Molecular Biology of Ethylene Biosynthesis and Its Application in Horticulture

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Summary

Ethylene is produced by all higher plants and in trace amounts regulates many aspects of growth and development, ranging from seed germination to flower fading, fruit ripening and leaf senescence; its effects are often spectacular and are commercially important in agriculture. Ethylene is biosynthesized via the following pathway: methionine $\rightarrow$ S-adenosylmethionine (AdoMet) $\rightarrow$ 1-aminocyclopropane-1-carboxylic acid (ACC) $\rightarrow$ C₂H₄. The committed steps in ethylene biosynthesis are the conversion of AdoMet to ACC catalyzed by ACC synthase, and the conversion of ACC to ethylene by ACC oxidase. ACC synthase is a pyridoxal phosphate (PLP) enzyme, which mediates a unique α , γ -elimination of the substrate AdoMet to form ACC. Biochemical and site-directed mutagenesis studies confirm that Lys-272 in apple ACC synthase is the active centre that binds the PLP coenzyme and functions as the base in the enzymic reaction. ACC oxidase, which catalyzes the aerobic oxidation of ACC to ethylene, CO₂ and HCN, requires Fe²⁺, and CO₂ as essential cofactors, and ascorbate as its co-substrate. The cloning of the ACC synthase and ACC oxidase genes has permitted scientists to use antisense RNA technology to genetically engineer perishable crops, such as tomato and melon fruits and carnation flowers, with suppressed ethylene production rate and thereby to extend the shelf-life and to reduce spoilage. Recent molecular genetic approach using *Arabidopsis thaliana* has resulted in the isolation of genes responsible for ethylene insensitivity. Transgenic biotechnology has demonstrated the feasibility to confer ethylene insensitivity in horticultural crops resulting in delay of the senescence process.

Key Words: ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid, senescence, ethylene insensitivity, transgenic biotechnology

Introduction

Ethylene is a gaseous plant hormone involved in the regulation of many physiological responses (Abeles et al., 1994). In addition to its recognition as a “ripening hormone”, ethylene is involved in other developmental processes from germination of seeds to senescence of various organs and in many responses to environmental stresses. The rate of ethylene production by plant tissue is normally low. An increase in ethylene production is induced during certain stages of growth as part of the normal life of a plant. Ethylene production can also be induced by auxin application or environmental stresses, such as physical wounding and cutting, chilling, drought, and water flooding. It has been recognized that this increased ethylene production, in turn, brings about many important physiological responses.

Although ethylene was recognized as a plant-produced hormone over 50 years ago, the biosynthetic pathway of ethylene in plants remained elusive until the

key intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) was shown to be the immediate precursor of ethylene (Adams and Yang, 1979). It has been established that ethylene is synthesized in plant tissues via the following pathway: methionine $\rightarrow$ S-adenosylmethionine (AdoMet) $\rightarrow$ ACC $\rightarrow$ C₂H₄ (Adams and Yang, 1979). In plant tissues ACC synthase (EC 4.4.1.14), which catalyzes the conversion of AdoMet to ACC and 5'-methylthioadenosine (MTA), is generally the rate-limiting enzyme in the ethylene biosynthetic pathway (Yang and Hoffman, 1984). The expression of ACC synthase gene is controlled by a variety of environmental and developmental factors, and has received a great deal of attention in relation to the regulation of ethylene biosynthesis. The enzyme has been characterized at both biochemical and molecular levels.

Unlike the biosynthetic pathway of ethylene which is now clear, the mechanism of the hormone action is just about to be unveiled owing to the recent isolation and epinastic studies of the ethylene response mutants from *Arabidopsis thaliana* (Ecker, 1995). Several mutated genes corresponding to these mutant phenotypes have been isolated and shown that a putative protein kinase cascade is responsible for the transduction of ethylene signal. Among those, the ethylene receptor gene, *ETR1*,

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is the best studied one both biochemically and genetically.

Purification of ACC Synthase and Cloning of Its Gene

To understand the genetic regulation of ACC synthase in ethylene biosynthesis, it is necessary to clone the gene encoding the protein. For years, progress in the purification of ACC synthase was slow because ACC synthase exists in very low abundance and is unstable. Partial purification of the enzyme was carried out in ripe tomato fruit after wounding. Toward this end various laboratories have undertaken different approaches.

In apple fruit, we first raised monoclonal antibodies (mAbs) against a partially purified apple enzyme preparation. Specific mAbs against ACC synthase were then screened based on their ability to precipitate the enzyme activity (Dong et al., 1991a), and these were in turn used to immuno-purify apple ACC synthase protein (Yip et al., 1991). A mAb was immobilized to protein A-agarose gel, which allowed the isolation of ACC synthase from a relatively crude preparation as a single band on SDS-PAGE (Yip et al., 1991). A partially purified apple enzyme ACC synthase preparation was radiolabeled with Ado[¹⁴C] or NaB₃H₄ and purified by immunoaffinity chromatography. Following trypsin digestion, the radioactive active-site peptide was isolated and sequenced (Yip et al., 1990). In addition, we also isolated and sequenced ten tryptic peptides derived from the purified apple ACC synthase protein (Dong et al., 1991b). From these sequences, three partial sequences including that of the active-site peptide were chosen to construct corresponding degenerate oligonucleotides which were employed as probes for screening an apple fruit cDNA library for ACC synthase cDNA clone.

Multigene Family of ACC Synthase and Differential Expression

To date, most of our molecular genetic knowledge on ACC synthase in fruit ripening is from tomato. In this fruit, the marked increase of ACC and ethylene production can now be attributed to the expression of ACC synthase genes *LE-ACS2* and *LE-ACS4*. Their cDNAs initially described by Van der Straeten et al. (1990) are greatly induced immediately prior to and during climacteric fruit ripening (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992). Both genes are inducible in mature-green fruit by treatment with 0.1 ppm of ethylene for 2 days. Both contain elements in their promoters that resemble an element from the ethylene induced gene E4 of tomato (Cordes et al., 1989). The *LE-ACS2* transcript is approximately tenfold more abundant than *LE-ACS4* during climacteric ripening (Olson et al., 1991; Lincoln et al., 1993). The expression of these transcripts causes the marked rise of ethylene during the

climacterium and the ripening of the tomato fruit. This is proven by *LE-ACS2* antisense tomato fruits which do not have detectable levels of these mRNAs and fail to ripen (Oeller et al., 1991).

Noteworthy, the expression of *LE-ACS2* in tomato is not limited to fruit tissue; it is observed in senescent flowers, in suspension cultures, pathogen infested leaves and flooded roots (Rottmann et al., 1991; Yip et al., 1992; Spanu et al., 1993; Olson et al., 1995; Oetiker et al., 1997).

ACC synthase gene expression patterns is complex. Two examples may illustrate this point. First example is the ACC synthase genes expressed in elicited suspension cultures. We treated tomato suspension cultures with a yeast elicitor and analyzed the expression pattern of seven ACC synthase genes (Oetiker et al., 1997). While *LE-ACS1A* was not detectable at any time, *LE-ACS1B* was strongly expressed. *LE-ACS1B* was enhanced by addition of the elicitor and remained high throughout the experiment. The transcript of *LE-ACS2* was near to the detection limit without the addition of the elicitor. However, it was strongly inducible and reached a maximum after 2 h. *LE-ACS3* was constitutively expressed at a low level. *LE-ACS4* was at the limit of detection at time 0, slightly induced by the elicitor after 20 minutes and leveled off after 1 h. *LE-ACS5* was present in the absence of elicitor at an amount similar to *LE-ACS2* and *LE-ACS5* was strongly inducible and continued to increase over 2 h. Only little *LE-ACS6* transcript was detectable at time 0, but it was induced by the elicitor, peaked at 1 h and then strongly decreased. Quantification of the radioactive RNA bands revealed two groups of expressed ACC synthases. The first group contains the genes *LE-ACS2*, *LE-ACS5* and *LE-ACS6* whose transcripts were stimulated and *LE-ACS6* whose transcripts were stimulated tenfold or more by the elicitor. Their change of abundance paralleled the increase of the rate of ethylene production. The second group contains *LE-ACS1B*, *LE-ACS3* and *LE-ACS4*, whose transcripts were stimulated less than threefold after addition of the elicitor, i.e. they were only weakly induced by the elicitor and nearly constitutive. Their abundance was not correlated with the increase of ethylene synthesis. Clearly, directed genetic manipulation of ethylene synthesis in this system would require better knowledge of how much each of the ACC synthase isoforms is contributing to the total of ACC synthesis.

A second example is the flooding of plant roots. The identification of the specific ACC synthase genes induced during flooding is important to ameliorate the deleterious effects of ethylene during flooding. For instance, flooding induced ethylene causes losses due to ethylene-mediated premature abscission of cotton bowls (Guinn, 1982). The ACC synthases *LE-ACS2* and *LE-ACS3* are induced by flooding (Olson et al., 1995). *LE-ACS3* was detected after 1 h of flooding, while *LE-*

Fig. 1. Phylogenetic relationship of the tomato ACC synthase isoforms. The potato ST-ACS2 gene is also included in this tree to demonstrate the high degree of similarity to LE-ACS7. The program Clustal and DNA sequences encoding the polypeptide between block 4 and 6 (Dong et al., 1991) were used to construct the tree.

ACS2 was only induced after 10 h, suggesting its induction is not a primary event after flooding. More recently, O.Y. Shiu et al. (unpublished) identified a novel ACC synthase gene, *LE-ACS7*, which is transiently induced and early after flooding. This gene is highly similar to *LE-ACS3* (Fig. 1). Thus, both genes, *LE-ACS3* and *LE-ACS7* are involved in the flooding response of the tomato.

A common feature of multigene families is that their members are expressed preferentially in response to an internal or environmental signal. Differential expression of various ACC synthase transcripts during ripening and in response to, IAA application (Yip et al., 1992).

Characterization of ACC Oxidase and Its Gene

The conversion of ACC to ethylene is carried out by an oxidative enzyme that is now known as ACC oxidase. The enzyme was formerly named as ethylene-forming enzyme (EFE) by Yang and his colleagues because the reaction mechanism was not known at that time. The ACC oxidase activity was first described by Adams and Yang, who trapped ACC in apple tissue incubated in a nitrogen atmosphere and demonstrated its conversion to ethylene under aerobic conditions. For more than a decade, authentic ACC oxidase activity could not be demonstrated in vitro.

Unlike ACC synthase, ACC oxidase was first identified by its cDNA. While studying gene expression during ripening, Davies and Grierson (1989) isolated a cDNA, pTOM13, by differential cloning techniques. Based on the observation that ACC oxidase activity in tomato plants was greatly reduced with pTOM13 anti-sense gene, Hamilton et al. (1990) suggested that the pTOM13 gene product was related to ACC oxidase.

Later work confirmed that pTOM13 confers ACC oxidase activity when expressed in yeast (Hamilton et al., 1991). The deduced amino acid sequence of pTOM13 shows homology to those of flavanone-3-hydroxylase and other known hydroxylases (Hamilton et al., 1990). Although Yang suggested as early as 1981 that ACC oxidase might be an ACC hydroxylase (Yang and Hoffman, 1984), attempts to assay ACC oxidase as a hydroxylase were not pursued. Ververidis and John (1991) first demonstrated authentic ACC oxidase activity in vitro by extracting melon fruit and assaying the enzyme under the conditions for flavanone-3-hydroxylase. Their enzyme preparation requires Fe^{2+} and ascorbate for full activity, and meets all the criteria for ACC oxidase in vivo. Others have since detected ACC oxidase activity in homogenates of apple (Fernandez-Maculet and Yang, 1992; Fernandez-Maculet et al., 1993) and avocado fruits (McGarvey and Christoffersen, 1992). All these enzyme preparations are soluble and require Fe^{2+} and ascorbate for activity. The failure of previous attempts to demonstrate ACC oxidase activity in vitro is now attributed to the loss of these factors during the enzyme extraction.

Recently, ACC oxidase has been purified to near homogeneity from apple fruits (Dong et al., 1992; Dupille et al., 1993). The enzyme is a monomer with a molecular mass of 35 kDa. Using the purified ACC oxidase, Dong et al. (1992) determined the stoichiometry of the reaction catalyzed by the enzyme as follows (where AH_2 and A stand for ascorbate and dehydroascorbate, respectively):

Ascorbate serves as a co-substrate, and is concurrently oxidized to dehydroascorbate in an amount equivalent to that of ethylene produced. The stoichiometry agrees with the results obtained for ACC oxidase in vivo by Peiser et al. (1984). The exact mechanism of the enzymic reaction and the activation by CO_2 are still unknown (Charnig et al. 1997a).

Regulation of Ethylene Biosynthesis in Ripening Fruits

In climacteric fruits, one of the earliest events of ripening is the onset of ethylene production. Ethylene production in pre-climacteric fruits is very low, but increases dramatically at the onset of ripening. Application of ethylene to pre-climacteric fruits such as tomato, banana, apple, and avocado hastens the ripening process. It is now recognized that ethylene plays an essential role in the ripening of climacteric fruits (Yang and Hoffman, 1984). Since the application of ACC to the pre-climacteric fruits results in only a slight increase in ethylene, the fruit at the pre-climacteric stage must lack the capabilities not only of synthesizing ACC

but also of oxidizing ACC to ethylene.

When ethylene is applied to mature, pre-climacteric fruits, the ripening process is triggered and accompanied by a massive synthesis of ethylene. The capability of tissues to synthesize large quantities of ethylene in response to ethylene is referred to as autocatalytic ethylene production. This ethylene effect was found to be due to the enhancement of both ACC synthase and ACC oxidase activities (Riov and Yang, 1982; Liu et al., 1985). Recent results establish that this increased synthesis of ethylene results from the expression of messenger RNAs for both ACC synthase and ACC oxidase (Oeller et al., 1991; Dong et al., 1992). When pre-climacteric fruit was treated with exogenous ethylene for a short period, ethylene production is not induced because there is no increased synthesis of ACC; this treatment, however, causes a marked increase in ACC oxidase transcript (Dong et al., 1992).

It is to be noted that ethylene production during fruit ripening and the autocatalysis of ethylene production are common features not only of ripening fruits, but also of other senescing organs and tissues.

Genetic Engineering of Ethylene Biosynthesis

The cloning of the genes involved in ethylene biosynthesis during fruit ripening has permitted scientists to use biotechnology to genetically engineer crop plants with reduced endogenous ethylene production and fruits with prolonged storage life. It is possible to reduce ethylene production by lowering the expression of either ACC synthase or ACC oxidase genes. Application of antisense RNA technology is such an example. In antisense RNA technique, a target gene (or cDNA) is inserted in the reverse orientation (antisense) to a plant through *A. tumefaciens*-mediated transformation. When both antisense and sense (parent) gene are expressed in the tissue, they tend to hybridize through sequence complementarily, and the subsequent translational process is prevented (Mol et al., 1990). Such an approach has previously been undertaken with success to inhibit the expression of polygalacturonase gene in tomato fruit (Smith et al., 1988; Sheehy et al., 1988).

So far, efforts to alter ethylene biosynthesis in plant tissue have been focused on ripening fruits. Hamilton et al. (1990) were the first to reduce ethylene biosynthesis in tomato plants by transformation with an antisense pTOM13 construct. Since ethylene production in the antisense-bearing plants was greatly reduced, they suggested that pTOM13 may be related to ACC oxidase. Ripening of the transgenic fruits was retarded and their storage life extended. A similar approach was undertaken by Oeller et al. (1991) who transformed tomato plants with the antisense ACC synthase genes. Fruits from transgenic plants bearing expressed antisense RNA produced little ethylene, and did not undergo ripening unless exogenous ethylene was provided. These results demonstrate that antisense technology can be success-

fully used to reduce ethylene production and thereby to extend the shelf-life of fruits and vegetables. Alternatively, ethylene biosynthesis can be reduced by lowering the cellular level of ACC by introducing a bacterial gene encoding ACC deaminase (Sheehy et al., 1991) into plants. Klee et al. (1991) cloned an ACC deaminase gene from *Pseudomonas* sp. and expressed it in tomato plants. Ethylene biosynthesis in the transformed plants was greatly reduced and ripening of fruits was similarly delayed. More recently, the ACC oxidase antisense gene has been used to delay the ripening of cantaloupe melon (Ayub et al., 1996). Antisense gene from heterologous source was also proven to be useful. Transgenic apple ACC oxidase antisense gene was shown to be able to reduce the ethylene biosynthesis in tomato and hence delay its ripening (Bolitho et al., 1997).

Molecular Cloning of Ethylene Receptors and Genetic Engineering of Ethylene Action

Although Burg and Burg (1967) have postulated 30 years ago that a metal bound-ethylene receptor might be responsible to initiate the ethylene action by interacting with the hormone, direct isolation of the receptor molecule was unsuccessful. Recently, the results from molecular genetic approach using *Arabidopsis thaliana* were shown to be successful. A number of mutants showing affected ethylene response have been generated by screening randomly mutagenized plants. Some of these mutants, such as *etr1*, *ein2*, and *ein3*, etc., are insensitive to ethylene, while some are constitutively expressing the hormone effect, such as *ctr1* (Ecker, 1995). Following the studies of the epinastic relationships between these genes, an ethylene signal transduction pathway was mapped out (Ecker, 1995), indicating that *ETR1* gene is involved in the initiation of ethylene response. The *ETR1* gene was isolated by chromosome walking and was shown to encode a protein resembling the two component signal transducers in prokaryote (Chang et al., 1993). The protein has three membrane spanning domains at the N-terminal portion, in which substitutions of amino acid residues results in dominant phenotype of ethylene insensitivity (Chang et al., 1993). At the C-terminal half locate the putative histidine kinase and receiver domains. In vitro ethylene binding studies have shown that mutation or truncation at the membrane spanning domains leads to the decrease or abolishment in ethylene-binding ability (Schaller and Bleeker, 1995). The biochemical properties of *ETR1* suggest that *ETR1* is at least one, if not the sole, component of the authentic ethylene receptor.

A number of *ETR1* homologous genes have been isolated from *Arabidopsis* and tomato by using the *ETR1* gene as a heterologous probe. In *Arabidopsis*, ERS shares high degree of identity with the amino-terminal domain and putative histidine protein kinase domain of *ETR1*, but lacks the receiver domain (Hua et al., 1995).

The long-existed Nr (never-ripe) phenotype in tomato was shown to be controlled by a gene encoding an ERS-like protein (Wilkinson et al., 1995), while tomato eTAE1 gene product is more similar to *ETR1* (Zhou et al., 1996). Recently, the *ETR1* family is growing quite rapidly by the joining members from other origins such as *OS-ERS* from rice (Yau and Yip, 1997), *DC-ERS* (Charng et al., 1997b) and *DC-ERS2* (Shibuya et al., 1998) from carnation, and *NT-ETR1* from tobacco (Knoester et al., 1997). While more basic studies on the ethylene signal transduction pathway are currently underway, the transgenic biotechnology employing a mutated receptor gene, *etr1-1* of *Arabidopsis*, has demonstrated the feasibility to confer ethylene insensitivity in tomato fruits and petunia flowers, resulting in delays of fruit ripening and flower senescence (Wilkinson et al., 1997). It is expectable that similar application on other important horticultural crops will soon emerge.

Research on ethylene biosynthesis and action is an excellent example illustrating that fundamental research has not only helped in our understanding of how ethylene regulates plant processes, but also opened the prospect for improving through biotechnology the quality of crop products by extending shelf-life and reducing spoilage.

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