17.5 Ethylene

In 1886, while a graduate student in St. Petersburg, Dimitry Nikolayevich Neljubow noticed that etiolated pea seedlings grew horizontally in laboratory air, and vertically in air from outside the laboratory.

After an extensive study to exclude cultural practices, light, and temperature as causative agents, he showed that **ethylene**, in the gas used for lighting, induced this abnormal growth.

Many of ethylene's physiological effects on plant growth and development, including its impact on seed germination, root and shoot growth, flower development, senescence and abscission of flowers and leaves, and the ripening of fruit, were discovered prior to 1940. Subsequent work has since shown that ethylene also participates in the modulation of plant responses to a range of biotic and abiotic stresses.

Effects

- The so called *triple response* (a decrease in stem elongation, a thickening of the stem and a transition to lateral growth)
- Maintenance of the apical hook in seedlings (*apical hook—a structure of dicotyledonous plants shaped by the bended hypocotyl that eases the penetration through the covering soil*).
- Stimulation of numerous defense responses in response to injury or disease.
- Release from dormancy
- Shoot and root growth and differentiation
- Adventitious root formation
- Leaf and fruit abscission
- Flower induction in some plants
- Induction of femaleness in dioecious flowers
- Flower opening
- Flower and leaf senescence
- Fruit ripening

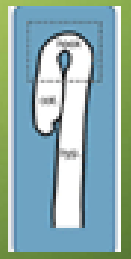


FIGURE 17.49 *The triple response to ethylene of six-day-old etiolated* P. sativum *seedlings and four-day-old etiolated* Vigna radiata *bean seedlings. (A) Untreated control* P. sativum *seedlings (0) and* P. sativum *seedlings grown for two days in air supplemented with ethylene at 0.1, 1.0, and 10 µl/ml. Note the concentration-dependent effects of ethylene on diageotropism, inhibition of epicotyl elongation, and lateral enlargement of the epicotyl. (B) Control* V. radiata *seedlings (0) and* V. radiata *seedlings grown for two days in air supplemented with 1 and 10 µl/ ml ethylene, which induces a concentration-dependent inhibition of hypocotyl elongation, lateral enlargement of the hypocotyl, and extreme bending of the apical hook. (C) Magnification of ethylene-treated etiolated* V. radiata *seedlings.*
Source: (A–C) H. Mori, Nagoya University, Japan; previously unpublished.

5.1 Ethylene is synthesized from S-adenosyl-l-methionine via the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC)

- Synthesis of ethylene from its immediate precursor 1- aminocyclopropane -1- carboxylic acid (ACC) is catalyzed by ACC oxidase (ACO).
- ACC is produced from SAM in a reaction catalyzed by ACC synthase (ACS).

These reactions are part of the methionine cycle or Yang cycle named after S. F. Yang, who carried out the early work in elucidating the pathway

In addition to its role in ethylene biosynthesis, SAM is involved in the biosynthesis of polyamines (PA) and a wide range of methylation reactions.

ACS has been isolated from a number of plant tissues following induction by factors that include exogenous IAA, wounding, lithium chloride stress, and climacteric fruit ripening.

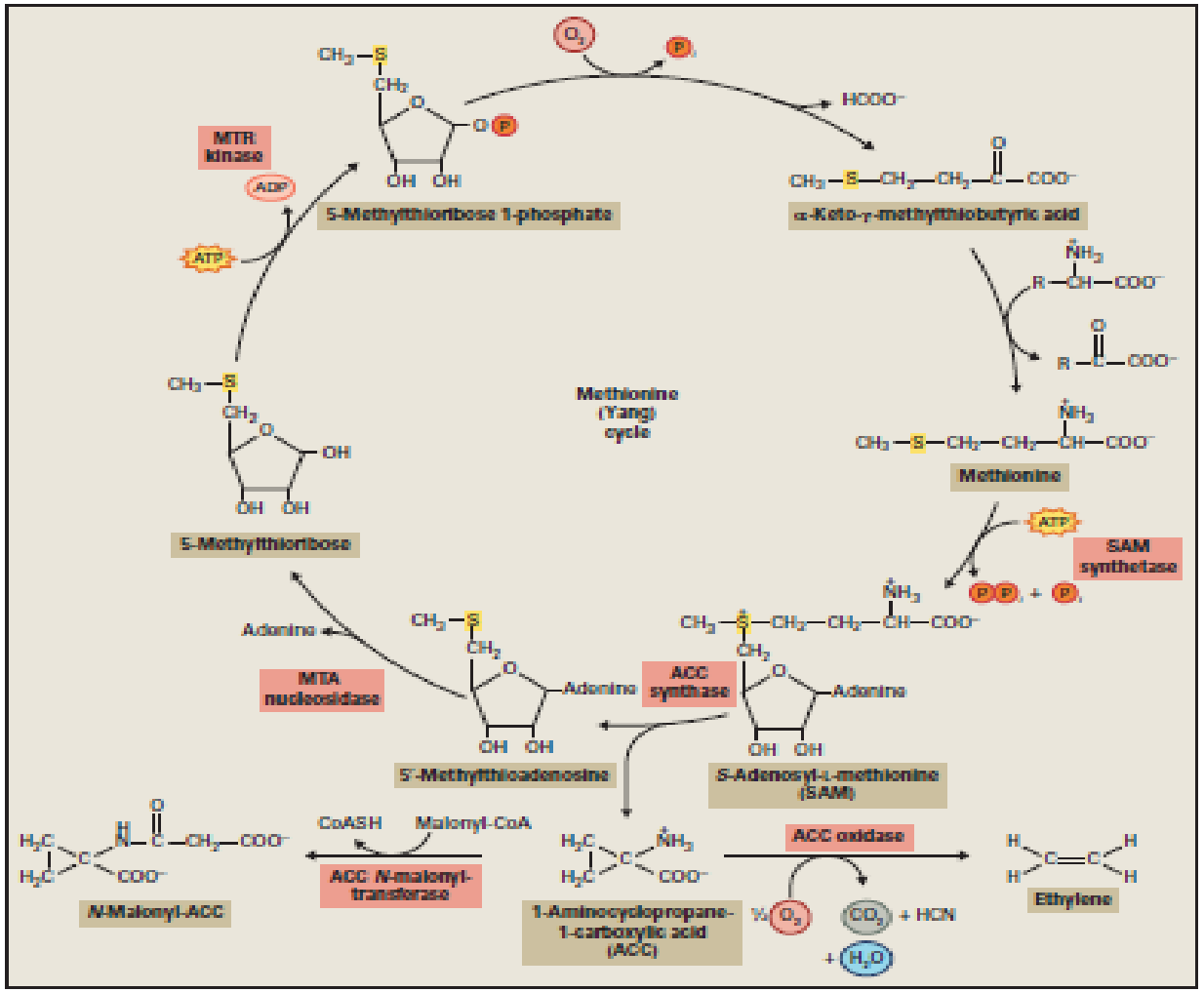


FIGURE 17.50 *The Met cycle and ethylene biosynthesis. Ethylene is synthesized from Met by way of SAM and ACC. The enzymes that catalyze these three steps are SAM synthase, ACC synthase (ACS), and ACC oxidase (ACO). 5′-Methylthioadenine, a product of the ACO reaction, is salvaged for the resynthesis of Met through the methionine cycle (see Chapter 7). If the methylthio group from SAM were not recycled, Met availability and ethylene biosynthesis would probably be restricted by sulfur availability. By converting ACC to N-malonyl-ACC instead of to ethylene, plants can deplete the ACC pool and thereby reduce the rate of ethylene production.*

5.2 ACSs are major regulators of ethylene biosynthesis

ACSs catalyze the rate-limiting step in ethylene biosynthesis.

ACSs levels are controlled by:

transcription

and

protein stability.

Increased ethylene production, associated with germination, ripening, flooding, and chilling, is **invariably** accompanied by increased ACC production due to induction or activation of ACS.

ACSs requires pyridoxal phosphate for activity and is sensitive to inhibitors of pyridoxal phosphate, especially aminoethoxy-vinyl glycine and amino-oxy acetic acid. These inhibitors allow investigators to distinguish between the effects of ACS and ACO.

The naturally occurring isomer of SAM, (–)-S-adenosyl-lmethionine, is the preferred substrate for ACS, while (+)-SAM is an effective inhibitor.

However, incubating the enzyme with **high** concentrations of **(–)-SAM** can irreversibly modify and **inhibit ACS**.

This "suicide inactivation" involves covalent linkage of a fragment of the SAM molecule to the active site of the enzyme. This substrate-dependent inactivation may be a contributory factor in the rapid turnover of ACS in plant tissues.

ACSs levels are controlled by: transcription and protein stability.

Transcriptional control

Ethylene biosynthesis rates are influenced by other plant hormones and by ethylene itself.

Auxins promote ethylene synthesis by enhancing the rate of ACC production. Transcript analysis showed that auxin application results in increased levels of certain ACS mRNAs, indicating transcriptional control.

The respective ACS genes have been shown to have *cis* acting auxin-response elements.

Unripe developing fruits have auto inhibitory ethylene production and certain ACS genes are transcribed. During ripening ethylene can promote (autocatalyze) its production and different ACS genes are transcribed.

Control via protein turnover

Early observations indicated that ACS stability in *S. lycopersicum* fruits varied between ripe and unripe fruits.

More recent experiments have shown that ACS turnover plays an important role in regulating ethylene production and that the C-terminal regions of the ACS protein act in regulating this turnover.

The stability of ACS proteins is influenced by their phosphorylation status at C-terminal regions with the phosphorylated forms being more stable.

Type 1 ACS proteins are phosphorylated by a MAPK in response to stress, for example, pathogen and wounding, and by a CDPK. Phosphorylation increases protein stability whereas unphosphorylated proteins are degraded by an undefined mechanism. Type 2 ACS proteins are phosphorylated by a CDPK and this prevents binding of the ETO (**Et**hylene **o**verproducing) gene product. ETO1 can catalyze the addition of ubiquitin to ACS. Once ACS is ubiquitinated it is targeted for proteolysis.

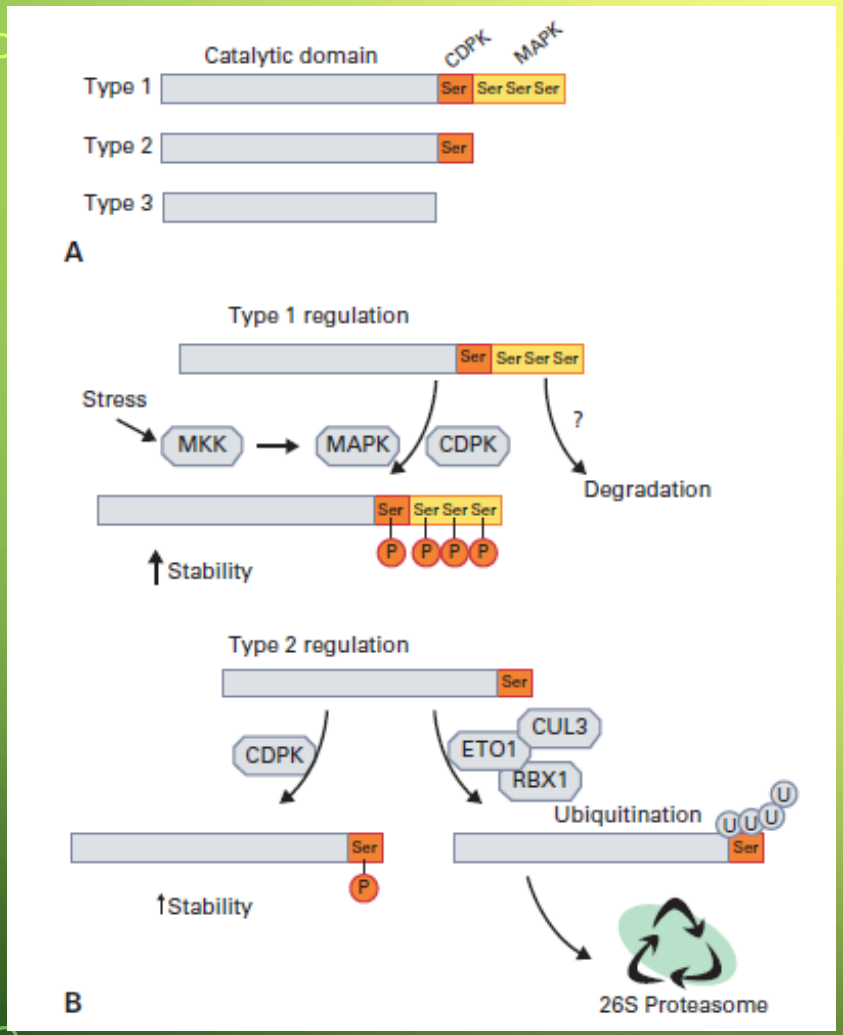


FIGURE 17.51 *ACS types and mechanisms of protein regulation. (A) ACS proteins fall into three major classes according to their C-terminal sequences. Type 1 have three conserved Ser residues (yellow) that can be phosphorylated by a MAPK and a single conserved Ser (red) that can be phosphorylated by a CDPK. Type 2 are slightly truncated and have a Ser that can be phosphorylated by a CDPK. Type 3 lack any conserved C-terminal Ser residues. (B) Type 1 regulation: CDPK and MAPK phosphorylation of C-terminal region increases ACS stability. The MAP kinase (MKK), induces MAPK activity when stimulated by stress e.g. wounding or pathogen attack. Unphosphorylated protein is removed by a yet to be defined mechanism. Type 2 regulation: CDPK phosphorylation of C-terminal Ser increases ACS stability by preventing binding of the ETO protein. Unphosphorylated ACS undergoes ETO binding and this facilitates the formation of a RING E ligase complex that includes a Cullin3 (CUL3) protein and a RING Box 1 protein (RBX1). The E3 ligase adds ubiquitin (U) moieties to the ACS protein thereby targeting it for degradation by the 26S proteasome.*

5.3 ACC oxidase resisted biochemical characterization and was cloned using molecular techniques

The conversion of ACC to ethylene is catalyzed by ACO, previously referred to as “ethylene-forming enzyme”.

- The ACO reaction can be summarized in:

$$ACC + O_2 + \text{ascorbate} \xrightarrow{Fe^{2+}} C_2H_2 + CO_2 + \text{dehydroascorbate} + HCN + 2H_2O$$

ACO is activated by one of its products, carbon dioxide. The cyanide generated by the reaction is detoxified by conversion to β-cyanoalanine, which is further metabolized to Asn or γ-glutamyl-β-cyanoalanine.

All plant tissues appear to contain ACO, as measured by the rate of ethylene evolution in the presence of a saturating concentration of ACC. Under stress conditions, in response to ethylene, and at selected stages of development (e.g., fruit ripening), ACO activity increases markedly. Both senescence and ripening-induced increases in ACO activity are a result of increased transcription.

FIGURE 17.52 *Effect of antisense ACO genes on ripening and spoilage of* S. lycopersicum *cultivar Ailsa Craig fruit picked three weeks after onset of ripening and stored at room temperature for three weeks. (Left) Fruits from the descendants of the original TOM13-antisense plants, which generate about 5% of the normal amount of ethylene. They ripen fully but do not overripen and deteriorate. (Right) Fruits from wild-type plants grown and stored under identical conditions. They produce normal amounts of ethylene and consequently exhibit severe signs of over-ripening.*

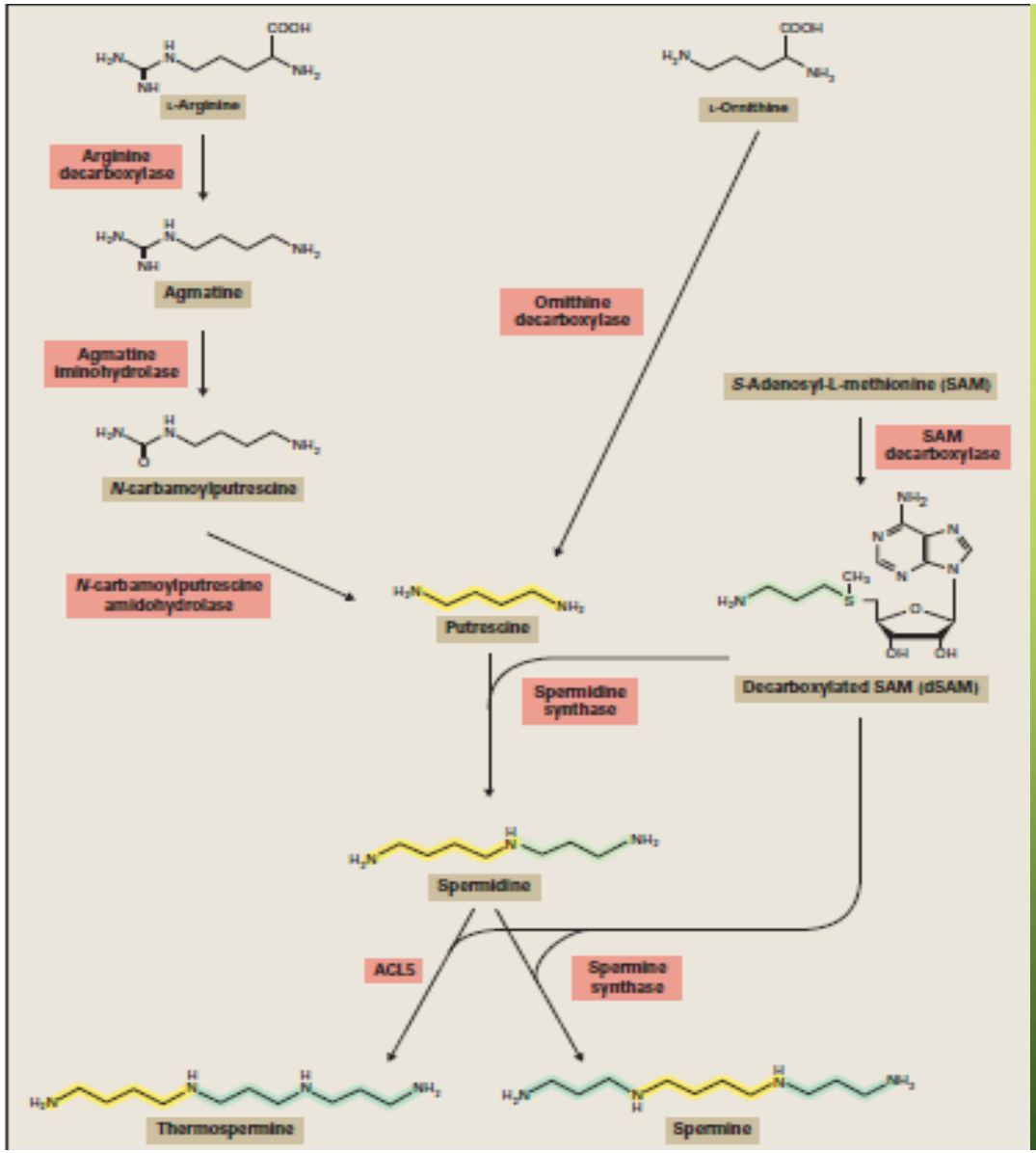
Source: D. Grierson, University of Nottingham, UK; previously unpublished.

5.4 When supply of available SAM is low, ethylene and polyamine biosynthetic pathways may compete for this shared substrate

- Biosynthesis of both ACC and PAs involves the incorporation of the aminopropyl group from SAM.

Under certain conditions, competition for SAM may restrict rates of ethylene or PA production. Inhibition of ACC synthesis by aminooxyacetic acid results in increased PA production. Conversely, inhibition of PA biosynthesis leads to increased concentrations of ACC and ethylene.

This implies that one SAM-dependent pathway is stimulated when the other is blocked. When competition for the available SAM is circumvented by low demand for PAs, or when ACC levels are increased by upregulation of 5′-methylthioribose-recycling enzymes, ethylene and PA production will not directly interact.


L-Arginine
Arginine decarboxylase
Agmatine
Agmatine iminohydrolase
N-carbamoylputrescine
N-carbamoylputrescine amidohydrolase
L-Ornithine
Ornithine decarboxylase
S-Adenosyl-L-methionine (SAM)
SAM decarboxylase
Decarboxylated SAM (dSAM)
Putrescine
Spermidine synthase
Spermidine
ACL5
Spermine synthase
Thermospermine
Spermine

5.5 Most hormones must be catabolized, but volatile ethylene can be released as a gas

Prior to 1975, ethylene metabolism by plants was considered to be an artifact, caused by bacterial contamination.

There is now evidence from plants grown in sterile conditions that [^{14}C]ethylene is oxidized to [^{14}C]CO2 or converted to [^{14}C]ethylene oxide and [^{14}C]ethylene glycol.

Ethylene metabolism exhibits a very high K_M indicative of a chemical reaction rather than a physiological process.

In peas, the concentration of ethylene yielding a half-maximal rate of ethylene metabolism is ≈1,000 times the concentration required for half-maximal response in the pea growth test.

It is likely that ethylene metabolism is largely a consequence of artificially elevated ethylene levels.

The major route by which plant tissues lose ethylene is probably diffusion to the surrounding atmosphere.

5.6 Repression of ethylene biosynthesis can delay over-ripening in fruit, and represents an important field of biotechnological research

Two different biotechnological strategies have been employed to generate transgenic tomato fruit that resist over-ripening.

1: the overexpression of a *Pseudomonas* gene encoding ACC deaminase, reduces ethylene levels in fruits by catalyzing the conversion of ACC to α-ketobutyric acid and NH3.

2: limiting ethylene biosynthesis involves use of antisense gene constructs against either ACO or ACS.

The phenotype of transgenic tomato fruit expressing antisense ACO:

- ethylene production is inhibited by about 95% during ripening.
- fruits grow normally and begin to change color, losing chlorophyll and accumulating lycopene, at the
- same stage of development as nontransformed fruit.
- exhibit reduced reddening and an increased resistance to over-ripening and shriveling when stored at
- room temperature for prolonged periods.
- do not soften as readily and can be left on the plant longer to ripen more fully.

ETHYLENE IN RIPENING

The role of ethylene in ripening of climacteric fruits has been known for more than 50 years.

Since then, considerable effort has been focused on the studies of:

- **ethylene biosynthesis** (S-adenosylmethionine, SAM; SAM synthetase; 1-aminocyclopropane carboxylic acid; ACC synthase; and ACC oxidase),
- **ethylene perception** (ethylene receptors, ETRs);
- **signal transduction** (ethylene response factor, ERFs);
- and **ethylene-regulated genes** such as cell-wall disassembling genes (endopolygalacturonase; pectin methyl esterase, PME; and pectate lyase).

six ethylene receptors have been isolated in tomato
(*ETHYLENE RECEPTOR1*, *LeETR1; ETHYLENE RECEPTOR2*, *LeETR2; ETHYLENERECEPTOR5*, *LeETR5; NEVER-RIPE, NR; ETHYLENE RECEPTOR4*, *LeETR4;* and *ETHYLENE RECEPTOR6*, *LeETR6*)

compared to

five members in *Arabidopsis*
(*ETHYLENE RECEPTOR1*, *ETR1; ETHYLENE RECEPTOR2*, *ETR2; ETHYLENE R*
ESPONSE SENSOR1, ERS1; ETHYLENE RESPONSE SENSOR2, ERS2; and ETHYLENE INSENSITIVE4, EIN4).

Five of the six tomato receptors have shown to bind ethylene but expression studies have been shown different profiles. Transcript levels of *LeETR1*, *LeETR2*, and *LeETR5* change little upon treatment of ethylene in fruit, where *NR*, *LeETR4*, and *LeETR6* are strongly induced during ripening

The existence of tomato-ripening mutants confirmed the genetic basis of ripening. The *Neverripe (Nr)* and *ripening inhibitor (rin)* fruit shown in Figure 3.1 were photographed 6 months after control fruit ripened and remained in good condition for a year or more, provided they were prevented from becoming dehydrated. After 1 year the *rin* seeds began to germinate but the flesh had still not changed their color, although there had been some softening.

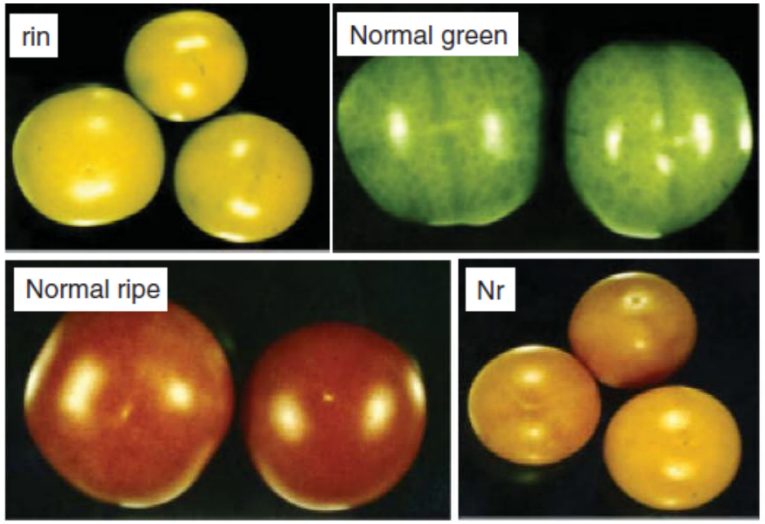


Figure 3.1 Mutants of ethylene action and ripening. Mature green and ripe Ailsa Craig tomato fruits, photographed at approximately 40 and 50 days respectively, together with fruit from near-isogenic lines of the *ripening inhibitor* (*rin*) *and Neverripe* (*Nr*) mutants, photographed at around 6 months old. As discussed in the text, the Nr mutation bred into the Pearson tomato cultivar (not shown) has a more severe phenotype and the fruits remain green.

The Nr mutation was shown to be due to a crucial amino acid change in a tomato ethylene receptor and the two other mutants had drastically reduced ethylene production, although ripening could not be restored by adding ethylene externally.

ETHYLENE PLAYS AN IMPORTANT ROLE IN RIPENING

This indicates that ripening and the eventual deterioration and rotting of normal fruit is a regulated process; it is not unavoidable event, but is genetically determined.

A range of fruit color mutants, in which no other aspect of ripening appeared to be affected, indicated that the ripening pathway had several independent branches (Fig. 3.2).

The production of individual enzymes involved in cell wall metabolism (e.g., polygalacturonase (PG), pectin esterase (PE), pigment synthesis (phytoene synthase (PSY)), and volatile production could each be inhibited independently by gene silencing without affecting other ripening attributes.

Thirty years before ethylene receptors were cloned and sequenced, Burg and Burg (1967) predicted the presence of a metal ion in the receptor based on the metal affinity of compounds that have ethylene-like or ethylene-antagonistic activities. For example, Ag(I), prevents ripening, by interfering with the ethylene-receptor interactions.

Figure 3.3 Ag^+ inhibits ethylene perception or action and consequently prevents ripening. Silver thiosulfate, which is a translocatable form of $Ag+$, was introduced to one side of the mature green fruit pedicel while it was still attached to the plant. A needle was used to insert a thread asymmetrically into the fruit stalk to act as a wick for the uptake of the solution. $Ag+$ was only transported to half of the vascular tissue and delivered to one-half of the fruit, preventing those cells from responding to ethylene and they did not undergo ripening changes. See Davies et al. (1988), (1990).

ETHYLENE AND CLIMACTERIC AND NONCLIMACTERIC FRUITS

All plants produce some ethylene during their life cycle.

Often there is a **low** basal level, in tomato this is around 0.05 $nL.g^{-1}.h^{-1}$, which can **increase** 100-fold or more at particular stages of the life cycle, for example in response to wounding or pathogen attack, ripening, senescence, or abscission.

Increased respiration and a burst of ethylene biosynthesis are characteristics of the ripening process in many fleshy fruits, such as tomato, avocado, apple, melon, and banana, and these are called climacteric fruits

In other fruits, such as strawberry, grape, and citrus, ripening control was **originally!** thought to be independent of ethylene.

It is **becoming** clear, however, that ethylene may be involved in the development and aspects of ripening of some nonclimacteric fruits, such as citrus, white grape, and strawberry, although it seems that only low levels of ethylene are required.

There is recent evidence for small changes in ethylene biosynthesis genes, and ethylene production during ripening of strawberry (Trainotti et al., 2005).

Fei et al. (2004) compared the ripening genes of climacteric tomato fruits and nonclimacteric grapes and showed that the two species shared a subset of common ripening transcription regulators, including members of the MADS-box, zinc finger, and bZIP transcription factor families, suggesting that these genes have been conserved for ripening control during evolution, and are active in both climacteric and nonclimacteric fruits.

Thus, it has gradually become clear that the distinction between climacteric and nonclimacteric fruits, and the view that ethylene is only involved in controlling ripening of climacteric fruits, is an oversimplification. In general, fruit with the highest respiration rate tend to ripen most rapidly. Ethylene hastens the ripening of climacteric fruits and the increase in ethylene production associated with the respiratory rise is autocatalytic. There is clear evidence that, at least in some fruits, ethylene causes the climacteric rise in respiration. Climacteric and nonclimacteric types can be found in the same species, however, suggesting that small genetic differences underlie the climacteric trait, and in other cases different fruit parts, such as skin and pulp, may behave differently. It is likely, therefore, that as our molecular understanding increases, and the ripening behavior of a wider range of fruits is investigated at the genetic level, we may need to modify our classification of fruit types. The view that is emerging is of a basic underlying genetic program that controls climacteric and nonclimacteric ripening, which is modulated by a range of factors, including ethylene, particularly in the climacteric fruit types, but also involving other regulators (Fig. 3.2).

Biochemical evidence suggests that ethylene production may be influenced or regulated by interactions between its biosynthesis and other metabolic pathways. One such example is provided by the fact that SAM is the substrate for both the polyamine pathway and the nucleic acid methylation; the competition for substrate was demonstrated by the finding that the overexpression of a SAM hydrolase has been associated with inhibited ethylene production during ripening (Good et al., 1994). On the other hand, the methionine cycle directly links ethylene biosynthesis to the central pathways of primary metabolism.

Many fruits can ripen on the plant or tree, but some, such as avocado, only ripen after they are picked. A "**tree factor" that inhibits ripening** has been postulated to explain this effect.

Also, in strawberry it was recognized over 50 years ago that auxin, coming probably from the developing achenes, actually delayed the onset of ripening.

Harvesting can also hasten the ripening of some fruits and in such cases wound- or dehydration stress-ethylene from the calyx or calyx scar may also stimulate ripening.

It would be wrong to assume, however, that ethylene is the only hormone that affects ripening.

Auxin inhibits the ripening of strawberry, which is nonclimacteric.

Auxin is also involved in the regulation of some ethylene response factors (ERFs, transcription factors), which are important in climacteric fruit ripening, and there is some evidence of interactions between auxin and ethylene signaling during ripening.