

One Rotten Apple Spoils the Whole Bushel: The Role of Ethylene in Fruit Ripening

Minireview

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Historical Perspective

Folklore says that sealing fruits in a bag encourages them to ripen. It is correct—the bag traps ethylene released by the fruit, and ethylene enhances ripening. The earliest record of human manipulation causing fruit to ripen is in the Old Testament: the prophet Amos described himself as a “piercer” of sycamore fig fruit. The Greek philosopher Theophrastus later recognized that sycamore figs do not ripen unless they are scraped with an iron claw. Twenty-three centuries later, we know that wounding induces ethylene production, resulting in fruit ripening (Abeles, 1973). The spectacular and commercially important effects of ethylene on plant growth can be found in the scientific literature as early as the 19th century, when the toxic effect of “illuminating” gas on plants was described (Burg, 1962): trees growing near broken gas pipes were severely injured; etiolated (dark-grown) pea seedlings grown in laboratory air contaminated with illuminating gas showed a radial swelling of the stem, inhibition of stem elongation, and absence of normal geotropic response (the “triple response”). In 1924, Denny identified ethylene as the component in combustion fumes from kerosene stoves that caused lemon degreening in California and described it as a ripening agent.

Ethylene is one of the simplest organic molecules with biological activity. This hydrocarbon gas, known as the fruit-ripening hormone, profoundly influences the growth and development of plants. Its effects include inhibition of growth, loss of geotropic sensitivity, onset of epinastic curvatures, acceleration of respiration, initiation of rooting, and modification of leaf and fruit pigments (Burg, 1962). Because large losses of fruits and vegetables are incurred annually (billions of dollars worldwide) due to ethylene's effects on plant senescence, the significance of a means to control the ripening process and prevent spoilage is clear. This review summarizes recent advances in cloning key genes in the ethylene biosynthetic pathway and manipulating them to prevent ethylene production and fruit ripening.

Biosynthesis of Ethylene

Ethylene is produced by plant tissues in amounts ranging from almost none up to 500 nI/g per hr (Burg, 1962). It is biologically active in trace amounts (as little as 10–100 nI/l of air). Ethylene production is induced during several developmental stages, including fruit ripening, seed germination, leaf and flower senescence, and abscission. It is also induced by external factors, such as wounding, anaerobiosis, viral infection, auxin treatment, chilling injury, drought, and Cd²⁺ and Li⁺ ions (Abeles, 1973; Yang and Hoffman, 1984).

Elucidation of the pathway for ethylene synthesis by Yang and associates (Yang and Hoffman, 1984) over the

course of 20 years is a major contribution to plant biochemistry. The simplicity of its chemical structure raised the possibility that many compounds could be potential precursors of ethylene, but it is clear that only carbons 3 and 4 of methionine give rise to ethylene in higher plants (Figure 1). The rate-limiting step in the pathway is catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (reaction 2 in Figure 1). The final step (conversion of ACC to ethylene, HCN, and CO₂) is catalyzed by ACC oxidase (reaction 3 in Figure 1). The pathway is designed to allow high rates of ethylene production without high intracellular concentrations of methionine (a less abundant amino acid). This is achieved by recycling 5'-methylthioadenosine (MTA) to methionine (Figure 1). The overall result is that the ribose moiety of ATP gives rise to the 4-carbon skeleton of methionine from which ethylene is derived. The methylthiol group, however, is conserved for continued regeneration of methionine. Thus, with a constant pool of the methylthiol group and available ATP, high rates of ethylene production can be achieved (Yang and Hoffman, 1984).

None of the ethylene biosynthetic enzymes have been purified to homogeneity because of their low abundance (Kende, 1989). It was molecular cloning and heterologous expression that allowed the isolation of the genes encoding AdoMet synthase (Peleman et al., 1989), ACC synthase (Sato and Theologis, 1989), and ACC oxidase (Slater et al., 1985; Hamilton et al., 1991; Spanu et al., 1991).

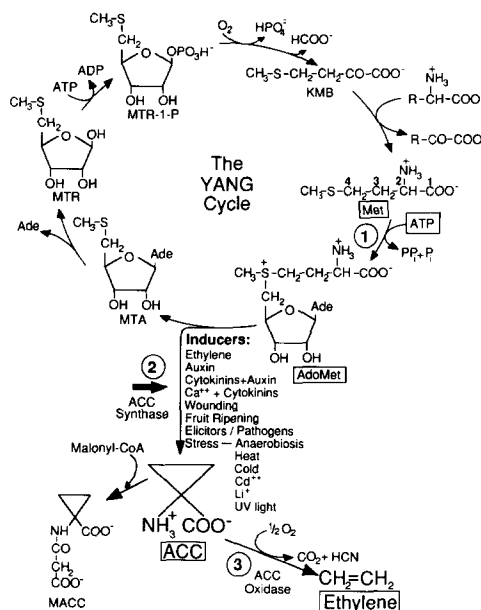


Figure 1. The Ethylene Biosynthetic Pathway of Higher Plants
AdoMet, S-adenosyl-L-methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; KMB, 2-keto-4-methylthiobutyrate; MACC, malonyl-ACC; MTA, 5'-methylthioadenosine; MTR, 5'-methylthioribose; MTR-1-P, MTR-1-phosphate (after Yang and Hoffman, 1984).

ACC Synthase

After the discovery that ACC is the immediate precursor of ethylene, it became obvious that the enzyme whose activity limits ethylene biosynthesis is ACC synthase. The induction of ethylene production by a variety of means is due to de novo synthesis of this enzyme (Kende, 1989). A fundamental question then arises: are there as many genes as inducers, or is there only one gene whose promoter is somehow activated by all inducers? A cDNA encoding ACC synthase has been cloned from zucchini using immunochemical approaches, and its authenticity has been confirmed by expression in *E. coli* and yeast (Sato and Theologis, 1989). Immediately thereafter, ACC synthase was cloned from a variety of plant species (Van der Straeten et al., 1990; Nakajima et al., 1990; Figure 2).

There is an emerging picture that ACC synthase is encoded by a highly divergent multigene family. In tomato, for example, ACC synthase is encoded by at least six genes, two of which are expressed during fruit ripening (Van der Straeten et al., 1990; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992). Similarly, in *Arabidopsis* and rice, ACC synthase genes are differentially expressed in response to developmental, hormonal, and environmental stimuli (Liang et al., 1992; Zarembinski and Theologis, 1992). Comparison of the structure and expression of 20 ACC synthase genes from various plant species (Figure 2) suggests that the extensive polymorphism and the distinct regulatory networks governing the expression of ACC synthase subfamilies arose early in plant evolution, prior to the divergence of monocots and dicots (Liang et al., 1992).

ACC synthase requires pyridoxal phosphate, and most such enzymes have a lysine residue in their active site. Lys-278 of a tomato isoenzyme, which is conserved in all ACC synthases so far, has been shown to be the site of pyridoxal phosphate attachment (Yip et al., 1990). Interestingly, the pyridoxal phosphate-binding site of several aminotransferases contains some of the residues surrounding Lys-278 (Figure 2). Furthermore, among various aminotransferases, only 12 amino acids are completely con-

served, and all but one of these residues are present in all ACC synthases identified (Figure 2). This suggests that aminotransferases and ACC synthase may be evolutionarily related (Rottmann et al., 1991).

ACC Oxidase

This enzyme is constitutively expressed in most vegetative tissues (Yang and Hoffman, 1984) and is induced during fruit ripening (Gray et al., 1992). A cDNA encoding ACC oxidase was first cloned from tomato fruit (Slater et al., 1985), and its authenticity was confirmed by antisense experiments in transgenic plants (Hamilton et al., 1990) and by expression experiments in yeast (Hamilton et al., 1991) and *Xenopus oocytes* (Spanu et al., 1991). ACC oxidase appears to be a dioxygenase that belongs to the superfamily of Fe²⁺/ascorbate oxidases (McGarvey et al., 1992).

Fruit Ripening and Its Inhibition by Antisense RNA

Ethylene is thought to regulate fruit ripening by coordinating the expression of genes responsible for enhancing a rise in the rate of respiration, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars, and increased activity of cell wall-degrading enzymes (Gray et al., 1992). It has always been a goal to prevent or delay fruit ripening in a reversible manner, and various methods have been employed, such as ventilation with air under hypobaric pressures or use of inhibitors of ethylene action, such as silver ions and carbon dioxide (Yang and Hoffman, 1984). However, these approaches are expensive and fail to prevent fruit senescence satisfactorily. (In a few instances, however, such as apple, controlled atmosphere storage has been successful.) A more desirable solution would be the construction of a mutant plant whose fruits do not ripen until treated with ethylene. Naturally occurring ripening mutants of tomato exist, but their phenotype is not reversible by ethylene.

The cloning of genes induced during fruit ripening and of genes involved in ethylene biosynthesis allowed the

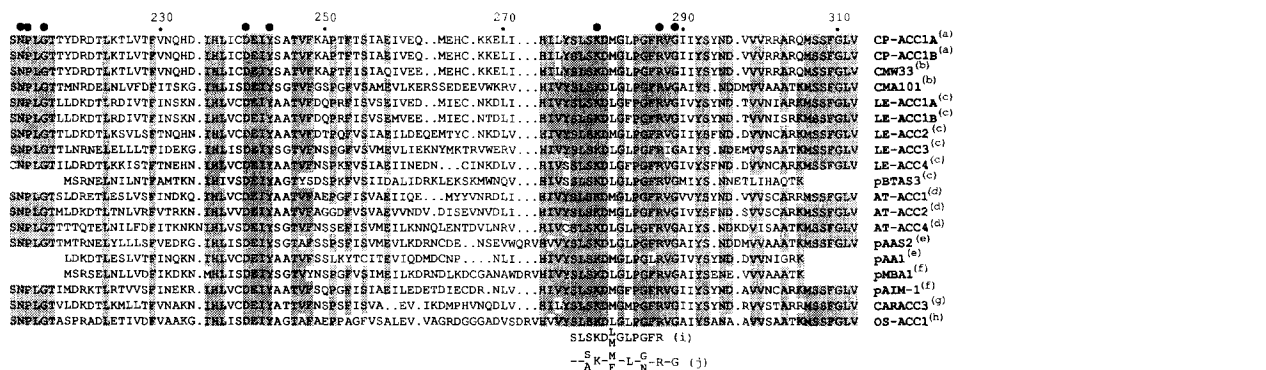


Figure 2. Comparison of a Highly Conserved Region of ACC Synthase among Various Plants

(a) Zucchini (Huang et al., 1991); (b) winter squash (Nakajima et al., 1990; Nakagawa et al., 1991); (c) tomato (Van der Straeten et al., 1990; Rottmann et al., 1991; Olson et al., 1991; Yip et al., 1992); (d) *Arabidopsis* (Liang et al., 1992); (e) apple (Kim et al., 1992, and references therein); (f) mung bean (Kim et al., 1992; Botella et al., 1992); (g) carnation (Park et al., 1992); (h) rice (Zarembinski and Theologis, 1992); (i) AdoMet and pyridoxal phosphate-binding site (Yip et al., 1990); (j) consensus of the pyridoxal phosphate-binding site in aminotransferases (Rottmann et al., 1991). Sequences derived from polymerase chain reaction products are missing the conserved regions at the N- and C-termini.

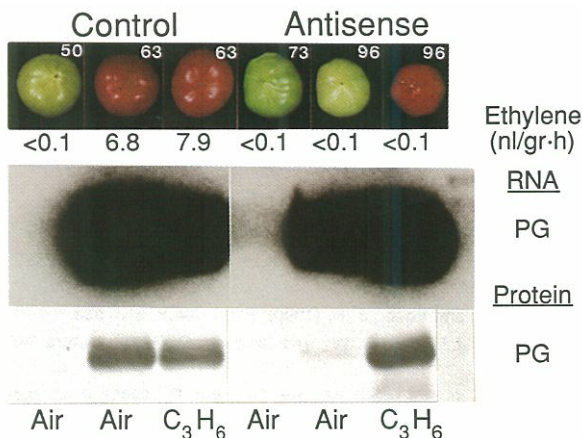


Figure 3. Inhibition of Tomato Fruit Ripening and Ethylene Evolution by Antisense ACC Synthase RNA
PG mRNA and polypeptide expression in control and antisense fruits is also shown (Oeller et al., 1991). Numbers in white indicate the age of the fruits in days.

construction of ripening mutants in tomato using reverse genetics. In the absence of gene replacement technology in plants, antisense RNA technology became the tool of choice. Initial attempts to inhibit tomato fruit softening by antisense polygalacturonase (PG) RNA, a gene thought to be responsible for cell wall hydrolysis during ripening, failed to give a strong effect (Smith et al., 1988; Sheehy et al., 1988). PG antisense RNA dramatically inhibited PG mRNA accumulation and enzyme activity, suggesting that PG is not the sole determinant of cell wall hydrolysis.

Another approach to prevent fruit ripening is to inhibit ethylene production. Attempts to either metabolize ACC by overexpressing the *Pseudomonas* ACC deaminase gene (Klee et al., 1991) or inhibit ACC oxidase activity with antisense RNA (Hamilton et al., 1990) were inconclusive as to whether ethylene was the key regulator of fruit ripening. Ethylene production was not sufficiently decreased to allow effective inhibition of the ripening process. The approach that was most successful in preventing fruit ripening was to inhibit ACC synthase with antisense RNA (Oeller et al., 1991), which led to the severe inhibition of ethylene production (<math><0.1</math> nl/g per hr) that is required of a ripening mutant. The striking phenotype of this mutant can probably be attributed to the short half-life of ACC synthase.

During tomato fruit ripening, two ACC synthase genes are expressed, LE-ACC2 and LE-ACC4 (Olson et al., 1991; Rottmann et al., 1991). Expression of antisense RNA derived from LE-ACC2 resulted in an almost complete inhibition of mRNA accumulation of both these genes (Oeller et al., 1991; Figure 3). Antisense fruits never ripen. Control fruits kept in air begin to produce ethylene 50 days after pollination and fully ripen 10 days later. The red color resulting from chlorophyll degradation and lycopene biosynthesis is inhibited in antisense fruits (Figure 3). Antisense fruits kept in air or on plants for 90–150 days eventually develop an orange color but never turn red and soft or develop an aroma.

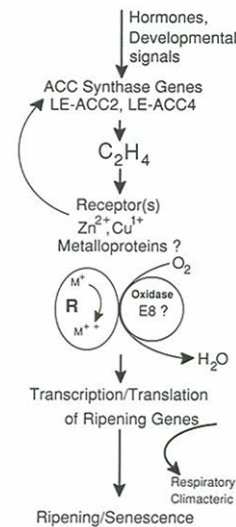


Figure 4. Ethylene-Mediated Signal Transduction Pathway Responsible for Fruit Ripening

The antisense phenotype can be reversed by treatment with ethylene or propylene, an ethylene analog (Oeller et al., 1991; Figure 3). Treated fruits are indistinguishable from naturally ripened fruits with respect to texture, color, aroma, and compressibility. Six days of ethylene treatment is required to reverse the antisense phenotype. Antisense fruits treated for 1–2 days with ethylene do not develop a fully ripe phenotype compared with control fruits. In addition, removal of ethylene from antisense fruits after they have fully ripened prevents overripening. Three important conclusions can be inferred from these results. First, ethylene-mediated ripening requires continuous transcription of the necessary genes, which may reflect a short half-life of the induced gene products. Second, ethylene is indeed autocatalytically regulated. Finally, the hormone acts as a rheostat rather than as a switch for controlling the ripening process. The accumulated experimental evidence from the antisense experiments indisputably demonstrates that the Yang cycle is solely responsible for ethylene synthesis during ripening and that ethylene is the key regulatory molecule for fruit ripening and senescence, not the by-product of ripening (Figure 4).

Antisense fruits producing low levels of ethylene have been useful in assessing which ripening-induced genes are indeed ethylene inducible. The expression of PG and ACC oxidase genes, which were thought to be ethylene regulated, was found to be ethylene independent (Oeller et al., 1991). While antisense fruits express large amounts of PG mRNA, they fail to accumulate the PG polypeptide, suggesting that ethylene controls the translation of PG mRNA or the stability of the PG polypeptide (Figure 3).

Antisense fruits also revealed that at least two signal transduction pathways are operating during tomato fruit ripening. The ethylene-independent (developmental) pathway is responsible for the transcriptional activation of genes such as PG, ACC oxidase, and chlorophyllase. The ethylene-dependent pathway, on the other hand, is responsible for the transcriptional and posttranscriptional

regulation of genes involved in lycopene and aroma biosynthesis, respiratory metabolism, ACC synthase gene expression, and translation of genes such as PG (Figure 3). Ethylene appears to have a dual role in senescence. First, it activates transcription of genes (yet to be identified) whose products are unstable but required for fruit senescence. Second, it regulates the translation of developmentally regulated mRNAs such as PG (Figure 3).

The mode of ethylene action at the molecular level remains a mystery. It has been suggested that, since ethylene is an olefin, its receptor may be either a Zn²⁺- or Cu⁺-containing metalloprotein (Burg and Burg, 1967; Figure 4). This proposition is still attractive, in view of the fact that some mammalian hormone receptors are known to be both transcriptional activators and Zn²⁺ metalloproteins. Since ethylene action requires oxygen, it has been postulated that the metalloprotein receptor is oxidized by an oxidase and, while in the oxidized form, is activated by ethylene to produce fundamental changes in the metabolism of plant tissue (Burg and Burg, 1967).

Based on the observations of Peñarrubia et al. (1992), I would like to propose that the putative oxidase of the ethylene metalloprotein receptor may be the E8 protein, a dioxygenase related to ACC oxidase. Inactivation of E8 gene expression by antisense RNA results in a 10-fold ethylene overproduction; inhibition of ethylene action with silver ions also results in ethylene overproduction in tomato fruits (Peñarrubia et al., 1992). Furthermore, ethylene-overproducing Arabidopsis mutants produce more ethylene after silver ion treatment (Guzman and Ecker, 1990). All this suggests that interference with the ethylene receptor system that results in lower levels of reception is interpreted by the cell as an absence of the hormone, leading to ethylene overproduction. According to this view, some of the ethylene-overproducing Arabidopsis mutants may be E8 mutations (Guzman and Ecker, 1990). The biochemical sensor that detects the level of functional receptor and that enhances or depresses ACC synthase activity is unknown.

The Future

The use of antisense is only the first step toward controlling fruit senescence. The development of gene transplacement by homologous recombination should allow the creation of nonleaky ripening mutants. Understanding the tissue- and cell-specific expression of the ACC synthase and ACC oxidase multigene families during plant development will offer new knowledge of the role of ethylene as a signaling molecule. The challenge in the 90s will be the isolation of the ethylene receptor and the elucidation of the components of the ethylene-regulated signal transduction pathways. Ethylene mutants in Arabidopsis may offer answers to these fundamental questions (Guzman and Ecker, 1990, and references therein).

Finally, the importance of gases as signaling molecules is just beginning to be appreciated in a variety of systems other than plants. The identification of nitric oxide and carbon monoxide as neurotransmitters (e.g., Bredt and Snyder, 1992) has created great excitement in the field of brain neurochemistry. The possibility exists that the biochemical mechanisms of sensing gases are conserved between plants and animals.

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