addition of previous FLAVR SAVR tomato lines. The enclosure contains the same type of data and information accepted for the most recent additions of FLAVR SAVR lines [60FR: 38788 (July 28, 1995) and 60FR 15284 (March 23, 1995)].

This request contains no confidential business information.

The ability to grow these lines commercially as soon as possible is very important to Calgene and our subsidiary Gargiulo, and we appreciate your prompt assistance. Please call either me at 916-753-6313 should you have questions or require additional information.

Sincerely,

Lori Malyj
Manager, Regulatory Affairs

Enclosure

Stamp:



CALGENE

Field Trial Report ~~95-089-10N~~, 95-089-11N
Ann Yanagi 3/19/96

Contains No Confidential Business Information

Field Trials Under USDA Notification

Locations	Gene(s)	Notification Number
Solano County, CA (Dixon)	asPG	95-089-10N
Solano County, CA (Dixon)	SPS	95-089-11N

Introduction

The objective of the trial was to evaluate genes in fresh market tomatoes. The genes and related construct numbers evaluated are antisense polygalacturonase (asPG) construct pCGN 4109, and Sucrose Phosphate Synthase (SPS), pCGN 3812 and pCGN 3815. The trial was planted on May 22, 1995.

Chronology

Site	Seeded in greenhouse	Planted	Harvested	Destruction	Final Monitoring
Dixon, CA	April 17, 1995	May 22, 1995	August 17, 24 & 28, 1995; September 21, 1995	October 10, 1995	March 18, 1996

Trial Entries, # 95-089-10N

Variety	Construct	Event
519A	4109a	4509
532A	4109a	5166
88F	4109a	2738

Trial Entries, # 95-089-11N

Variety	Construct	Event
UC82B	3812	9
UC82B	3815	12
UC82B	3815	13

The trials also contained deregulated FLAVR SAVR tomato lines and non-transgenic controls.

Maintenance of Transgenic Plant materials

To prevent the dissemination of propagules, the following precautions were taken. All transplants were grown in Calgene greenhouses. Seed was maintained and handled under the control of Calgene personnel within Calgene facilities. Transport of transplants to the field was conducted within an enclosed trailer.

Field Operations

Planting of the transplants was done by hand. Planting was supervised by Calgene personnel. Any leftover transplants were destroyed and left in the field. Standard tomato cultural practices were conducted by the farmer during the seedling and vegetative stages. Plants were grown on poles. Pesticides and herbicides were applied by ground application. Harvesting and subsequent transport of the transgenic fruit was conducted by Calgene personnel within sealed boxes in enclosed vehicles to Calgene facilities.

Containment and Safety

Bare earth buffers of 30 feet each were maintained on all sides of each trial to enhance the prevention of pollen transfer and seed dissemination. This zone was kept weed free by periodic cultivations and herbicide applications. No evidence of horizontal movement into other organisms by the transgenic tomatoes was observed during the growing season.

Plant Observations

During the growing season, the field trial was monitored regularly to check for potential problems with insects, diseases, stresses, and abnormal phenotypes: The types of insects and diseases plants were checked for are as follows:

INSECTS: Stink bug, army worm, pin worm, tomato fruit worm and horn worm, aphids, russet mites, thrips, white flies, leaf miners and flea beetle.

DISEASES: Phytophthora, fusarium, crown gall, stem canker, powdery mildew, tobacco mosaic, early and late blight, tomato spec., and leaf spot.

Specifically, each trial was monitored weekly by a Pest Control Advisor (PCA) or cooperator (Paul Lum) hired by Calgene. As the plants approached maturity, the frequency of monitoring was increased; generally 2 to 3 times per week.

The field was walked and observations were made comparing the transgenic plants to their respective non-transgenic controls on nine separate occasions including the dates of harvest, by Calgene research staff. Plant observations were made regarding plant morphology, flowering, fruit set, fruit ripening, and general plant health with respect to disease and insects. Inspections were made on 5/29, 6/23, 7/21, 8/7, 8/11, 8/17, 8/24, 8/28 and 9/21, whereas the field was walked through and observed on a plant by plant, line by line basis, comparing the transgenic plants to their non-transgenic siblings. On these dates no crown gall disease or cauliflower mosaic virus (CaMV) was observed. Verticillium Race 2 was observed in the trial on 8/7/95 inspection. No insects were observed on any of the inspection dates. Plants in each portion of the trial were examined for any abnormalities arising from the transformation process utilizing the controls included for each transgenic

line to aid the observation process. These observations were conducted throughout the entire growing cycle. Some stunting and retarded or enhanced fruit ripening occurred in some lines due to water stress. The advanced lines grown in the trials did not exhibit abnormal appearance or characteristics.

Plants transformed with the asPG construct appeared normal with respect to plant morphology and fruit development when compared to their non-transgenic parents.

Harvest and Gene Function

Fruit from the trial was harvested on 8/17, 8/24, 8/28 and 9/21.

Fruit were harvested on 8/17 & 8/28/95 for laboratory analysis for the reduction of the enzyme polygalacturonase, and 9/21/95 for seed. Fruit were harvested at stage 6 (red) and were placed into plastic zip lock bags, and the bags containing fruit were placed into a cardboard tomato packing box. Fruit were transported to Calgene laboratory or seed production facilities in an enclosed van.

At completion of all evaluations, fruit were either destroyed (autoclaved) in Davis or shipped to Galt, CA for seed collection and destruction of remaining fruit parts.

Trial Destruction

Upon completion of the field trial harvest, the poles and string were then pulled and a flail mower was used to chop the plants. The field was disked and stubble disked four times on October 10, 1995 using the cooperating farmer's equipment. To prevent seed dissemination, field equipment was scraped and water tanks equipped with high pressure pumps were used to clean both the tractor and implement to remove all reproductive plant parts. This procedure was performed within the field trial boundaries.

The field was disked October 10, 1995. Beds were made and the field lay fallow during the winter 1995. The site was monitored March 18, 1996, and no volunteer tomato plants were present.

FLAVR SAVR™ Tomato Line for Addition to the Determination

Calgene requests that the following FLAVR SAVR tomato transformation event (line) be added to the existing Determination of Nonregulated Status:

532A 4109a 5166

Tomato lines produced using the identical construct (pCGN4109) and methods have been previously deregulated by APHIS. The safety of FLAVR SAVR tomatoes and the lack of potential for weediness have been previously addressed. The genetic sequences in pCGN4109 and their gene products have been extensively characterized. The tomato lines which are the subject of this current request also meet the definition of FLAVR SAVR tomato and demonstrate the same characteristics as previous lines.

The data supplied in this attachment was produced using the same protocols as the data in support of the previous addition of FLAVR SAVR lines [e.g. January 26, 1995 request, finalized in 60FR 15284 (March 23, 1995)]. Presence of the antisense PG gene is demonstrated by reduced polygalacturonase enzyme activity (Section A, below). The presence of the *kan^r* gene is confirmed by the transformation process used for all FLAVR SAVR lines: selection on media containing kanamycin. Results of Southern analysis show there was no transfer beyond the left and right borders for this FLAVR SAVR line (Section B, below). Line 532A 4109a 5166 has been field tested under Release Notification # 95-089-10N, and a field trial report has been submitted previously to USDA APHIS BBEP. Trial observations demonstrate that this FLAVR SAVR (asPG) line was normal in morphology, development and disease resistance (copy provided in Section C, below).

Section A. Polygalacturonase (PG) Enzyme Activity

Line 532A 4109a-5166 shows markedly reduced PG enzyme activity as compared to the non-transgenic control. This is the predicted, intended effect of the FLAVR SAVR gene.

Generation	Genotype	PG Activity ¹
	532A control	0.98
T ₁	532A 4109a-5166	0.195 ²

¹Assay procedure previously provided. The procedure is based on Sheehy, et al. (1988. PNAS 85:8805).

² Average of two replicates

Section B. Southern analysis for DNA transfer beyond T-DNA borders

The data supplied in this attachment was produced using the same protocols as the data in support of the previous additions of FLAVR SAVR lines. The results of analysis confirm that there was no transfer beyond the left and right borders in 532A 4109a-5166.

The protocol used for analysis has been extensively used by Calgene to test FLAVR SAVR lines for the presence of backbone sequences. Data generated using this protocol has been previously reviewed by APHIS in regard to the deregulation of FLAVR SAVR lines, as follows:

60 FR: 38788-38789, July 28, 1995

60 FR: 15284, March 23, 1995

59 FR: 59746, November 18, 1994

In addition, data generated using this protocol were reviewed by FDA in connection with the concluded consultation on food safety of FLAVR SAVR tomato (FDA. 1994 (May 23). Calgene, Inc.; Availability of Letter Concluding Consultation (Docket No. 91A-0330). Federal Register. 59:26647-26648).

This method is a very sensitive analytical technique to detect whether backbone sequences have been integrated into the transformed plant. Plasmid pCGN1532 contains identical backbone sequences to the constructs used to produce FLAVR SAVR tomatoes (pCGN1436; pCGN4109), as it is the common backbone to Calgene's 7 binaries. The pCGN1532 DNA is used as the probe template to generate a nested set of uniformly labeled probes (via random primed DNA labeling) that cover all backbone sequences in the constructs used to produce the transgenic plants.

Figure 1 following page) provides Southern hybridization data for line 532A 4109a-5166, as well as analyses for other lines not under consideration for this request. The blots shown in the Figure were hybridized to the same pCGN1532 probe at the same time and exposed to film for 3 days to produce the autoradiograms.

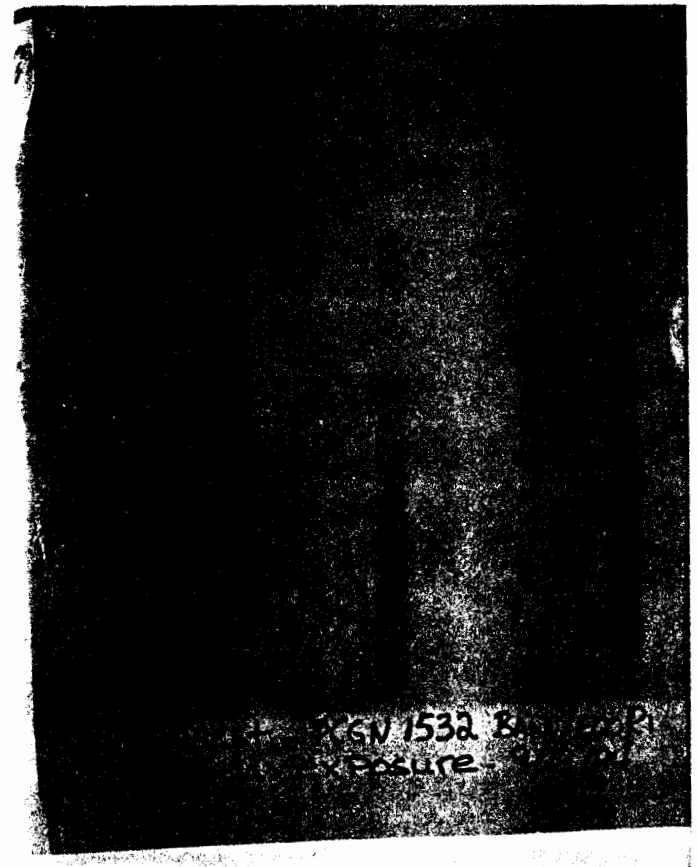
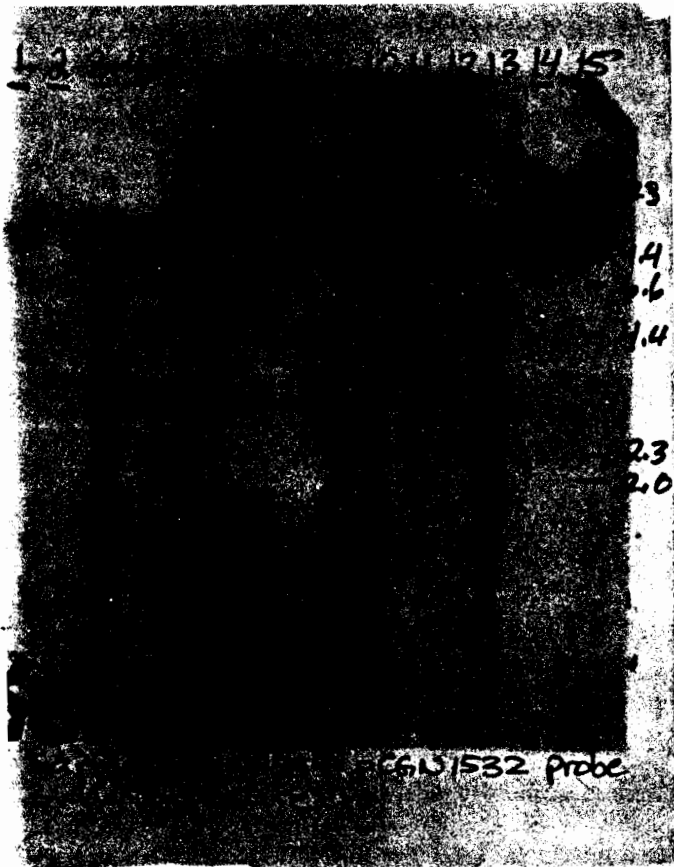
Analysis of results shown in Figure 1:

Blot A: Lane 3 containing DNA from line 540A 4109a-1650, a plant known to contain transfer beyond the T-DNA borders based on previous analyses, was loaded with an equal amount of DNA as used in the other lanes. It shows hybridization to the pCGN1532 probe, as expected. Blot B contains results from the positive control plasmid DNA and an additional lane of 540A 4109a-1650 DNA, both of which hybridized to the pCGN1532 probe. Lane 1 contains DNA from a non-transformed control plant and does not show hybridization to the pCGN1532 probe. Transgenic plant line 532A 4109a-5166 in Lane 14, the subject of this request, did not show any hybridization to the pCGN1532 probe, indicating no transfer occurred beyond the T-DNA borders.

Blot B. Lane 15, containing one copy of pCGN1436 plasmid DNA (with the same backbone structure as pCGN4109a) was used for a positive control and showed the 5 bands expected when cut with the restriction enzyme BglII and hybridized to the pCGN1532 probe. Lane 9, containing DNA from line 540A 4109a-1650, a plant known to contain transfer beyond the T-DNA borders based on previous analyses, was loaded with about half the DNA used in the other lanes. It also shows hybridization to the pCGN1532 probe, as expected. Lane 8 contains DNA from a non-transformed control plant and does not show hybridization to the pCGN1532 probe. Lane 1 contains DNA from line 519A 4109a-4645, a line previously granted deregulation, and did not show hybridization to the pCGN1532 probe.

FIGURE 1: Southern Blot of tomato lines transformed with 4109a and 1436 (asPG).
 BLOT A

BLOT B



Blot A. Plant DNA was digested with the restriction enzyme BglIII. The blot was probed with random-primed labelled pCGN1532 (linearized with the restriction enzymes BamHI and EcoRI before labelling) and exposed to X-ray film for 3 days. Lane assignments are as follows:
 (1) **105F Control**, (2) 137F 4109a-71, (3) **540A 4109a-1650**, (4) 540A 4109a-1739, (5) 105F 1436-2062 (6) 105F 1436-2072, (7) 105F 1436-2077, (8) 105F 1436-2114, (9) 105F 1436-2126, (10) 105F 1436-2181, (11) 35F 4109a- 3013, (12) 35F 4109a- 3023, (13) 42F 4109a-4050, (14) **532A 4109a-5166**, (15) molecular weight markers.

Blot B. Southern Blot of tomato lines transformed with 4109a (asPG), conducted as above. One copy of BglIII restriction enzyme-digested pCGN1436 plasmid DNA (containing the same backbone structure as pCGN4109a) was utilized as a positive control. Lane assignments:
 (1) **519A 4109a-4645**, (2) 519A 4109a-4509, (3) 540A 4109a-1819, (4) 540A 4109a-1776 (5) 540A 4109a-1772 (6) 540A 4109a-1763, (7) 540A 4109a-1759, (8) **540A non-transformed control**, (9) **540A 4109a-1650**, (10) 540A 4109a- 1555, (11) 540A 4109a-1526, (12) 137F 4109a-143B, (13) 137F 4109a-30, (14) molecular weight markers, (15) **pCGN1436**.

Section C: Field Trial Report

A copy of the previously filed report on Release Notification # 95-089-10N is provided. This trial included the tomato line which is the subject of this request, and demonstrates that the characteristics are as expected for tomatoes.