Euphytica **79:** 293–297, 1994.

Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVR^{TM} tomato story

Matthew G. Kramer & Keith Redenbaugh
Calgene, Inc., 1920 Fifth Street, Davis, CA 95616, USA

Key words: Fruit ripening, antisense, polygalacturonase

Abstract

The FLAVR SAVR^{TM} tomato was developed through the use of antisense RNA to regulate the expression of the enzyme polygalacturonase (PG) in ripening tomato fruit. This enzyme is one of the most abundant proteins in ripe tomato fruit and has long been thought to be responsible for softening in ripe tomatoes. The FLAVR SAVR^{TM} tomato is the first genetically engineered whole food to be sold in commerce. The history of the development of this product is discussed beginning with the results of the original antisense work (including conclusions regarding the role of PG in ripe tomatoes) and will be followed by a description of the regulatory and food safety assessment. Finally, the development of an operating business to produce, market and distribute a genetically engineered whole food product is discussed.

Introduction

The FLAVR SAVR^{TM} tomato is the first genetically engineered whole food to be sold in commerce following FDA approval on May 18, 1994. FLAVR SAVR^{TM} tomatoes (*Lycopersicon esculentum* Mill.) are defined as tomato cultivars or progeny of tomato lines genetically engineered using an antisense polygalacturonase gene isolated from tomato (Sheehy et al., 1987). These tomato cultivars were developed to improve flavor and taste in fresh market tomatoes. The polygalacturonase (PG) gene was isolated from tomato and reintroduced in the antisense orientation. PG is the major enzyme involved in pectin metabolism during fruit ripening and has historically been associated with fruit softening (Hobson, 1965; Brady et al., 1985). The use of an antisense strategy to reduce the expression of the PG gene in tomatoes causes decreased pectin solublization in the ripening fruit which in fresh market tomatoes results in ripe fruit that remain intact for extended periods of time (Kramer et al., 1992). In terms of a commercially viable product, the technology allows for the production of fresh market tomatoes which can be vine-ripened for enhanced flavor and have a longer shelf life yet still survive the traditional distribution system intact.

Development and characterization

Plant material used for both research and product development has included both processing and fresh market tomato varieties. Initial transgenic lines were generated by *Agrobacterium*-mediated transformation to develop a set of commercially viable breeding lines which have been used in an ongoing breeding and variety development program. In addition, the developmental material has allowed for what is probably the most extensive safety evaluation of tomato that has ever been undertaken. This was necessary because not only has the antisense technology never before been applied to a whole food product, but in addition it has been necessary to demonstrate the precision and safety of techniques which utilize *Agrobacterium*-mediated transformation as a method of creating new and novel genotypes and genotypic combinations. As a result, the FLAVR SAVR^{TM} tomato currently available in the market represents a unique combination of tradition-

al plant breeding technologies and the techniques of molecular biology and genetic engineering.

Initial tomato lines for both phenotypic characterization and product development were produced through transformation with a PG antisense construct pCGN 1416 (Sheehy et al., 1988). Approximately 50 independent transformation events were generated for line selection in each variety transformed. Plants were then selected for further evaluation based on the levels of PG activity, whole plant morphology and *kan*^{r} segregation ratios. Plant material selected based on these criteria served as the initial population on which the bulk of phenotypic characterization was carried out (Kramer et al., 1990). Homozygous progeny of selected transformants were then used to produce seed for further field testing and development.

More than 10 experimental field trials and over 400 acres of commercial production have been conducted by Calgene, Inc. with FLAVR SAVR^{TM} tomato cultivars in the principal tomato producing regions of California, Florida and Mexico. The experimental field trials have been conducted both to determine the phenotypic effect of an antisense PG gene on fresh market and processing tomatoes as well as to determine the effect of the transgene and the transformation process on overall horticultural performance.

In terms of phenotypic evaluation, it has been demonstrated that the absence of PG in the ripening fruit imparted improved field holding and firmness as well as improved resistance to certain post harvest fungal pathogens (Kramer et al., 1990, 1992). In addition, when processing characteristics were evaluated, it was discovered that the absence of PG resulted in significant improvements in both juice consistency and serum viscosity as measured by Bostwick and Ostwald values respectively (Kramer et al., 1990).

With respect to horticultural performance, observations conducted during all trials and commercial production demonstrate that cultivars developed through transformation with the antisense PG gene exhibit similar horticultural traits when compared to the identical non-transformed genotypes. No unpredicted changes were observed to have occurred, as documented in the field trial reports submitted to the USDA APHIS, subsequent publications (Kramer et al., 1990, 1992), and Calgene's Request for Advisory Opinion filing with the FDA.

More detailed analysis of the fruit expressing the antisense PG gene demonstrated that expression of the antisense PG gene affects only the composition of pectin in the fruit (Kramer et al., 1990, 1992). The gene has no effect on levels of vitamins and nutrients, on production of potential toxins (tomatine), on taste, on non-pectin related processing traits, on horticultural traits (growth form, time to flowering, time to fruit set, etc.), fruit pH and acidity, and fruit color and size (Redenbaugh et al., 1992). In fact, in its environmental assessments, the USDA concluded, 'The antisense PG gene does not provide the transformed tomato plants with any measurable selective advantage over nontransformed tomato plants in their ability to be disseminated or to become established in the environment' (USDA APHIS, 1991).

Safety assessment

Environmental release

Historically, the USDA APHIS BBEP (United States Department of Agriculture, Animal and Plant Health Inspection Service, Biotechnology, Biologics, and Environmental Protection) has regulatory oversight of release of genetically engineered plants into the environment, as per Vol. 7 Code of Federal Regulations, Part 340. Using these regulations, the USDA has approved over 1100 field trials of genetically engineered plants in the United States, without any adverse effects.

In terms of release of genetically engineered plants into the environment, the greatest concern has always been the potential to inadvertently produce a new weed or somehow increase the competitiveness of existing weeds (Colwell et al., 1985). Plant breeders have a long history of using a variety of plant breeding techniques to select and produce plant cultivars with improved resistance or tolerance to external factors that inhibit their inherent productivity and/or competitiveness. Examples include such traits as resistance to insect and disease pests, heat, cold and drought tolerance as well as earliness and winter hardiness. In theory, such improved cultivars are better adapted to persist in the presence of disease, insects and a variety of environmental conditions which would normally decrease productivity and competitiveness. Although there is no evidence that demonstrates that incorporating these types of traits into crops has created cultivars which pose a risk of enhanced weediness (USDA APHIS, 1991), each gene/crop combination has been evaluated on a case by case basis. Since the techniques of molecular biology and genetic engineering are highly specific in terms of what genes are being transferred,

Table 1. Nutritional components for FLAVR SAVR tomatoes and controls as compared to normal ranges for tomato.

Constituent	Normal Range	Measured Range for FLAVR SAVR Lines	Measured Range for Control Lines	Unit
Protein	0.85 (.015 se)*	0.75–1.14	0.53–1.05	g
Vitamin A	192–1667	330–1600	420–2200	IU
Vit. B_1 (Thiamin)	16–80	38–72	39–64	μg
Vit. B_2 (Riboflavin)	20–78	24–36	24–36	μg
Vitamin B_6	50–150	86–150	10–140	μg
Vitamin C	8.4–59	15.3–29.2	12.3–29.2	mg
Nicotinic acid (Niacin)	0.3–0.85	0.43–0.70	0.43–0.76	mg
Calcium	4.0–21	9–13	10–12	mg
Magnesium	5.2–20.4	7–12	9–13	mg
Phosphorus	7.7–53	25–37	29–38	mg
Sodium	1.2–32.7	2–5	2–3	mg
Iron	0.2–0.95	0.2–0.41	0.26–0.42	mg

* Protein measurement is mean ± standard error (se) from USDA Handbook No. 8. Range is based on ripe fruit constituents per 100 g fresh tissue.

products developed using these methods will be more easily defined in terms of the traits being introduced into the plants (USDA APHIS, 1991).

Because of the history of safe field trials in the U.S. and the development of new cultivars containing transgenes, the USDA implemented a petition process for removing genetically engineered cultivars from regulatory oversight. As an integral step in the process of commercialization, Calgene, Inc. submitted such a petition to the USDA APHIS requesting a determination that FLAVR SAVR tomatoes do not present a plant pest risk and are not otherwise deleterious to the environment. These tomato cultivars contain specific gene sequences introduced into the plant genome via the binary vectors (pCGN1547, pCGN1548, pCGN1549, pCGN1557, pCGN1558, pCGN1559, or pCGN1578) plus the antisense polygalacturonase gene with its associated promoter and terminator (Sheehy et al., 1987, 1988). These sequences, as used in producing FLAVR SAVR tomatoes, do not cause these tomatoes to become a plant pest risk the USDA determined on October 19, 1992 (Federal Register. 57: 47608–47616) that the FLAVR SAVR tomatoes are not a plant pest risk, are not deleterious to the environment, and are no longer a regulated article under 7 CFR 340 for the following reasons:

1. Tomato is not a regulated article.
2. Genetic sequences from regulated articles used to produce FLAVR SAVR^{TM} tomatoes have been disarmed and do not pose a plant pest risk.
3. Genes from regulated articles, introduced into tomato, do not confer characteristics that would present FLAVR SAVR^{TM} tomato as a plant pest risk (e.g. cause tomato to become a weed pest risk).
4. No new compounds have been measured in FLAVR SAVR^{TM} tomato that pose a hazard or are deleterious to the environment.

Food safety

The data package developed by Calgene, Inc. to demonstrate the safety of the FLAVR SAVR^{TM} tomatoes was submitted to FDA as two separate requests for advisory opinions. The first, submitted November 26, 1990, and entitled "*kan*^{r} Gene: Safety and Use in the Production of Genetically Engineered Plants" (FDA Docket #90A-0416) and addressed the safety of the use of the *kan*^{r} gene as a selectable marker in food. The second, submitted August 12, 1991, entitled 'FLAVR SAVR^{TM} Tomato: Status as Food' (FDA Docket #91A-0330/API) provided a detailed safety assessment of the FLAVR SAVR tomato.

From a food safety perspective, one issue of concern was the APH(3′)II protein produced by the *kan*^{r} gene. APH(3′)II is a protein and proteins are generally not known to have toxicity in humans. In fact, the protein is produced naturally by bacteria found in the human gut. Calgene, Inc. conducted an acute toxicity study which showed no toxicity, mortality or gross necropsy in rats fed FLAVR SAVR^{TM} tomatoes which contain the *kan*^{r} gene. Experimental results demon-

Table 2. Comparison of FLAVR SAVR tomatoes with nontransformed controls.

Parameter	Changed	Unchanged
Tomatine levels		+
Taste (at same stage)		+
Viscosity	Increased	
Other processing traits		+
Horticultural traits		+
Fungal resistance	Increased	
Color (pigmentation)		+
Softening rate	Decreased	

strated that the enzyme was inactivated by pepsin in simulated gastric and intestinal fluids, as is the case for any other typical protein. Glycosylation and subsequent increase in the antigenic capacity of APH(3′)II also would not occur since APH(3′)II does not contain the necessary sequence information for transport to the subcellular locations at which glycosylation reactions take place. Finally, APH(3′)II was shown not to have significant homology with known toxins and allergens.

To demonstrate qualitative and nutritional equivalence, nutrient components of FLAVR SAVR tomatoes were measured. These included protein, vitamins A, B_1, B_2, B_6, and C, niacin, calcium, magnesium, phosphorus, sodium and iron (Table 1). In all cases, the range of variation of these nutrients in FLAVR SAVR^{TM} tomatoes was the same as found in control fruit. In addition, the range of tomatine levels (a glycoalkaloid related to solanine) was measured (Table 2). These measurements demonstrated that there were no changes in nutrients or potential toxins as a result of the process involved in the production of these new cultivars.

The extensive compositional analyses, which showed no changes in the components essential for a safety evaluation and the verifying feeding studies, establish that FLAVR SAVR^{TM} tomatoes are as safe for human consumption as other tomatoes that are currently part of the human diet. A complete characterization was done of all DNA sequences between the border of the T-DNA with regard to potential open reading frames and any potential corresponding proteins. The structure of the inserted T-DNA into FLAVR SAVR^{TM} tomatoes remained intact and the inserted T-DNA locus behaved predictably, based on Mendelian genetics, over five generations. These data provided specific evidence that the T-DNA region was stably integrated into the tomato genome. FLAVR SAVR^{TM} tomatoes, stored to the end of their shelf life, had the same levels of pro-vitamin A and vitamin C as compared to controls or compared to the normal range of these vitamins in other tomato varieties. The additional time the FLAVR SAVR^{TM} tomato will stay in the field to reach the vine-ripened stage will not result in any significant changes in agricultural practices that will impact the environment. The harvest practices for FLAVR SAVR^{TM} tomatoes will be the same as those used for non-engineered fresh market vine ripened tomatoes. The FLAVR SAVR^{TM} tomato is less perishable and more durable than tomatoes that do not contain the FLAVR SAVR^{TM} gene and will provide better quality tomatoes to the consumer. Finally, Calgene, Inc. has in place a quality assurance program for future transformation events of FLAVR SAVR^{TM} tomatoes to determine the levels of provitamin A and vitamin C and the glycoalkaloid tomatine, and ensure that these levels are within the ranges measured in nontransformed tomatoes.

An additional concern is the potential for horizontal gene flow from the engineered plant to soil and/or gastrointestinal microorganisms. Arguments have been made concerning this potential risk, but no data have been published to support such a concern. There are no known mechanisms for transfer of genes from plants to microorganisms and no cases of such transfer have been reported. No mechanism by which plant DNA could be incorporated into the genomes of the microorganisms has been proposed. In addition, Zambryski et al. (1982) provided evidence that once inserted DNA is integrated into the plant host genome, it cannot be remobilized even if acted on again by *vir* genes. To date, such horizontal gene flow remains speculative with no actual examples.

In conjunction with its review of Calgene's data, FDA developed a policy regarding the safety of genetically engineered foods (Department of Health and Human Services, Food and Drug Administration, Docket No. 92N-0139, Statement of Policy: Foods Derived from New Plant Varieties). The new policy applies the same regulatory standards to transgenic crops and foods as for those produced using conventional technology. The policy requires companies to consult with FDA on any safety issue and to thoroughly evaluate food safety with respect to allergenicity, toxins, nutrition, and any newly produced substances.

Commercial release

Sale of FLAVR SAVR^{TM} tomatoes began on May 21, 1994. This was a historic event in that this product represented the first time a genetically engineered whole food had been sold in the public marketplace. Initially fruit was available in only two stores, one in the midwest (Chicago area) and the other in California (Davis). Consumer acceptance was instantaneous and overwhelmingly positive. The first three days of sales saw over 3,000 pounds sold from each store. This resulted in a shortage of fruit on the store level which actually required rationing of fruit for a time so that all the consumers who wanted to try a FLAVR SAVR^{TM} tomato were able. Store numbers are being increased at a very slow rate in an effort to insure quality and to keep consumers satisfied. The emphasis of the FLVR SAVR^{TM} tomato will continue to be quality and flavor.

Concluding remarks

The FLAVR SAVR^{TM} tomato was developed through the use of antisense RNA to regulate the expression of the enzyme polygalacturonase (PG) in ripening tomato fruit in order to create a commercial vine ripened tomato product with superior consumer quality and flavor. Extensive field testing as well as environmental and food safety assessments determined that FLAVR SAVR^{TM} tomatoes are unchanged in terms of nutrition, potential toxins, and horticultural traits and that no unintended technical effects were observed. Data supporting these conclusions were submitted as two 'Requests for Advisory Opinion' to the FDA and as a 'Petition for Determination' with the USDA. The USDA issued a determination that FLAVR SAVR^{TM} tomatoes which had previously been field tested under USDA regulations 'will no longer be considered regulated articles under APHIS regulations' (7 CFR Part 340) on October 19, 1992. On May 18, 1994, FDA concluded that FLAVR SAVR ^{TM} tomatoes were as safe as any other tomato produced through conventional means. As a result of these determinations, it has been concluded that these tomato lines pose no risk to either the environment or consumers. The sale of FLAVR SAVR^{TM} tomatoes began May 21, 1994. Consumer acceptance has been overwhelming positive.

References

Brady, C., W. McGlasson, J. Pearson, S. Meldrum & E. Kopeliovitch, 1985. Interaction between the amount and molecular forms of polygalacturonase, calcium, and firmness in tomato fruit. J. Am. Soc. Hort. Sci. 110: 254–258.

Colwell, R., P. Brayton, D. Grimes, D. Roszak, S. Huq & L. Palmer, 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for the release of genetically engineered microorganisms. Bio/Technology 3: 817–820.

Hobson, G., 1965. The firmness of tomato fruit in relation to polygalacturonase activity. Hort. Sci. 40: 66–72.

Kramer, M., R. Sanders, H. Bolkan, C. Waters, R. Sheehy & W. Hiatt, 1992. Post-harvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness and disease resistance. Post Harvest Biol. Technol. 1: 241–255.

Kramer, M., R. Sanders, R. Sheehy, M. Melis, M. Kuehn & W. Hiatt, 1990. Field evaluation of tomatoes with reduced polygalacturonase by antisense RNA. In: Bennett, A. & S. O'Neill (Ed.), Horticultural Biotechnology, pp. 347–355. Wiley-Liss, Inc., New York.

Redenbaugh, K., W. Hiatt, B. Martineau, M. Kramer, R. Sheehy, R. Sanders, C. Houck & D. Emlay, 1992. Safety Assessment of Genetically-Engineered Fruits and Vegetables: A Case Study the FLAVR SAVR^{TM} Tomatoes. CRC Press, Boca Raton, FL.

Sheehy, R., M. Kramer & W. Hiatt, 1988. Reduction of polygalacturonase activity in tomato fruit by antisense RNA. Proc. Natl. Acad. Sci. USA 85: 8805–8809.

Sheehy, R., J. Pearson, C. Brady & W. Hiatt, 1987. Molecular characterization of tomato fruit polygalacturonase. Mol. Gen. Genet. 208: 30–36.

USDA APHIS, 1991. Environmental Assessment and Finding of No Significant Impact on Tomato Containing an Antisense Polygalacturonase Gene. Permit Number 91–268–01.

USDA APHIS, 1992 (October 19). Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status of FLAVR SAVR^{TM} Tomato (Docket No. 92–087–2). Federal Register. 57: 47608–47616.

Zambryski, P., A. Depicker, K. Kruger, & H. Goodman, 1982. Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. J. Mol. Appl. Genet. 1: 361–370.