INTRODUCTION

In 1994, the first “genetically modified” food was approved by the FDA to go to market. The tomato, Flavr Savr, was modified by Calgene (a biotechnology company) using antisense technology resulting in altered ripening. Traditional tomatoes must be picked from the vine while still green in order to maintain their firmness during transport to the supermarket. The tomatoes are then sprayed with ethylene, their natural ripening agent, in order to turn the tomatoes red. Flavr Savr tomatoes are designed so they can ripen on the vine longer while maintaining firmer skin, thus producing a fuller flavoured tomato on the supermarket shelves. (Figure 1).

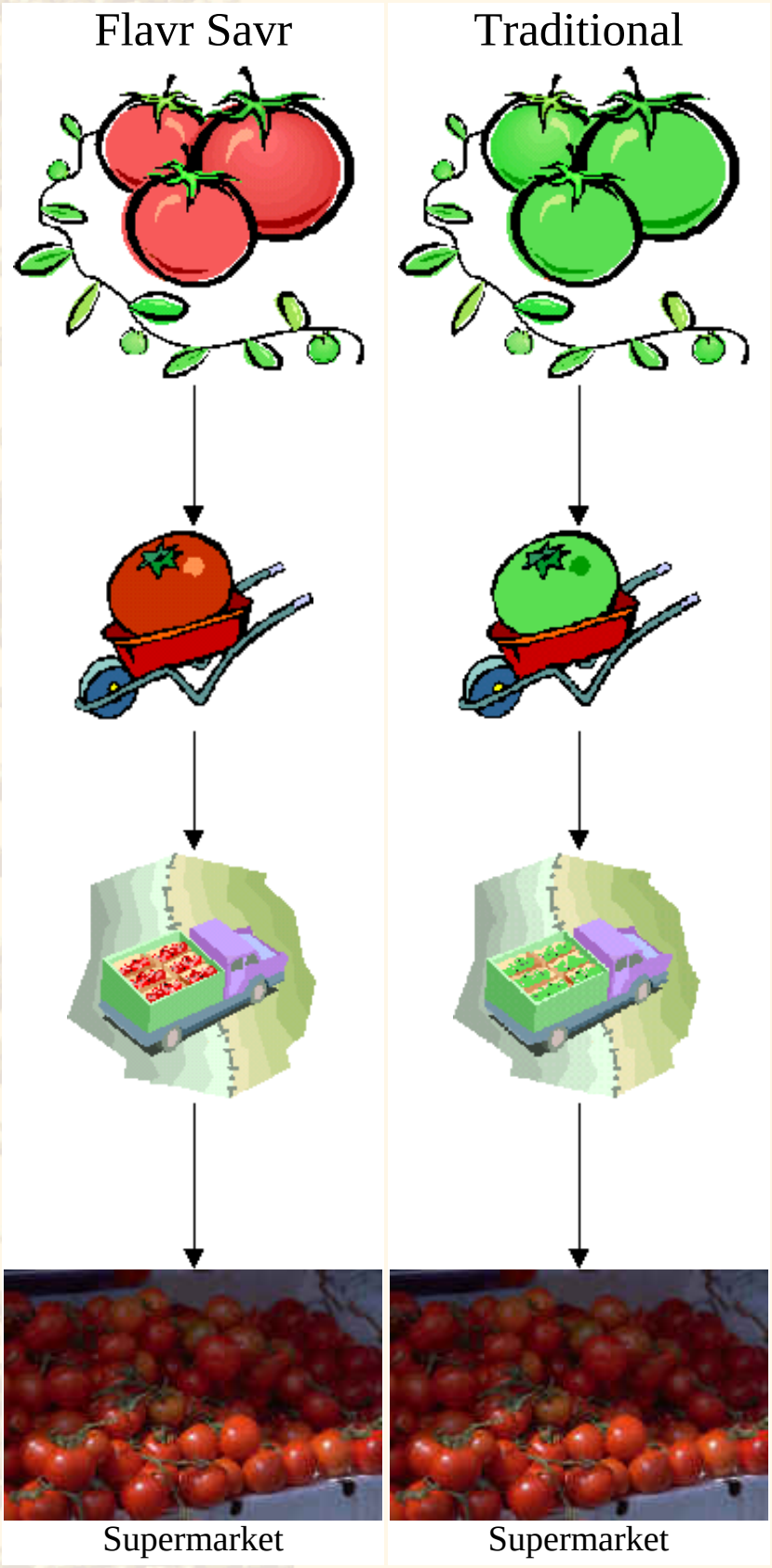
Public concern surrounded Flavr Savr’s introduction to the market. Debate raged across North America. How would this change the tomato? Was this tomato dangerous to our health? Should we have concerns about allergies? Nutrition? Toxins? What were the dangers to the environment? What about gene transfer across different organisms? Had Calgene created a “Frankenfood”?

In 1997, the tomato was pulled from the market. Supporters of the tomato claimed that the company required specialized transportation equipment which was not economically feasible for Calgene. Those opposed to genetically modified foods hailed it as a victory.

Was the public concern founded?

(Figure 1)

The Flavr Savr tomato ripens on the vine – resulting in fuller flavour. It is modified so that it remains firm after harvesting.



The traditional tomato must be harvested while it is still green and firm so that it is not crushed on the way to the supermarket.

The traditional tomato is sprayed with ethylene after shipping to induce ripening.

Ripe and Increased Flavour.

Ripe but decreased Flavour.

Fame or Shame: The Story of the Flavr Savr Tomato

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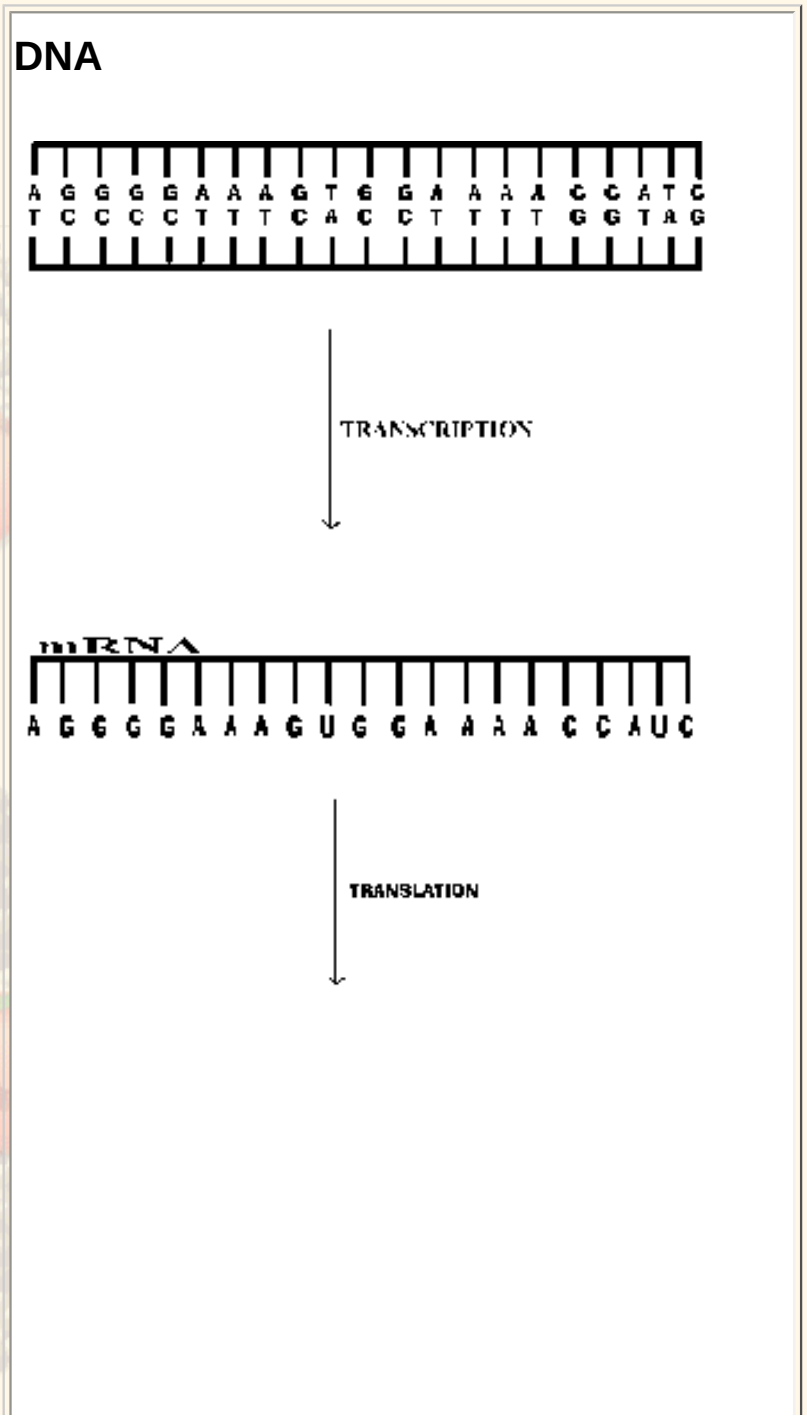
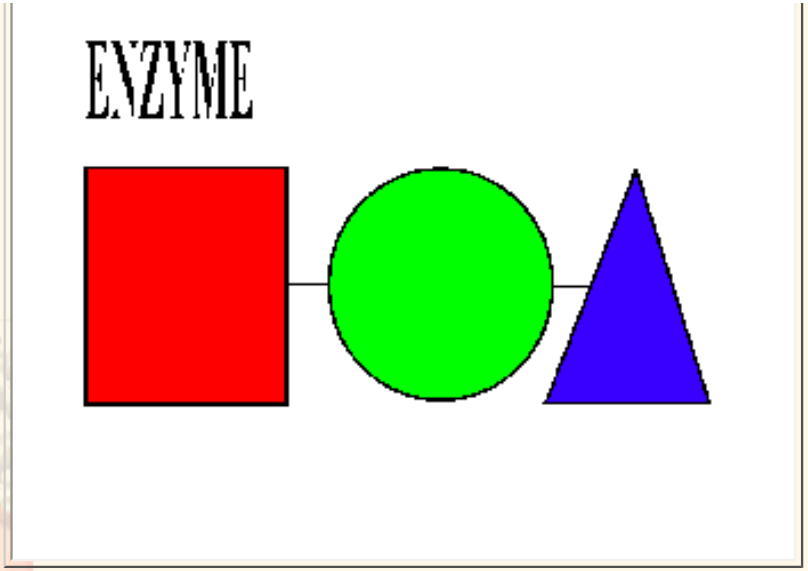
What is antisense Technology?

In basic terms it is a method of gene silencing. If one is to understand antisense one must first understand how an enzyme is made? The first step in making an enzyme is the translation of a particular area of the DNA into a mRNA. Then the mRNA is transcribed to make an enzyme. This same procedure also applies to protein production. For the simplicity of this example let us assume that DNA is linear.

How is an enzyme made?

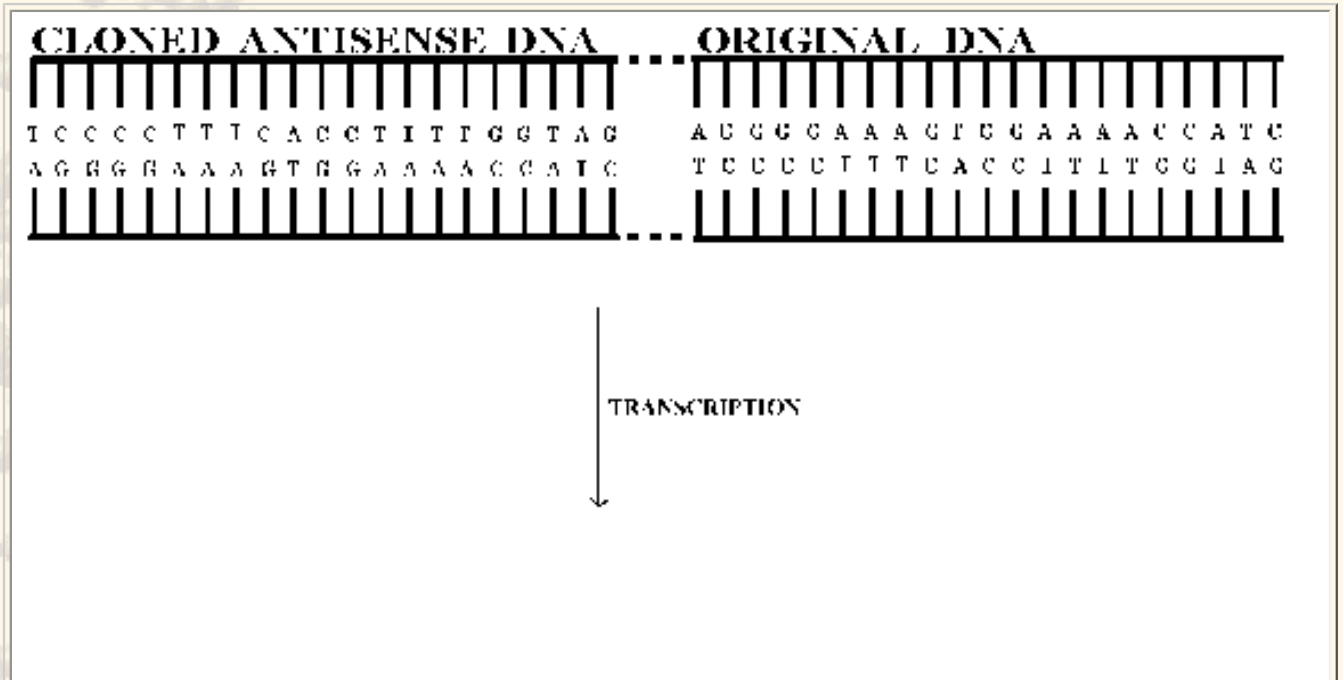
(Figure 2)


DNA
A G G G G A A A G T G G A A A A C C A T C
T C C C C T T T C A C C T T T T G G T A G
TRANSCRIPTION
mRNA
A G G G G A A A G U G G A A A A C C A U C
TRANSLATION

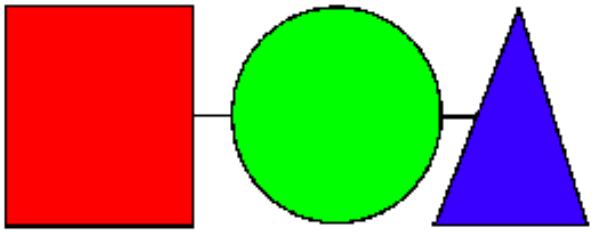


What Happens When A Cloned Antisense DNA Is Added To The Original DNA?

(Figure 3)



ENZYME NOT PRODUCED



Summary:

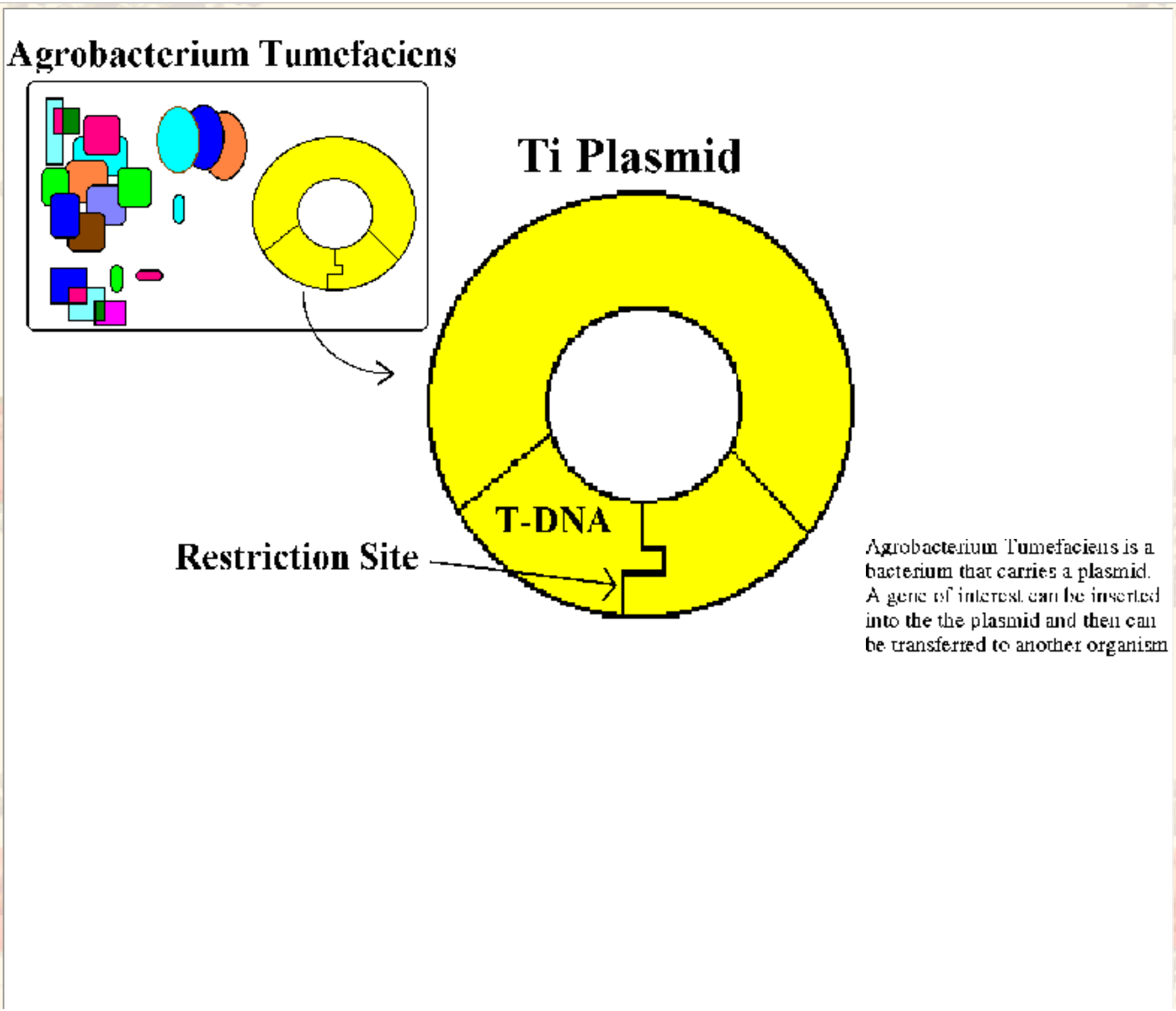
In order to understand how antisense works, one must first understand the central dogma of genetics. Information flows from DNA---> RNA ---> Proteins/Enzymes (generally). This is depicted graphically in the first diagram. DNA is transcribed into mRNA. Introns are removed from the primary transcript. Then the mRNA is processed by ribosome's and is translate into functional a protein.

How is the antisense PG gene inserted into the plasmid of the Agrobacterium Tumefaciens? The antisense polygalacturonase cDNA sequence is cut with restriction enzymes and fused in the inverted orientation to a plasmid (i.e. double-stranded, closed DNA molecule) containing a upstream promoter and a downstream terminator sequence. The cauliflower mosaic virus (CaMV) promoter is chosen because it produced the right amount of antisense RNA to provide an adequate delayed softening time. The degree of production of antisense RNA in the plant cells is dependent on a number of factors and the type of promoter sequence chosen is one of them. This plasmid is then transferred to Escherichia Coli (E-Coli) bacteria. E-Coil serves as the plasmid host.

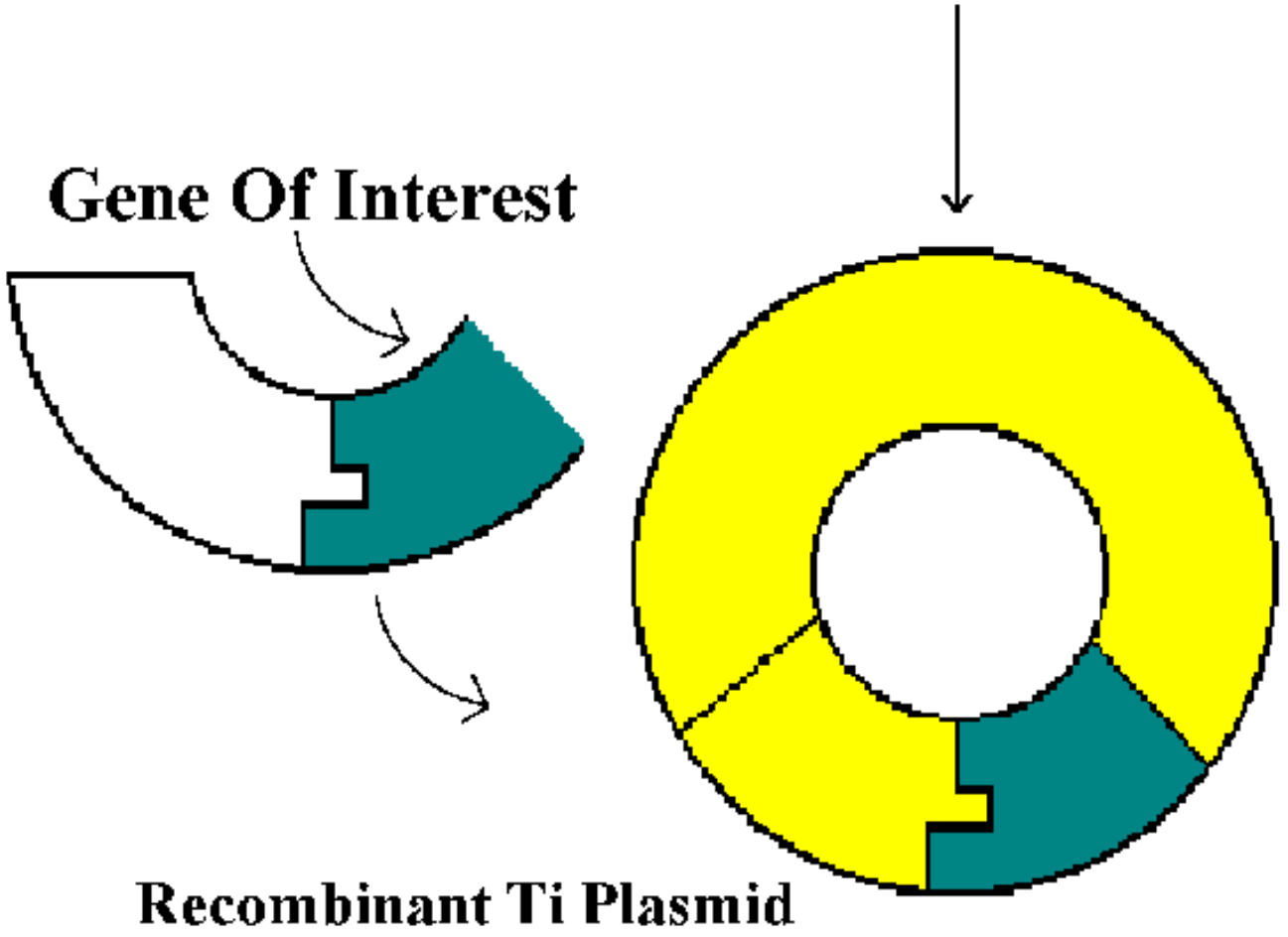
Afterwards the hybrid gene is transferred to the Agrobacterium Tumefaciens by triparental mating with E-Coil. This type of mating is a recombinant DNA method which allows genetic information to exchange from one parent to the other. The gene of interest is now present in Agrobacterium Tumefaciens plasmid (Ti plasmid) and is situated beside the T-DNA.

What does antisense technology accomplish? In the simplest terms antisense technology interferes with the production of specific proteins. This is depicted diagrammatically in the second diagram. In the diagram we are interested in suppressing the expression of the right hand side of the sequence. How is this accomplished? A complimentary DNA sequence is cloned and inserted in front of the sequence of interest. This means that the last base of the inserted DNA is complimentary to the first base of the original DNA, the second last base is complimentary to the second base and so forth. When transcription takes place in this modified strand of DNA, the mRNA becomes double stranded. Depending on the size of the inserted DNA, the mRNA could be double stranded along all its length or could be partially double stranded. As a result of the double strand mRNA, ribosome's find it difficult to process mRNA and very little protein is produced.

(Figure 4)

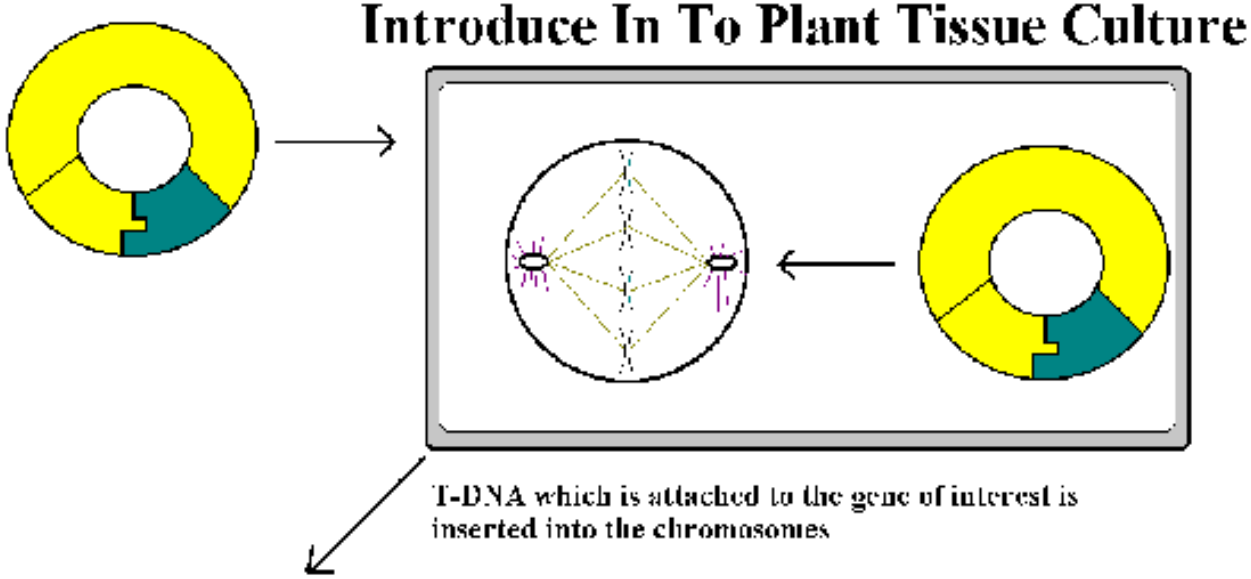


Agrobacterium Tumefaciens is a bacterium that carries a plasmid. A gene of interest can be inserted into the the plasmid and then can be transferred to another organism


Gene Of Interest
Recombinant Ti Plasmid
This plasmid vector now contains the gene of interest and will be used to infect the host cell

(Figure 5)

The bacterium enters dead/broken plant cells and then transfers the plasmid into the adjacent living cells. Once the plasmid is inserted into the cell, they are grown in tissue cultures.



After a period of time the cells in the tissue culture replicate and are generated into new plants containing the antisense gene with the use of appropriate hormones

For more details on exact procedure and methods outlined above refer to the following articles:

I.. Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Molecular Biology 14:369-379 (1990)

II. The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. Plant Molecular Biology 11:651-662 (1998)

III. Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. Nature 334:724-726 (1988)

For Further References Refer To References On Each Paper Listed Above.

Links To Relevant Websites:

http://opbs.okstate.edu/~melcer/MG/MGW4/MG4373.html

http://opbs.okstate.edu/~melcer/MG/MGW4/MG4372.html

http://opbs.okstate.edu/~melcer/MG/MGW1/MG1234.html

http://www.ejb.org/content/vol1/issue3/full/1/index.html

http://helios.bto.ed.ac.uk/bto/microbes/crown.htm

Summary :

How is antisense DNA inserted into plants? The first step is to clone the antisense DNA. Then insert this DNA into the plasmid of an agrobacterium. Next introduce the bacterium to plant cells. The bacterium then transfers the gene of interest into plant cells. The last step is to re-grow the plant cells by adding specific hormones. The re-generated plants will express the antisense DNA. As a result when the mRNA is made through the process of transcription the sense mRNA will bind to the anti-sense mRNA. This interferes with protein production.

The Flavr Savr Tomato Story

The objectives of Calgene, (then Monsanto) was to create a Tomato that would have a ripened taste, yet survive shipping. Current practice is to pick tomatoes from the vine before they ripen. These unripe tomatoes have a firm structure. This allows for undamaged transport. When the tomatoes arrive at their destination, they are sprayed with ethylene. Ethylene allows the tomato to take on a ripened look. Unfortunately, ethylene just causes only a cosmetic change. The tomato remains mostly unripe.

In a bid to create a transportable tomato, that would be ripe on delivery, Calgene created Flavr Savr. The Flavr Savr tomato is designed to ripen on the vine, with minimal softening. This means that ripe tomatoes can be transported to consumers, a definite plus for grocery stores.

How Calgene accomplished this feat is through antisense technology. The tomato cell's PG (polygluconase) enzyme was identified as the cellular machine responsible for the degradation of its' cells' walls. Calgene's approach is to disable this enzyme during its construction stage. This will dramatically slow down PG production, and allow the plant to ripen, without the loss of structural stiffness.

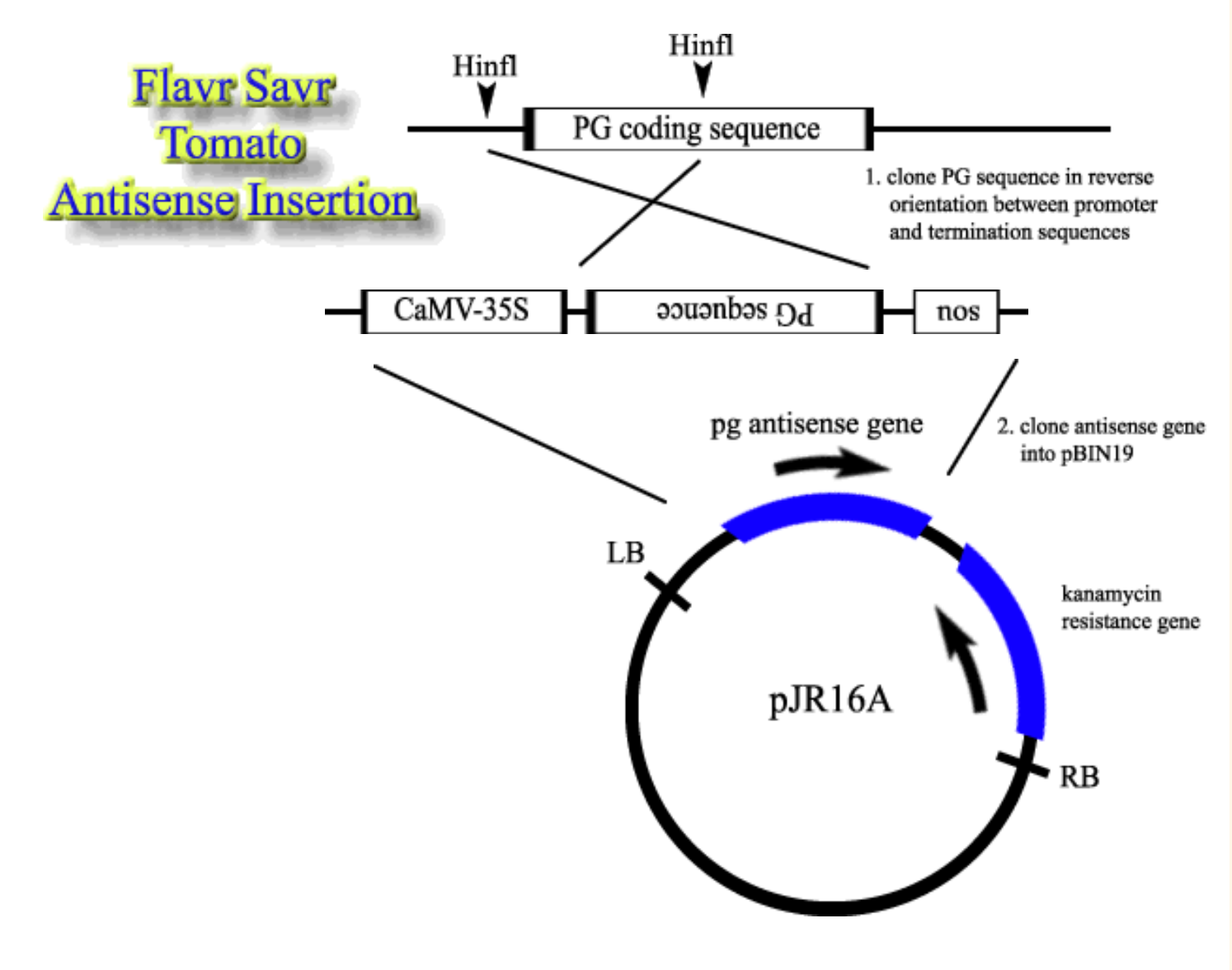
Calgene attacked PG construction at the template level. A reversed, "antisense" copy of the PG DNA was added to the tomato genome. When this gene is transcribed, it produces an RNA, that has the complementary bases of the actual PG RNA sequence. These two RNA strands base pair, in effect, disabling the RNA that would produce the PG enzyme protein. This disabling is enough to slow down softening of the tomatoes, allowing them to be transported to their consumer destinations ripe.

Indepth look at antisense and how it applies to Flavr Savr:

The first step is to create a cDNA of the PG gene. This involves generating an mRNA, then re-transcribing it back into a cDNA. This cDNA contains the entire PG gene (Figure 6, top). Next a 730 base pair (bp) region, including a 50bp non-coding region is excised called the Hinf1 fragment. This is cloned into a plasmid. Next the cloned fragment was excised and ligated into a second plasmid. The insertion occurred just after the CaMV35s (Cauliflower Mosaic Virus Promoter, Figure 6, middle). Finally, this plasmid was inserted into an

agrobacteria, where the complete fragment was inserted yet again, into another plasmid, this one containing the KanR gene (Figure 6, bottom).

(Figure 6)



Why antisense PG gene:

The PG enzyme is responsible for the breakdown pectin. Pectin is a building block in cell walls, and is what gives tomatoes their firmness. In an attempt to slow the softening process, the Flavr Savr employs antisense technology to block PG enzyme production. Figure 5 describes how the antisense gene (fragment is inserted into the tomato).

The use of antisense PG RNA is because the mRNA it generates is complementary to the mRNA produced by regular PG genes, it will actively inhibit PG enzymes by disabling their mRNA. This disabling is accomplished by having the small antisense fragment mRNA bind to the regular PG mRNA. This partial double-stranded complex will not for PG protein, and the complex is quickly degraded.

The CaMV35s promoter is used because of its constitutive effects. The CaMV35s is always expressed inside the tomato plants. This means the cell has readily large supply of antisense mRNA present. This means, that when tomato cells reach the ripening stage where PG enzymes are to be made, the normal PG mRNA is easily bound by the abundant supply of antisense mRNA. This is the key to disabling the PG gene/future enzyme.

The final stage plasmid is in an agrobacteria. The bacteria infects the plant, thereby passing the genes between the LB & RB restriction site in to the plants genome. This genetic material is then expressed with

The use of a kanamycin resistant gene, is to create a selective marker. What occurs, is the plants thought to be transformed are grown on a kanamycin containing medium. If the plant has the antisense gene which would mean it also has KanR, then it will grow on this selective medium. If the plant does not have the KanR gene (this means the antisense sequence was not incorporated into the genome of the plant), then it will not grow on the growth medium.

Antisense technology in the Flavr Savr tomato does work. Levels of PG mRNA are 6% of those experienced in normal tomatoes (not shown). For information on expression verification see related sections. Safety aspects and comparative reports are also present in other sections.

Testing the Expression of the Antisense Construct

After the plants have been selected on the kanomyosin medium, further tests are performed to examine if the endogenous gene is expressed appropriately in the organism. To ensure that the antisense gene is still intact, a Southern Blot is performed to locate the newly incorporated genomic sequence. The DNA is digested with a restriction enzyme and broken into smaller fragments which are subjected to gel

electrophoresis and separated according to their size. The fragments are then transferred onto a nitrocellulose filter or nylon membrane and incubated with a radiolobelled cDNA probe that is complementary to the endogenous gene. This probe binds to the fragment of the digested DNA corresponding to the newly inserted sequence and can be detected by autoradiography. This demonstrates that the DNA has incorporated the endogenous gene into its genome.

The next step that is essential in determining if the gene is being expressed is to test the activity of the endogenous sequence. First, a Northern Blot analysis is performed to determine if the RNA complement to the new gene is present. A Northern Blot is performed like the Southern Blot, except the probe used is an RNA complement to the transcribed gene. Although this is an effective test to determine if the RNA is present in the cell, it does not reveal the amount of RNA expressed. To test if transcription is occurring at a sufficient level, Nuclear Run-on experiments are performed. The nuclei are isolated from cells and allowed to incorporate ^{32}P into the growing RNA chains. The resulting labeled RNA is allowed to hybridize with the same probe as in the Northern Blot, and by autoradiography, the amount of RNA can be determined.

The expression of the engogenous gene can also be examined at the level of the translated protein. This is done by Western Blotting, or Immunoblotting. A mixture of the cellular protein is separated on a SDS-polyacrylamide gel, then transferred to a nitrocellulose membrane. The membrane is soaked in a solution of antibody that is specific only for the protein from the endogenous gene. The membrane is then soaked in a

second antibody that is labeled, and specific for the first antibody. This reveals the antibody:protein of interest complex, and is used as an indicator of gene expression.

The final test in assessing the activity of the endogenous gene is to ensure that integration of the antisense gene has not affected the expression of adjacent genes. This is accomplished using Nuclear Run-on experiments. As in the tests for the level of RNA transcription, ^{32}P is incorporated into growing RNA chains. The resulting RNA fragments can be separated according to size, and probed with an RNA probe. If any of the resulting fragments are longer than the expected length compared to the gene and/or the probe, it is indication that transcription run-through has occurred, and the gene has affected the expression of adjacent genes. This is an undesired result that must be carefully monitored during antisense experiments.

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How is the phenotype affected?

How safe is the Flavr Savr?

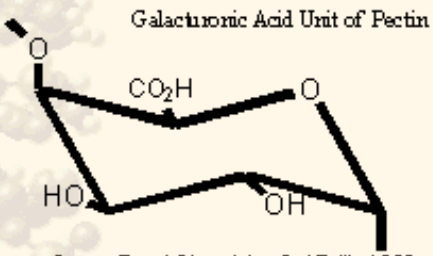
What are the toxins affecting Flavr Savr?

What are the environmental issues surrounding Flavr Savr?

What Are Pectins?

Pectins are a class of complex polysaccharides which make up approximately 35% of the plant cell wall. The other components of the cell wall include cellulose (30%), hemicellulose (30%), and proteins (5%). Pectin is responsible for the textural qualities of plants such as firmness. It is made up primarily of long chains of galacturonic acid interspersed occasionally with rhamnose residues.

(Figure 7)

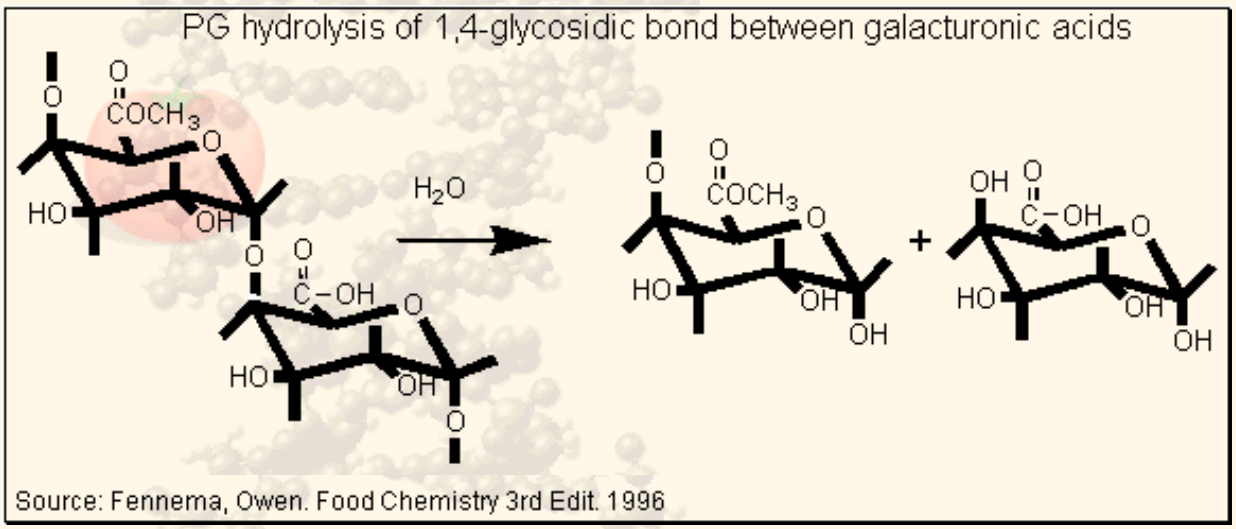


Source: Fennema, Owen. Food Chemistry 3rd Edit. 1996

What Happens to Pectin During Ripening?

At the onset of ripening, the hormone ethylene activates several biochemical pathways. One of these pathways produces the protein called polygalacturonase (PG). This cell wall enzyme catalyzes the hydrolytic cleavage of a-(1,4)-glycosidic bonds between galacturonic acid units of pectin. This causes pectin to depolymerize. The degradation of pectin is responsible for the softening of the cell wall of foods such as tomatoes. Other enzymes also responsible for the softening of the cell wall are cellulases, galactanases, and pectin methylesterases (PME).

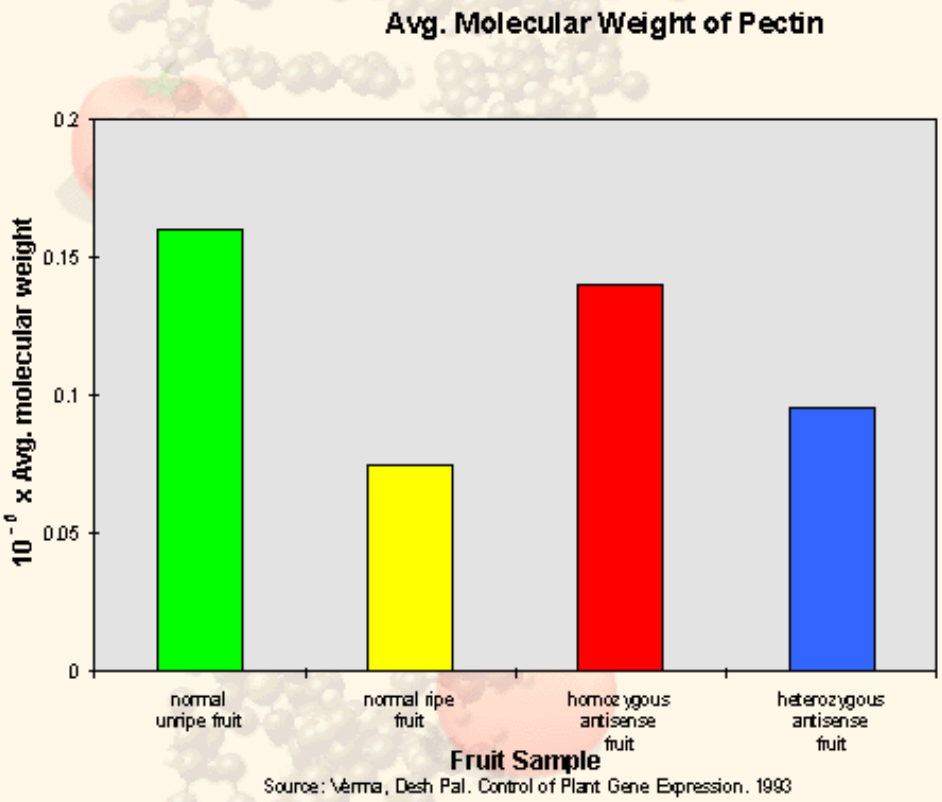
(Figure 8)



Changes to Pectin Caused by Antisense PG Gene in the Flavr-Savr Tomato

The downregulation of PG inhibits pectin depolymerization causing pectin to have a larger molecular weight. The reduced degradation of pectin causes the cell wall to maintain it's rigidity well after the tomato has ripened. The following graph compares the weight of pectin from several different types of tomatoes. Notice that pectin from the homozygous antisense tomato has an average molecular weight similar to a normal unripe tomato.

(Figure 9)



It should be noted that the antisense PG gene in the Flavr-Savr tomato does not alter other characteristics of the tomato. This is because the antisense gene does not effect the production of the hormone ethylene which acts upstream of the antisense gene. Thus, the other biochemical pathways controlled by ethylene are not effected.

Benefits from Reduced Pectin Degradation in the Flavr-Savr Tomato

A common practice with normal tomatoes is to harvest them before they have ripened so that the tomatoes are firm for shipping. Soft tomatoes, on the other hand, are prone to bruising during the shipping process. This practice does not produce a tasty tomato since flavour components accumulate during the ripening process on the vine. The insertion of the antisense PG gene in the Flavr-Savr tomato allows it to be harvested after it has started to ripen on the vine. The ripe Flavr-Savr tomato has more flavour and is firm enough to be shipped.

Pectin is also a major determinant of tomato juice viscosity. The larger the pectin molecules, the greater the viscosity of the juice. Since the antisense Flavr-Savr tomato has larger pectin molecules compared to a normal tomato, it will produce high-quality processed tomato products such as paste.

HOW SAFE IS THE FLAVR SAVR™ TOMATO?

Now that we know how to improve the quality of the tomato through genetic engineering, the next important step is to address the safety of consuming the tomato. The major concerns regarding the safety of the tomato are as follows:

1) Does insertion of the DNA into the tomato genome change tomato in any way to disqualify it as food?

2) Do levels of Toxin fall in acceptable range?

3) Do gene products produced by the Kan^r gene within acceptable limits?

4) Will this genetically modified tomato be safe enough for human consumption?

5) How different in terms of taste and nutrition is this tomato from the natural grown tomato?

Calgene has asked the FDA (Food and Drug Administration) to conduct an extensive

research regarding the safety of the Flavr Savr Tomato. In the following paragraphs, you will learn that the Flavr Savr is actually similar and just as safe as naturally grown tomatoes!

Stability in the integration and structure of inserted DNA

Since a DNA sequence is inserted into the tomato genome one has to test to see whether this sequence will either remain or falls off the tomato genome! In other words, the stability of inserted DNA sequence must be tested. If the sequence were not stable, that would mean the insertion is not successful and would also indicate a problem in controlling the inserted sequence.

Results indicate that the insertion of the gene into the plant genome is stable.

Level of Toxins present in tomato is within acceptable range

Tomatoes contain naturally occurring toxins called *Glycoalkaloids* of which *Solanine* and *alpha- Tomatine* are the two main toxins present in tomatoes.

Solanine is the major naturally occurring toxicant. It is relatively more toxic than tomatine yet its concentration is much lower. Research has indicated that its concentration is too low to cause any health concerns.

The other toxic compound, alpha-tomatine, is commonly used as an insect repellent. It is the most abundant glycoalkaloid in tomato. Animal tests have proven that even ingesting an abnormally high concentration would not cause health hazard. Thus the toxins present in the natural tomato and hence Flavr Savr will yield no health hazard.

Gene Product produced by Kan^r gene does not cause health hazard upon consumption.

As mentioned before, the only new component inserted into the Flavr Savr tomato is the Kan^r gene, Kanamycin (antibiotic)-resistant gene. Its function is to isolate or identify the cells that have the inserted antisense gene from other non-Kan^r gene by being able to grow in a kanamycin environment.

The protein product that is produced from the Kan^r gene is *Aminoglycoside3'-Phosphotransferase II* (APH 3'II). It is a type of enzyme commonly found in edible plants and animals that can inactivate the effects of antibiotic, Kanamycin and Neomycin.

Tests have confirmed that this protein:

a) Show no similarity to any proteins that would cause allergic reactions

b) Occur at very low concentration in foods

c) Is quickly broken down by stomach acids and digestive enzymes

Thus FDA concluded that APH 3'II does not have any characteristics of food allergens nor contributes to any hazardous toxic consequences that would otherwise cause health concerns upon consumption.

Nutritional aspects of Flavr Savr Tomato as compared to natural grown tomato

Calgene has compared the nutritional values of Flavr Savr to natural grown tomatoes and found that nutrient levels of the Flavr Savr tomato are all within normal range and is

comparable to natural grown tomatoes.

In addition, there is no difference in *lycopene* ***or *beta-carotene* ***content nor taste difference in the Flavr Savr tomato. The following charts summarizes the nutrients present in natural grown tomato and in Flavr Savr tomatoes.

Comparison of selected tomato containing Flavr Savr gene with Normal Controls

(naturally grown tomato)

(Table 1)

Component	Changed	Unchanged
Recommended Daily Intake		Ö
Potential Toxin		Ö
Taste		Ö
** Serum Viscosity	Ö	
Processing traits (others)		Ö
Fungal Resistance	Ö	
Colour		Ö
Softening Rate	Ö	

** Serum Viscosity = indicate the concentration and size of pectin molecules in the tomato

Nutritional Components between Modified and Control Tomato

(Table 2)

¯ = lower than normal rage (compared to highest limit)

–= higher than normal range. (compared to highest limit)

Component	Normal Range	Measured Range for tomatoes with coding region of antisense PG gene	**Measure range for Control, natural grown tomatoes	Within Normal?
Protein	0.85g	-	-	Yes
Vitamin A	192 - 1667	slightly higher	-	Yes
Vitamin B1	16 – 80	–	–	Yes
Vitamin B2	20 – 78	–	–	Yes
Vitamin B6	50 – 150 µg	lower limit is slightly higher	–	Yes
Vitamin C	8.4 - 59 mg	–	–	Yes
Niacin	0.3 - 0.85 mg	–	–	Yes
Calcium	4 - 21 mg	–	–	Yes
Magnesium	5.2 - 20.4 mg	–	–	Yes
Phosphorus	7.7 - 53 mg	–	–	Yes
Sodium	1.2 - 32.7 mg	–	–	Yes
Iron	0.2 - 0.95 mg	–	–	Yes

*** and * range was based on ripe fruit constituents per 100g fresh tissue*

FDA Policy for Flavr Savr Tomato

(Table 3)

Safety Tests Performed	Results
♦ Tested environmental safety of the use of Kanamycin resistance gene	à No environmental hazard present
♦ Compared Nutritional Profiles of Flavr Savr to the Natural variety (especially on Vitamin C and A)	à Found no significant difference between Flavr Savr with control parental line & no difference in Lycopene level or Beta-Carotene (vit A) level.

♦ Tested Toxicity of naturally occuring toxin (ie Tomatine).	à Tomatine occur in green plants, its concentration decrease during ripening, Calgene found no significant difference in glycoalkaloid content between Flavr Savr Tomatoe & commerial Tomato
♦ Tested the " new introduced substance" - APH 3' II	à It's found to be quickly degraded/inactivated by stomach acid & digestive enzymes
♦ Calgene also tested whether a large amount of of orally administered antibiotic can be inactivated by APH 3'II under abnormal stomach condition :	à used high ATP containing food and low dose of antibiotic to test the effect and found only a small fraction of antibiotic was inhibited ∴ no significant inactivation of kanamycin was observed in vitro.
♦ Calgene also tested whether Resistance gene can "leak" out to pathogenic microbes in the intestinal track or in soil.	à No known mechanism is found to test this aspect. (ie found no method that gene can be transferred from a plant chromosome to a microbe, thus, low or no possibility of transfering of gene)

In terms of processing characteristics, only those characteristics related to *pectin* (plant cell wall component) are altered. Tests have found that Flavr Savr Tomato has:

1) Greater serum viscosity (means higher concentration in size and amount of pectin molecules and

2) There is no significant difference in pH, acidity, colour nor sugar content as compared to natural grown tomato.

Moreover, experimental results has shown that the Flavr Savr tomatoe has the same physical appearance as natural grown tomato except Flavr Savr lacked 99% of normal polygalacturonase activity.

Principal difference between Flavr Savr and Natural-Grown Tomato

There is one major difference between the genetically modified tomato (Flavr Savr) and

natural tomato. Flavr Savr tomatoes :

Show reduction of polygalacturonase activity which causes:

- Changes in field quality and overall quality of fresh fruit
- Longer Shelf life (remain fresh longer)
- Firmer after maturity
- Greater resistance to fungal disease

Summary

The results obtained from these safety tests have thus proven that the Flavr Savr are genetically stable, nutritionally comparable to natural grown tomatoes, show no compositional change and most importantly does not have adverse health affects upon consumption. These strongly indicate that Flavr Savr tomatoes are safe and are just as good and enjoyable as natural grown, non-genetically modified tomatoes!

Naturally Occurring Toxins

- "Tomatine" found in unrippened tomato (its concentration decrease during ripening process
- Tomatine is the most abundant glycoalkaloid in tomato
- "Solanine " the major naturally occuring toxicant is also found in Red tomato but its level is too low to be significant

Taste Test

No significant difference were detected

Processing Characteristics

- in processing characteristics, only those related to “pectin” are changed
- Flavr Savr Tomato has

a) Greater serum viscosity

b) No difference in pH, acidity, color, or biochemical parts including sugar and organic acid

- transformed tomato w/ Flavr Savr gene are *Phenotypically* identical to nontransformed control except (difference = it lacked 99% of normal PG activity
- Flavr Savr are *firmer* after maturity
- *greater* resistance to fungal disease

Factors that are influenced by Lack of PG Activity

- Extended Shelf live
- Increased Yield_
- Greater Serum Viscosity_
- Reduce fungal Pathogens_

Nutritional Components

No significant difference reported/ detected in nutritional components

Naturally Occuring Toxins

- Glycoalkaloids (ie Solanine and Tomatine)
- toxicity due to anticholinesterase activity on CNS and membrane disruption activity w/ affects the digestive system
- alpha tomatine is toxic & a insect repellent_
- tomatine is 100x LESS TOXIC than potato glycalkaloids_

Principal difference between Flavr Savr and control

- reduction of PG activity
- reduction decrease changes 1) Field quality 2) overall quality of fresh fruit

Human Exposure

no increase in tomato consumption or chnage in tomato consumption patterns are shown

Summary of Risk Characterization

1) Nutri value, taste, procesing , are unchanged except for things related to pectin
2) # of Kan r gne is less than 10
3) composition of Flavr Savr is not changed
4) No adverse pleiotropic straits
5) No stomatoes are genetically stable

What is Tomatine?

Tomatine is a steroidal glycoalkaloid in tomato fruits and other *Lycopersicon* and *Solanum* (potato) species. This glycoalkaloid induces cytotoxic and anti-fungal activity by binding to the 3-beta-hydroxy sterols in the fungal membrane, acts as an inhibitor of cholinesterase, and is thought to present resistance to microorganisms and insects in the tomato.

Tomatine is distributed all over the fruit but most concentrated in leaves and opening flowers. Since tomatine is not mobile, the concentration depends on the synthesis and degradation of tomatine as well as the ripeness of the fruit. As the fruit turns from green to red, the concentration declines. When left on the vine for 2-3 days, the ripe red tomato loses most of its tomatine. Concentrations found in non-transgenic tomatoes at different ripening stages are 0.87mg, 0.45mg and 0.36mg of tomatine/g fresh weight in green fruit, yellow fruit and red fruit respectively. The level of tomatine is also strongly influenced by environmental factors and the variety of the plant.

TOMATINE AND ANTI-PG TOMATOES

Tomatine is used as an indicator for safety assessment by the FDA. According to the WHO, "there should be thus no variation in the amount of the major constituents in a transgenic tomato as compared with the host tomato control and natural range."

How to measure Tomatine?

Common methods to measure tomatine are the high performance liquid chromatography (HPLC), and the absorptiometric method. There is no standardized method to measure tomatine due to its low presence in the tomato. The complex structure and high molecular weight of tomatine also contribute to the difficulties encountered when measuring tomatine content.

High Performance Liquid Chromatography (HPLC) with Pulse Amperometric Detection (PAD)

HPLC with PAD is used for direct analysis of tomatine in different parts of the tomato plant (in store bought, field grown, transgenic). The average percent of recovery of tomatine from green tomato is 71-91% while the average percent of recovery from red tomato is 65-114%. It was found that tomatine in ripe red tomatoes was 0.03-0.6mg/100g fresh weight and unripe green tomatoes was 4-17mg/100g fresh weight.

ABSORPTIOMETRIC METHOD

The anti-PG and normal tomato used by Furui et al. Were grown in the Research Institute of Kagome Co.. The tomato was harvested at mature and turning stage for tomatine measurement. The PG activity of the anti-PG tomato was first measured by the spectrophotometric method by Gross. The PG activity at mature stage was found to be 0.6% of normal. The tomato samples were then homogenized and frozen and then lyophilized for 2 days. The resulting product was made into a powder and stored at –20ºC until needed.

Homogenize and lyophilize sample

30ml of 2% AcOH/50% MeOH

Extract at 50ºC

Hydrolyze with 1.5N HCl (80ºC for 2hr)

Extract with $CHCl_3$

Wash with sat. $NaHCO_3$ and H_2O

Evaporate

Make up to 10ml with CH_2Cl_2

5ml of 0.2mM BTB (Na salt)

Shake and separate

Lower layer

1ml of 0.01M NaOH in MeOH

Measure at 620nm

A calibration curve was prepared with a tomatidine standard that had been obtained from tomatine to assess the tomatine content in the fruit.

TOMATIDINE STANDARD PREPARATION

100ml H_2O and 50ml 10% sulfuric acid added to 735mg Tomatine which was then hydrolyze for 2 hr at 100 ^oC. This reaction mixture was extracted with dichloromethane and washed with saturated sodium carbonate and H_2O. The dichloromethane phase was chromatographed in a column of silica gel with 100:1 dichloromethane methanol to afford 266mg of tomatidine. The curve was prepared with tomatidine being linear in the range of 0-3mg/100g.

Results

(Table 4)

Tomato	Tomatine Content (mg/100mg fresh wt)	
	Turning Stage	Mature Stage
Anti-PG	1.7	0.4
Non-transgenic	1.4	0.5

The tomatine content in both tomatoes was comparative at both stages when measured by HPLC with PAD and the absorptiometric method.

PHYSIOLOGICAL EFFECTS OF TOMATINE

Tomatine can cause minor inactivation of acetylcholinesterase. When compared to potato glycoalkaloid intoxication which includes gastro-intestinal disturbances, increased heartbeat, hemolysis and neurotoxic, the toxicity of tomatine is far less severe. Tomatine was also found to interfere with membrane structure and function. This interaction induces leakage of cell content such as ions and proteins in studies done on plant cells, fungi and mammalian cells.

In a study done with erythrocytes and intestinal epithelial cells which are both expected to be target cells of tomatine, it had disruptive effects on the membranes of both types of cells. Tomatine was able to cause complete hemolysis in erythrocytes by primarily interacting with the lipid part of the membrane whereas the intestinal epithelial cells lost their barrier function after exposure.

When tomatine is administered by gavage to a hamster, severe gross changes in the gastric

glandular mucosa and intestinal mucosa resulted. In the same experiment done with mice, diets of tomatine resulted in reduced liver weight.

OTHER POTENTIAL TOXICANTS FOUND IN TOMATOES

Other potentially toxic substance that may have adverse effects besides tomatine are tomatidine aglycone of tomatine, lectins, oxalic acid, protease inhibitor, histamine, and dehydrotomatine. However, only tomatine is considered to be able to cause any harmful effects.

Environmental Assessment of the FLAVR SAVR Tomato

One of the greatest concerns associated with the introduction of genetically modified crops into the environment is whether they will pose a risk to other organisms, such as plants, animals, and soil
microorganisms, in the area where they are grown. Careful environmental assessments must be undertaken before such crops can be grown for the consumer market. Extensive field tests and laboratory experiments were conducted by the Food and Drug Administration (FDA) and the Animal and
Plant Health Inspection Service (APHIS) to determine the impact of the FLAVR SAVR tomato on environmental biosafety and consumption. Commercially grown tomatoes, otherwise known as Lycopersicon resculentum, are self-pollinating, partly due to an inserted stigma, and are highly inbred. They do not cross-pollinate with other plants growing in their vicinity and do not maintain themselves in the environment without human aid. Insect pollination does not generally occur and tomatoes are not wind pollinated. Field tests in which tomatoes are

crossed with wild Lycopersicon species result in the production of inviable seeds. There is no evidence that the FLAVR SAVR tomato can cross-pollinate with other plants, and if such an event did occur, it would not contribute to the weedy properties of those plants any more than traditionally bred tomatoes. Tomato weeds that have occurred from traditionally bred tomatoes are easily controlled with herbicides. The FLAVR SAVR is also not known to have any selective advantage over other commercially grown tomatoes.

Beneficial insects, earthworms, bees and other animals, including humans, have not been negatively effected by the FLAVR SAVR tomato since the crops retains the essential characteristics of traditionally bred tomatoes. The modified tomato has produced no new toxins or pathogenic proteins. The kan-r selectable marker used in FLAVR SAVR also does not pose a threat to soil microorganisms since there is no natural mechanism in which the tomato genome can be horizontally transferred and incorporated into their genomes, and thus will not pose a plant pest risk. The FDA and APHIS have concluded that the FLAVR SAVR tomato poses no threat to the environment and other organisms and thus is safe to be commercially grown and sold on the market.

Fame or Shame: The Story of the Flavr Savr Tomato
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How do antisense tomatoes compare to transgenic tomatoes?

Transgenic Tomatoes – Advantages

§ Genes controlling for salt tolerance, and resistance to drought, flood, and extreme temperatures have been identified. Tomatoes will soon be able to fix nitrogen with greater efficiency, thereby, reducing the need for fertilizers. In a press release from Michigan State University in April, molecular geneticists announced identification of a gene that regulates a plant's ability to cope with cold weather.

§ Some tomato juice is made from tomatoes containing enzymes from the Arctic Flounder – this will assist tomatoes to withstand low temperatures.

§ Soon we can grow tomatoes in places where it is not normally grown, even in hot dry places.

§ Tomatoes may be cheaper.

§ Tomatoes may look better.

§ Scientists have already succeeded in breeding a vitamin-rich tomato which they hope can eventually help prevent heart disease and cancer. This tomato has increased levels of carotenoids that are important to health. It has about four times the normal levels of beta-carotene, which the body uses to make vitamin A, and twice the levels of lycopene (a close cousin of beta-carotene), the compound that

helps make tomatoes red. With reports that increased levels of lycopene reduce the risk of some cancers, scientists have found the gene that encodes the enzyme to produce lycopene and inserted it into a ripening tomato. Bacteria was used to deliver the new gene into the tomato.

§ There is a lot of development in herbicide-resistant, virus-resistant and pest-resistant crops. Bacillus thuringiensis (Bt) is a naturally occurring soil bacteria which produces a protein that kills a range of common insects once it is ingested. The Bt gene has been isolated and inserted into crops including tomatoes. Another example is the Roundup tomatoes. Roundup is a herbicide, also known as glyphosate, which kills weeds but may also kill crops. It works by inhibiting amino acid biosynthesis and prevents plants from making aromatic amino acids (EPSP is the inhibited enzyme). Scientists wanted a plant that was resistant to glyphosate. They isolated bacteria that were resistant and cloned the resistant gene (called AroA). They took the AroA gene from the resistant bacteria and inserted it into the plant thus the glyphosate resistant gene was produced in the plant. Field tests showed that the weeds died but not the glyphosate resistant plants when sprayed with glyphosate. This is in its development stages for tomato by Monsanto.

§ Scientists in the UK at Axis Genetics have modified plant viruses and directed genetic modification of edible plants to stimulate the body's immune system. Their Epicoat technique modified plant viruses so that they become living vaccine factories presenting biologically active polypeptides on their surfaces. These vaccines can in turn be inserted into edible plants to be eaten. This technology is still in its developmental stages for tomatoes. There may be rabies protection in the future. Tomatoes are producing the necessary G proteins in current experiments.

§ Edible vaccines and vitamin-rich tomatoes can benefit countries where naturally vitamin rich vegetables are scarce. Edible vaccines are cheaper and more accessible (shots are no longer needed).

§ Generally, the insertion of genes from different species into tomatoes has enabled it to greatly exceed its potential (e.g. grow in extreme climatic conditions, pest resistance, etc.). Transgenic tomatoes can do things that people could only imagine (e.g. fight cancer and disease).

Transgenic Tomatoes – Disadvantages

§ The following is a list of potential hazards of transgenic foods (possibly, including tomatoes):

i) The inserted gene may itself have adverse effects._

ii) The inserted gene may code for a protein that is toxic to human beings or that produces an allergic reaction._

iii) The inserted gene may alter the way existing genes in a plant or an animal express themselves, which may in turn increase the production of existing toxins or switch on the production of previously silent genes. Proteins that are normally produced at low levels may become toxic at high levels._

iv) The inserted gene may alter the behavior of a micro-organism which is carrying it to make it potentially harmful._

v) The inserted gene may be transferred from a micro-organism which is carrying it to other micro-organisms, in the human gut or respiratory tract or to animals or humans._

vi) The consumption of a GM micro-organism may alter the balance of existing micro-organisms in the human gut._

§ The following are a list of **possible** health concerns:

i) *Communicable Disease* – pathogens could ‘ jump’ from one species to another.

ii) *Antibiotic Resistance* – antibiotic resistance genes may be transferred from GM organisms.

iii) *Non-communicable disease, including chronic disease and fetal abnormalities* – diseases that are not cause by infections (e.g. cancer, diabetes, mellitus, heart disease, etc.) could be increased.

iv) *Nutritional Imbalance Effects* – the use of a genetically modified crop for food may result in the composition of the final food product being different to that of the conventional food it would replace.

v) *Altered Immune Response* – some chemicals can alter the immune responsiveness, either increasing it, leading to allergy, or depressing it. With the edible vaccines, too much exposure to too much protein may cause a tolerance

instead of an immune response.

vi) *Indirect Effects* – the effects of these foods on the environment may lead to health problems indirectly.

§ Vaccines may be destroyed. When plants are cooked the proteins may denature (many proteins are not heat stable).

§ There are environmental concerns as well. There may be a rise of superweeds (weeds that are resistant to herbicides and pesticides and viruses) and resistant insects. With Bt, this protein is not selective therefore it may create a population of resistant insects as a result of constant exposure.

§ Biology is very complex therefore there is an inability to control or predict the effects of DNA recombination (e.g. new proteins may be produced that humans have never encountered, thus scientists will not know the effects they may have on human health).

Antisense Tomatoes – Advantages

§ There has been an improvement of flavor, texture, viscosity (for purees, ketchup, and soups), bio-absorbability, nutritional content and elimination of genes for toxic substances and allergens.

§ Calgene researchers removed the gene that produced polygalacturonic acid (PG), copied it and reinserted the copy into the plant backwards. This "antisense" gene cancels most of the PG enzyme production, allowing the tomatoes to soften more slowly.

§ The tomatoes can remain longer on the vine to develop their natural flavor, but stay firm enough to be shipped to the market.

§ Scientists have genetically altered the levels of auxin, a tomato hormone for growth and ripening. It's the best known – and probably the most important – of the five major plant hormones (its been studied for more that 120 years). Scientists had been able to change auxin levels, but the changes were expressed throughout the

plant, not just in the fruit. The aim was to control the hormone production so that it can be introduced into specific, targeted tissues – such as the fruit – without affecting the growth processes in other parts of the plant. The scientists have inserted a copy of iaglu backwards (a corn gene) to turn off auxin. Because the gene was put in with a fruit-specific promoter, only the tomato fruit was affected. The resultant fruit ripened slowly. Another plus for auxin: Decreasing the gene's level of expression throughout receptor plants caused them to easily form large numbers of roots from cuttings and spurred rapid root growth in germinating seedlings. This could be significant for plants that are difficult to root from cuttings and could increase the survival rate of seeds planted in dry soils.

§ Generally, antisense technology has exploited pre-existing processed in tomatoes to enhance their quality (e.g. manipulate levels of enzymes during ripening). The gain over normal tomatoes is significant but not as much as transgenic tomatoes.

Antisense Tomatoes – Disadvantages

§ If the antisense modification involves the insertion of foreign DNA into the tomato genome, then the "list of potential hazards" (see above) could possibly apply.

§ The use of antisense technology to reduce PE and PG levels has led to tomatoes that are more resistant to splitting and have a prolonged ripening period. However, there are still plenty of features of a tomato that are unaffected by reduced PG and PE during the ripening process (e.g. PG and PE have no effect on softening). Therefore, there are some limitations to antisense tomatoes. To completely prolong all the processes during ripening, we must fully understand all of the genes and enzymes involved. It is definitely not as simple as inserting a new gene into the tomato genome.

What's on the Market?

(Table 5)

Product	Company	Altered Trait	Purpose	Sources of New Genes	Agency Action[1]
Tomato	Calgene	Delayed Ripening	Enhance fresh market value	Tomato, Bacteria, Virus	USDA approved[2] FDA approved[3]
Tomato	DNA Plant Technology	Delayed Ripening	Enhance fresh market value	Tomato, Bacteria, Virus	USDA approved FDA approved
Tomato	Monsanto	Delayed Ripening	Enhance fresh market value	Bacteria	USDA approved FDA approved

Tomato	Zeneca / PetoSeed	Thicker Skin, altered pectin	Enhance processing value	Tomato, Bacteria, Virus	USDA approved FDA approved

1. Agency action may respond to wither voluntary or required submissions from companies

2. USDA approval means that the product has been approved to enter market by the Food Safety and Inspection Service (FSIS) division of the United States Department of Agriculture.

3. FDA approval means that the Food and Drug Agency has completed consultations with a company and will allow the product to enter the market once regulatory requirements are met at other agencies. Except for the Calgene tomato approved in 1994, FDA consultations are abbreviated reviews of company safety assessments.

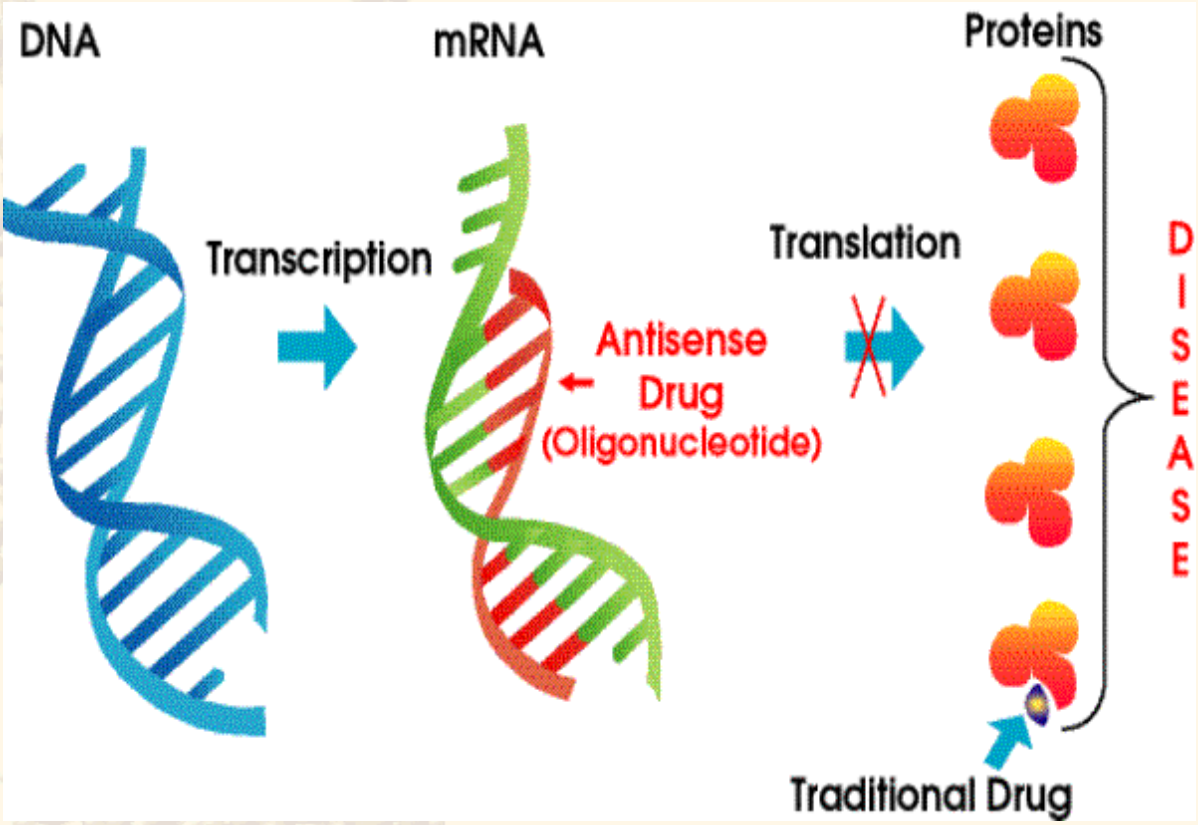
Provided by The Gene Exchange - A Public Voice on Biotechnology and Agriculture: 7 (1), Union of Concerned Scientists, Dec 1996.

Flavr Savr Around the World

ANTISENSE IN DISEASE RESEARCH

The antisense technology is used in disease research to inhibit the production of disease-causing proteins. They can be designed to treat a wide variety of diseases including viral, inflammatory and cardiovascular diseases, as well as cancer. These antisense drugs have the potential to be more selective and therefore more effective and less toxic than traditional drugs.

(Figure 10)



Creating Antisense Drugs:

Nucleotides are linked together in short chains or oligonucleotides. Antisense drug technology uses the same insertion process as our Flavr Savr tomato uses. The antisense drug is designed to bind to a specific sequence of nucleotides in its mRNA target to inhibit the production of the protein encoded by the target mRNA, therefore, the disease causing protein is stopped by acting in this early stage.

Drugs in the Works:

There are several drugs currently being worked on, including drugs for AIDS, CMV retinitis, Crohn's disease, psoriasis, ulcerative, colitis (enema),

hepatitis C, inflammation and all forms of cancer. All of these drugs use the same technology, and several of them are very close to being put on the market. These antisense drugs can not kill the cells which are already infected, providing more of a therapy than a cure.

Although the therapeutic applications of antisense technology are still waiting for approval, many scientists are hopeful that this form of gene therapy will provide patients with a painless alternative treatment in the future.

Conclusion:

After conducting this study it becomes apparent that the public concern, specifically targeted at Flavr Savr Tomatoes, is unfounded. Antisense technology effectively modifies and enhances tomatoes without

significantly altering the contents of the tomato or threatening public health.

THE END