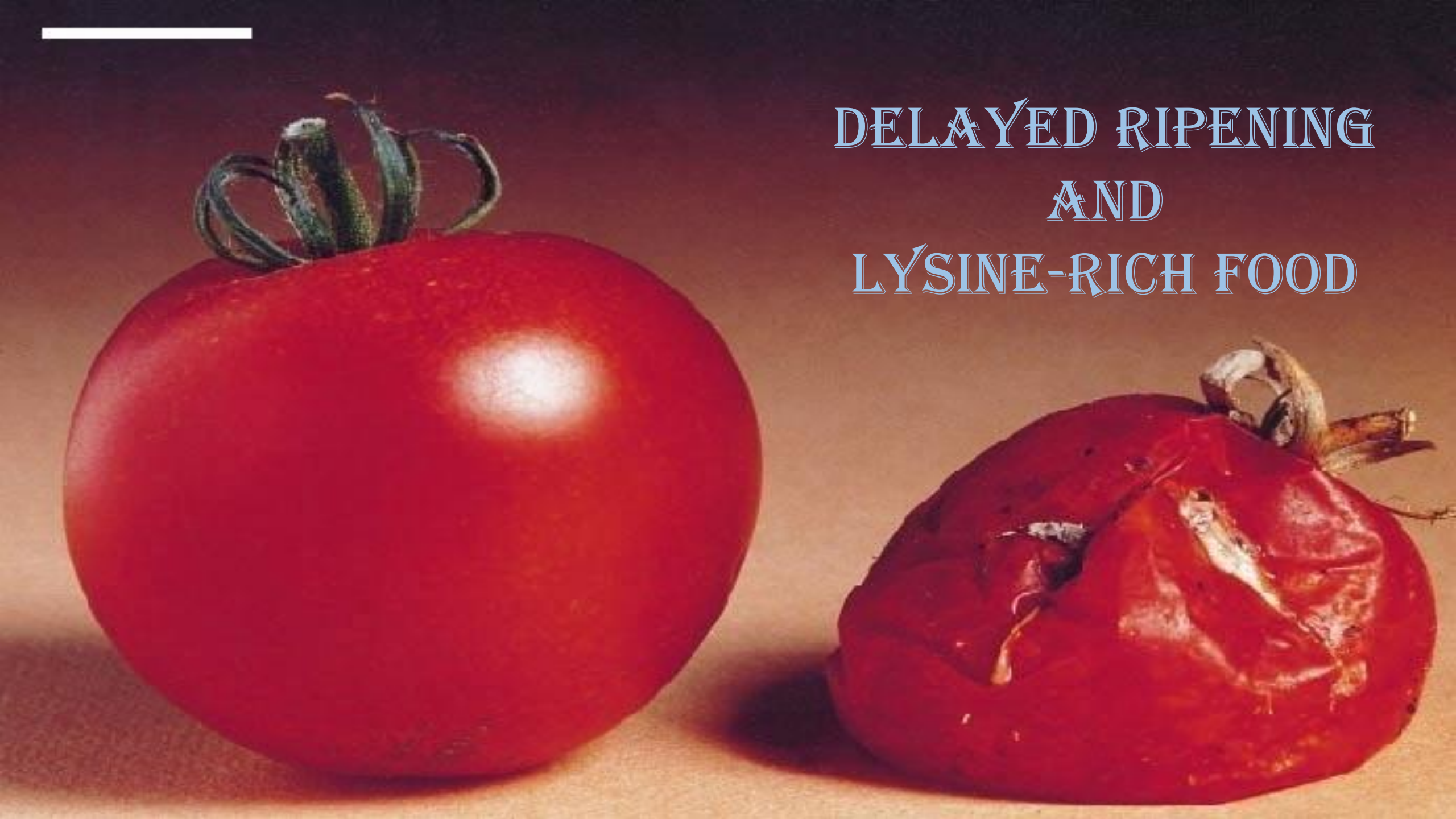
DELAYED RIPENING AND LYSINE-RICH FOOD



PURPOSE

- There are a wide range of crops that have been manipulated by scientists for improved yield and quality.

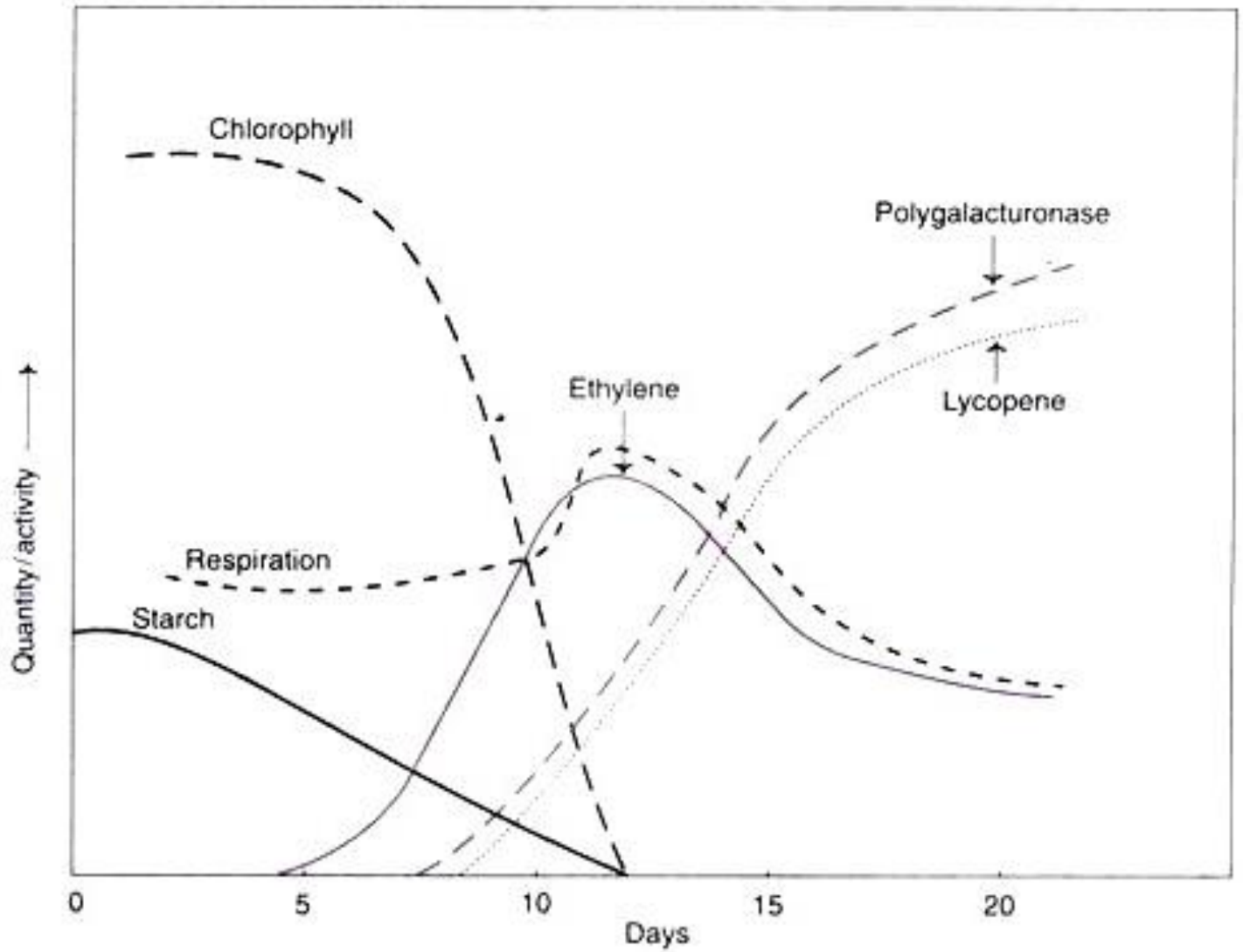
- The genetic manipulation of fruit ripening has become an important commercial aspect. Delay in fruit ripening has many advantages
  1. It extends the shelf-life, keeping the quality of the fruit intact.
  2. Long distance transport becomes easy without damage to fruit.
  3. Slow ripening improves the flavor.

Biochemical Changes during Tomato Ripening

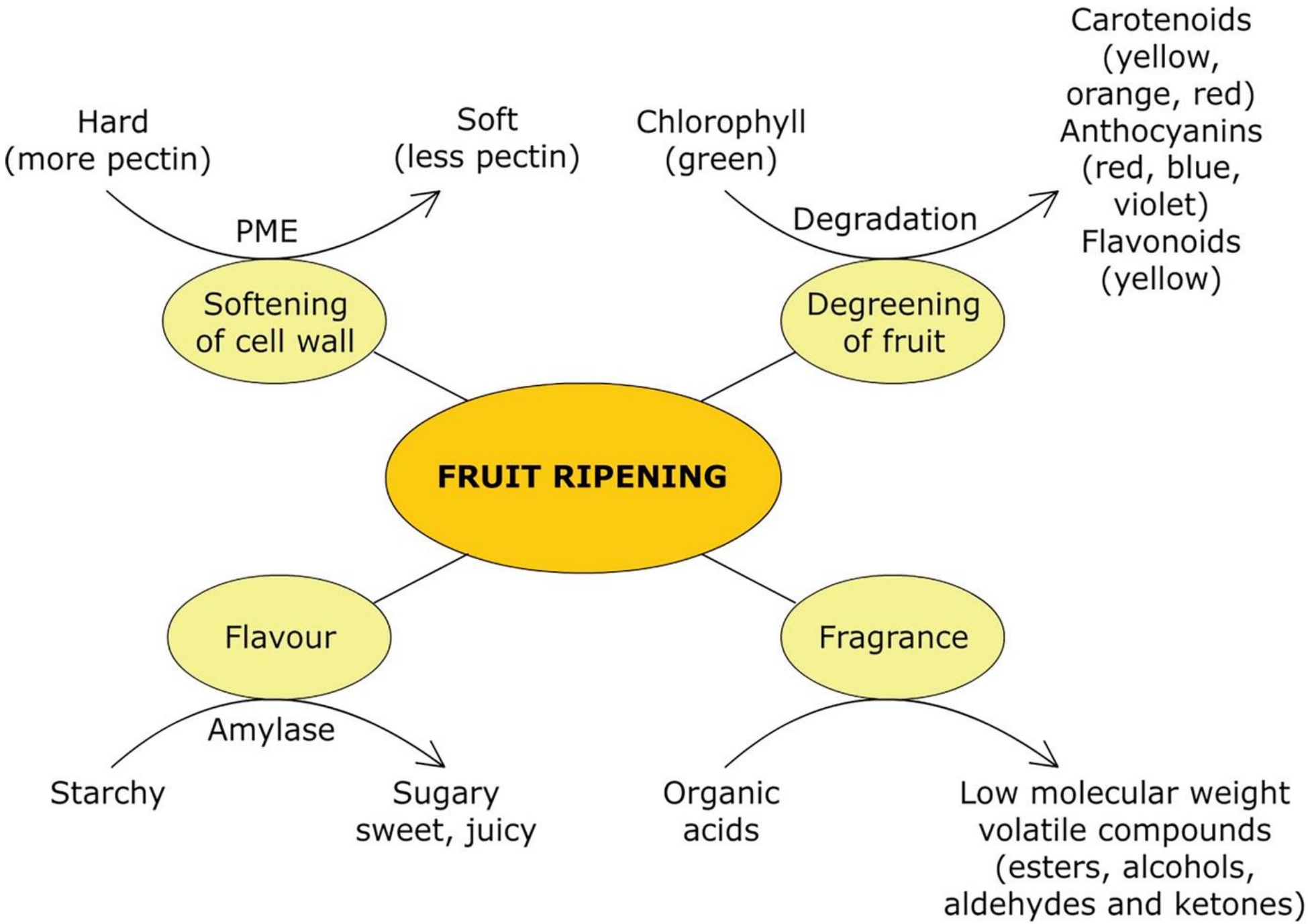
- Genetic engineering work has been extensively carried out in tomatoes.

- Fruit ripening is an active process. It is characterized by increased respiration accompanied by a rapid increase in ethylene synthesis.
- As the chlorophyll gets degraded, the green color of the fruit disappears, and a red pigment, lycopene is synthesized.

- The fruit gets softened as a result of the activity of cell wall degrading enzymes namely polygalacturonase (PG) and pectin methyl esterase.



Biochemical changes during the process of tomato ripening


Hard
(more pectin)
Soft
(less pectin)
PME
Softening
of cell wall
Chlorophyll
(green)
Carotenoids
(yellow,
orange, red)
Anthocyanins
(red, blue,
violet)
Flavonoids
(yellow)
Degradation
Degreening
of fruit
FRUIT RIPENING
Flavour
Amylase
Starchy
Sugary
sweet, juicy
Fragrance
Organic
acids
Low molecular weight
volatile compounds
(esters, alcohols,
aldehydes and ketones)

- The phytohormone ethylene production is intimately linked to fruit ripening as it triggers the ripening process of fruit. Addition of exogenous ethylene promotes fruit ripening, while inhibition of ethylene biosynthesis drastically reduces ripening.
- The breakdown of starch to sugars, and accumulation of a large number of secondary products improves the flavour, taste and smell of the fruit.
- Three distinct genes involved in tomato ripening have been isolated and cloned.
- The enzymes encoded by these genes and their respective role in fruit ripening are given in table below

Gene clone	Enzyme synthesized	Function in ripening
pTOM5	Phytoene synthase	Lycopene synthesis that gives red coloration
pTOM6	Polygalacturonase	Degradation of cell, resulting in fruit softening
pTOM13	ACC oxidase	Ethylene formation that triggers fruit ripening

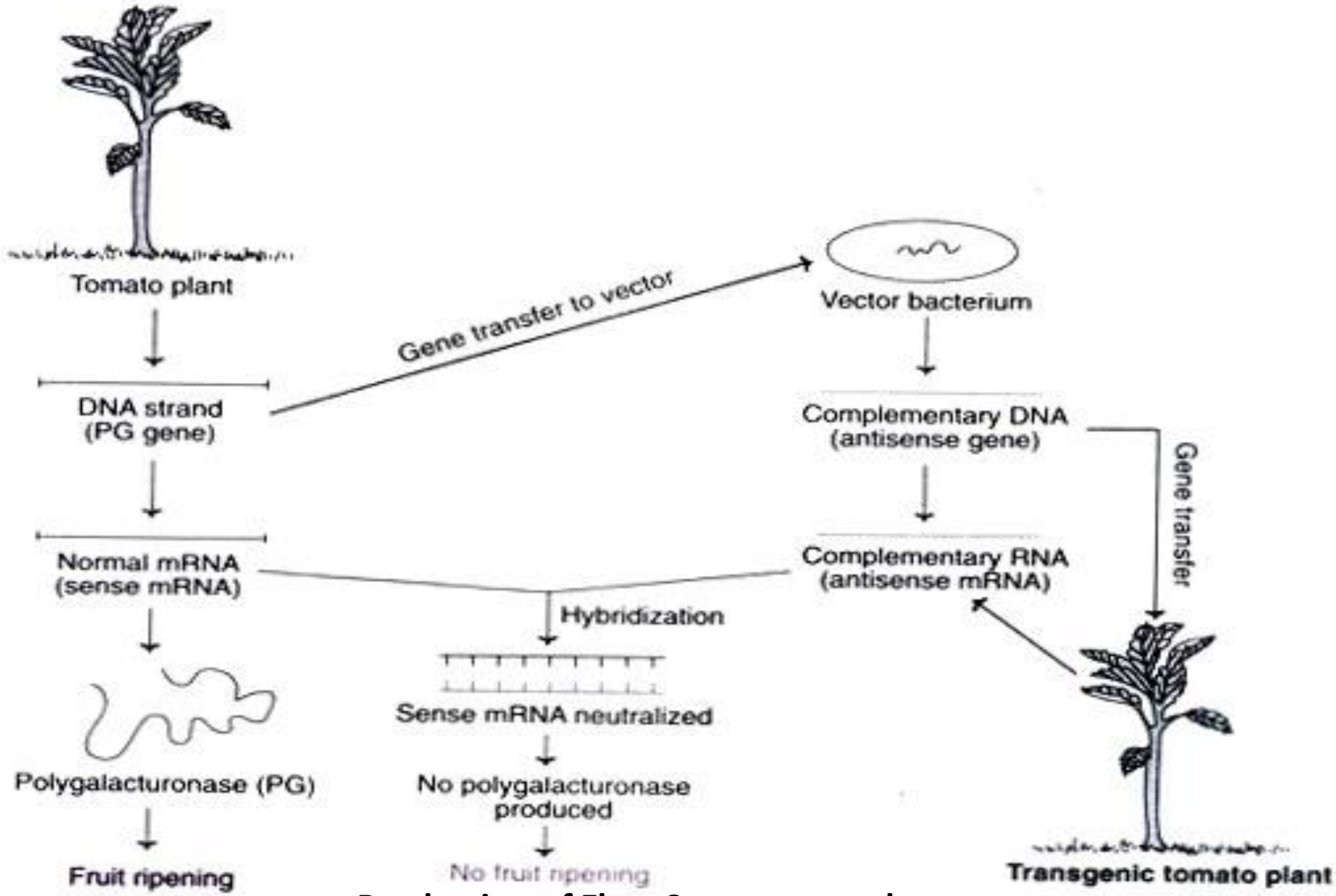
Genetic Manipulations of Fruit Ripening

1. Manipulation of the enzyme polygalacturonase (development of Flavr Savr tomato):
   - Softening of the fruit is largely due to degradation of the cell wall (pectin) by the enzyme polygalacturonase (PG). The gene responsible for PG, the rotting enzyme, has been cloned (pTOM 6). The genetic manipulation of polygalacturonase by antisense RNA approach for the development of Flavr Savr tomato was done by Calgene Company in USA. It involves the following steps-

   i. Isolation of the DNA from tomato plant that encodes the enzyme polygalacturonase (PG).

   ii. Transfer of PG gene to a vector bacteria and production of complementary DNA molecules.

   iii. Introduction of complementary DNA into a fresh tomato plant to produce a transgenic plant.



Production of Flavr Savr tomato plant

Mechanism of PG antisense RNA approach:

- In the normal tomato plant, PG gene encodes a normal (sense) mRNA that produces the enzyme polygalacturonase that is actively involved in fruit ripening.
- The complementary DNA of PG encodes for antisense mRNA, which is complementary to normal (sense) mRNA.
- The hybridization between the sense and antisense mRNAs renders the sense mRNA ineffective.
- Consequently, no poly-galacturonase is produced, hence fruit ripening is delayed.

The rise and fall of Flavr Savr Tomato:

- The genetically engineered tomato, known as Flavr Savr (pronounced flavour saver) by employing PG antisense RNA was approved by U.S. Food and Drug Administration on 18th May 1994.
- The FDA ruled that Flavr Savr tomatoes are as safe as tomatoes that are bred by conventional means, and therefore no special labeling is required.
- The new tomato could be shipped without refrigeration to far off places, as it was capable of resisting rot for more than three weeks (double the time of a conventional tomato).
- Although Flavr Savr was launched with a great fanfare in 1995, it did not fulfill the expectation for the following reasons:
    i. Transgenic tomatoes could not be grown properly in different parts of U.S.A.
    ii. The yield of tomatoes was low.
    iii. The cost of Flavr Savr was high.

The rise and fall of Flavr Savr Tomato:

- It is argued that the company that developed Flavr Savr, in its overenthusiasm to become the first Biotech Company to market a bioengineered food had not taken adequate care in developing the transgenic plant.
- And unfortunately, within a year after its entry, Flavr Savr was withdrawn, and it is now almost forgotten.
- Mainly havoc created by anti-GM groups and Kan^R gene inserted also created problems for the company as people stopped buying the product due to this gene.

Delayed Fruit Ripening

d) Tomatoes have been engineered to produce less ethylene so they can develop more taste before ripening, and shipment to markets.

e) What happened to the Flavr Savr tomato?

i. Produced by Calgene by blocking the polygalacturonase (PG) gene, which is involved in spoilage. PG is an enzyme that breaks down pectin, which is found in plant cell walls.

ii. Plants were transformed with the anti-sense PG gene, which is mRNA that base pair with mRNA that the plant produces, essentially blocking the gene from translation.

iii. First genetically modified organism to be approved by the FDA, in 1994.

iv. Tomatoes were delicate, did not grow well in Florida, and cost much more than regular tomatoes.

v. Calgene was sold to Monsanto after Monsanto filed a patent-infringement lawsuit against Calgene, and the Flavr Savr tomato left the market.

Flavr Savr Tomato

Normally, tomatoes are picked while green and transported many miles before being sprayed with ethylene to ripen them.

This prevents damage and perishing on the journey.

The Flavr Savr tomato is a genetically engineered tomato which has a gene inserted to extend shelf-life by slowing down the rotting process.

The Flavr Savr tomato was the first GM fruit to be sold in the World.

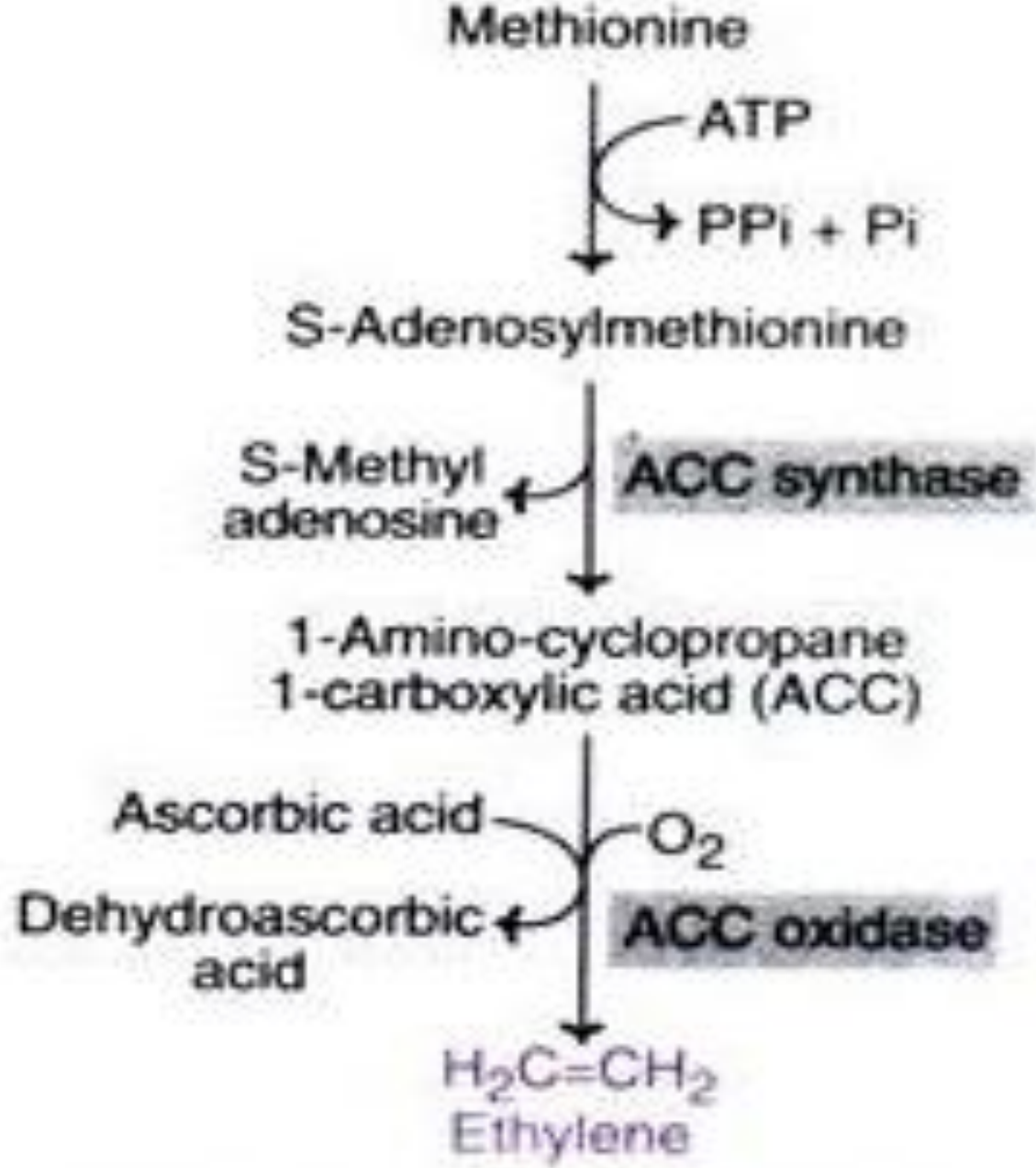
Is it better to spray tomatoes with ethylene than genetically engineer them?

Plant Hormones - Ethylene

- Found in
  - Tissues of ripening fruit
  - Nodes of stems
  - Aging leaves and flowers
- Major functions
  - Changes of ovary to become fruit
    - Degradation of cell walls; softening
    - Dropping from plant
  - Leaf abscission
    - Loss of leaves to prevent water loss
    - Tissue at base of petiole dies
  - Senescence (aging)
    - Autumn leaves; withering flowers

2. Manipulation of ethylene biosynthesis:

- It has been clearly established that ethylene plays a key role in the ripening of fruits.
- Ethylene is synthesized from S-adenosyl methonine via the formation of an intermediate, namely 1 -aminocyclopropane-1 carboxylic acid (ACC), catalysed by the enzyme ACC synthase.
- The next step is the conversion of ACC to ethylene by ACC oxidase.


Methionine
ATP
PPi + Pi
S-Adenosylmethionine
S-Methyl adenosine
ACC synthase
1-Amino-cyclopropane 1-carboxylic acid (ACC)
Ascorbic acid
O2
Dehydroascorbic acid
ACC oxidase
H2C=CH2
Ethylene

- **Strategies developed to block ethylene biosynthesis are as follows-**

  i. Antisense gene of ACC oxidase:

  Transgenic plants with antisense gene of ACC oxidase have been developed. In these plants, production of ethylene was reduced by about 97% with a significant delay in fruit ripening.

  ii. Antisense gene of ACC synthase:

  Ethylene biosynthesis was inhibited to an extent of 99.5% by inserting antisense gene of ACC synthase, and the tomato ripening was markedly delayed.

  iii. Insertion of ACC deaminase gene:

  ACC deaminase is a bacterial enzyme. It acts on ACC (removes amino group), and consequently the substrate availability for ethylene biosynthesis is reduced. The bacterial gene encoding ACC deaminase has been transferred and expressed in tomato plants. These transgenic plants inhibited about 90% of ethylene biosynthesis. The fruit ripening was delayed by about six weeks.

Longer Shelf-Life of Fruits and Vegetables:

- The spoilage of fruits, vegetables and senescence of picked flowers, collectively referred to as post- harvest spoilage is major concern in agriculture. This hampers the distribution system particularly when the transport is done to far off places.
- The successful manipulations to delay ripening, senescence and spoilage of various foods will significantly contribute to the appropriate food distribution and thus good economic practices in agriculture.
- Suppressing the biosynthesis of ethylene appears to be a promising area to reduce the spoilage of fruits, vegetables and senescence of flowers.
- The three different strategies to block ethylene synthesis described in tomato can be successfully used for other fruits, vegetables etc., to achieve longer shelf- life.

Transgenic Plants with Improved Nutrition

Transgenic Plants with Improved Nutrition:

- Create crops that are tailored to provide better nutrition for humans and their domestic animals. Ex. Improve lysine content in Cereals and Methionine content in Legume seeds.
- Genetic manipulations for improving the nutritional quality of plant products are of great importance in plant biotechnology. Some success has been achieved in this direction through conventional cross-breeding of plants.

- However, this approach is very slow and difficult, and many a times will not give the traits with the desired improvements in the nutritional quality.

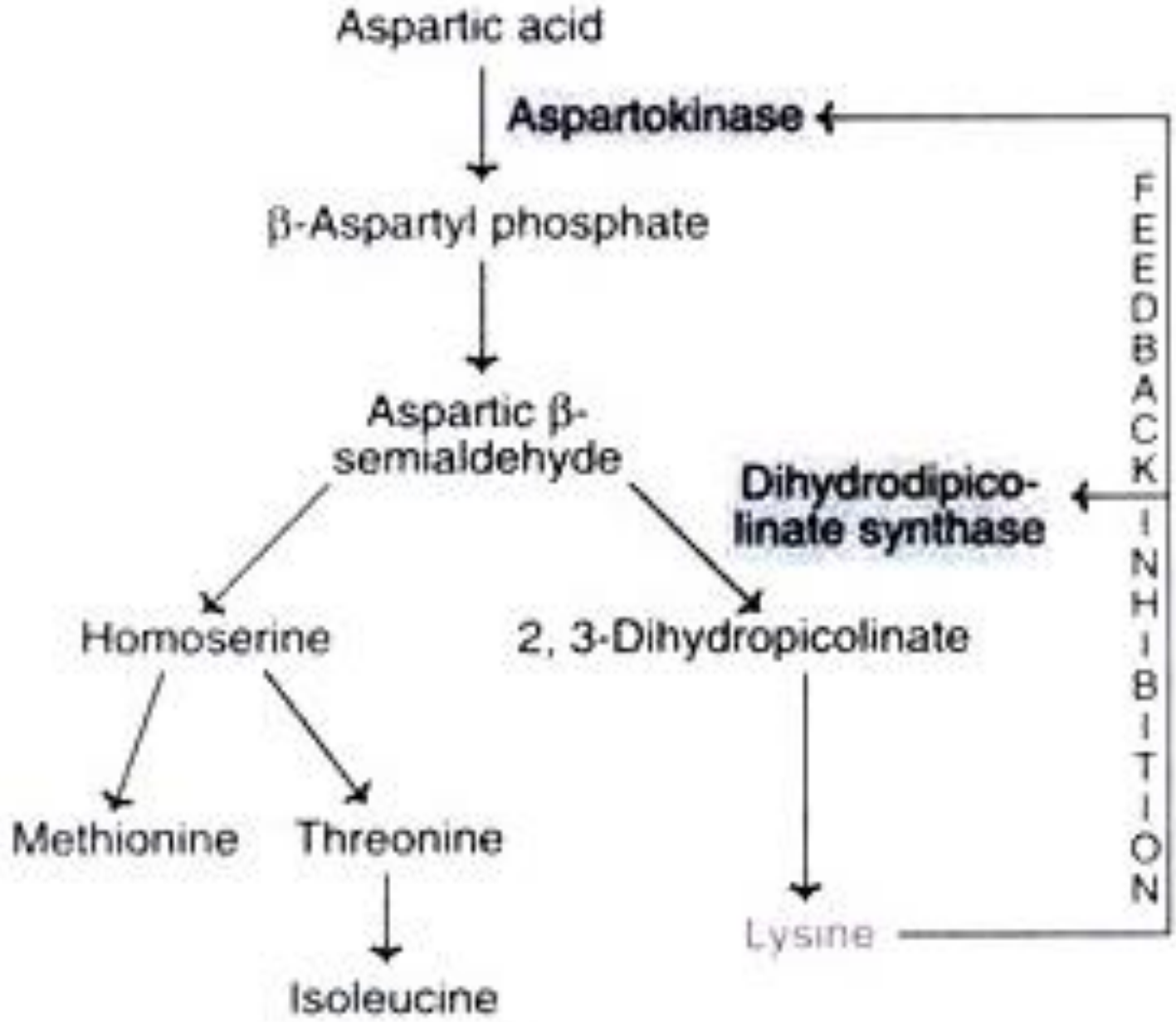
- In the following slides selected examples of genetic engineering with improved nutritional contents are described.

1. Amino Acids of Seed Storage Proteins:

- Of the 20 amino acids present in the humans, 10 are essential while the other 10 can be synthesized by the body.
- The 10 essential amino acids (EAAs) have to be supplied through the diet.
- Cereals (rice, wheat, maize, corn) are the predominant suppliers of EAAs. However, cereals do not contain adequate quantity of the essential amino acid lysine.
- On the other hand, pulses (Bengal gram, red gram, soybean) are rich in lysine and limited in sulfur-containing amino acids (the essential one being methionine).
- Transgenic routes have been developed to improve the essential amino acid contents in the seed storage proteins of various crop plants.

i. **Overproduction of lysine by deregulation:**

- The four essential amino acids namely lysine, methionine, threonine and isoleucine are produced from a non-essential amino acid aspartic acid.
- The formation of lysine is regulated by feedback inhibition of the enzymes aspartokinase (AK) and dihydrodipicolinate synthase (DHDPS).
- Theoretically, it is possible to overproduce lysine by abolishing the feedback regulation.
- The lysine feedback-insensitive genes encoding the enzymes AK and DHPDS have been respectively isolated from *E. coli* and *Cornynebacterium*.
- With appropriate genetic manipulations, these genes were introduced into soybean and canola plants. The transgenic plants so developed produced high quantities of lysine.


Aspartic acid
Aspartokinase
β-Aspartyl phosphate
Aspartic β-semialdehyde
Dihydrodipico-linate synthase
Homoserine
2, 3-Dihydropicolinate
Methionine
Threonine
Isoleucine
Lysine
FEEDBACK INHIBITION

ii. Transfer of genes encoding methionine-rich proteins:

- Several genes encoding methonine-rich proteins have been identified:
  a. In maize, 21 KDa zein with 28% methionine.
  b. In rice, 10 KDa prolamin with 20% methionine.
  c. In sunflower, seed albumin with 16% methionine
- These genes have been introduced into some crops such as soybean, maize and canola.
- The transgenic plants produced proteins with high contents of sulfur-containing amino acids.

Transfer of genes encoding methionine-rich proteins:

- Isolate the gene for a naturally occurring, sulphur-poor seed protein and to modify its nucleotide sequence so that it encodes a protein with an increased Sulphur AA composition

Ex. Gene for Beta-Phaseolin from *Phaseolus vulgaris* was modified by addition of a 45bp nucleotide sequence encoding a methionine rich region from a maize 15KDa Zein seed storage protein

The added peptide was predicted to form a Alpha-helical structure and was inserted into a alpha-helical region of Phaseolin

Modification increased the M residues from 3 to 9.

- Creation of entirely synthetic gene sequences encoding artificial proteins with high Meth content

Transfer of genes encoding methionine-rich proteins:

- Genes encoding novel proteins that contain large amounts of EAA like Ly, Trp and Met have been synthesized in *E.coli* to test stability.
- When expressed in transgenic tobacco, protein containing 31%Lys residues and 22% Met residues resulted in upto 20% increase in seed Met.
- Similar level can be expressed in legume seeds – overall Met content could be improved in seeds.
- Sweet potato – experiments were done – A synthetic gene encoding a protein rich in several EAA including 13% Met residues – resulted in significant increase in EAA concentration in the storage roots.
- Sweet potato – important part of diet in many countries – human nutrition
- Although animal feeding experiments – necessary to confirm the digestibility of the synthetic protein.

iii. Production of lysine-rich glycinin in rice:

- Glycinin is a lysine-rich protein of soybean. The gene encoding glycinin has been introduced into rice and successfully expressed.
- The transgenic rice plants produced glycinin with high contents of lysine.
- Another added advantage of glycinin is that its consumption in humans is associated with a reduction in serum cholesterol (hypo- cholesterolaemic effect).

iv. Construction of artificial genes to produce proteins rich in EAAs:

- Attempts are being made to construct artificial genes that code for proteins containing the essential amino acids in the desired proportion. Some success has been reported in the production of one synthetic protein containing 13% methionine residues.

Genetic Engineering for Improving Palatability of Foods:

- More than the nutritive value, taste of the food is important for attracting humans. It is customary to make food palatable by adding salt, sugar, flavors and many other ingredients. It would be nice if a food has an intrinsically appetizing character.

- A protein monellin isolated from an African plant (*Dioscorephyllum cumminsii*) is about 100,000 sweeter than sucrose on molar basis. Monellin gene has been introduced into tomato and lettuce plants. Some success has been reported in the production of monellin in these plants, improving the palatability.