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Physiological, Biochemical, and Molecular Aspects of Ethylene Biosynthesis and Action

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I. INTRODUCTION

Ethylene (C_2H_4) is a simple gaseous plant hormone. It is produced by higher plants, bacteria, and fungi and influences many aspects of plant growth and development (Abeles et al., 1992; Mattoo and Suttle, 1991). This hydrocarbon gas, well known as the ripening hormone, is biologically active in trace amounts (as little as 10 nL L^{-1} air). It promotes leaf and flower senescence and abscission, acceleration of respiration and modification of leaf and fruit pigments, onset of epinastic curvatures, and root initiation, and it causes loss of geotropic sensitivity (Abeles et al., 1992; Mattoo and Suttle, 1991). It also controls plumular expansion and maintains the plumular hook, which facilitates the emergence of germinating seedlings through the soil, a process vital to successful seed germination. In pea (*Pisum sativum* L.), exogenous ethylene exaggerates the curvature of the apical hook,

inhibits stem elongation, and prevents a normal geotropic response, effects known as the "triple response" (Abeles et al., 1992). Ethylene is produced in response to a plethora of abiotic and biotic stresses (Morgan and Drew, 1997), including flooding (Drew, 1997; Grichko and Glick, 2001b; Jackson, 1985; Kennedy et al., 1992; Voesenek et al., 1993), wounding (Hyodo, 1991; O'Donnell et al., 1996), viruses, bacteria, fungi, insects, and nematodes (Abeles et al., 1992), other plant hormones such as IAA, ABA, cytokinins, and methyl jasmonate; and small metabolites such as carbohydrates, orthophosphate, and polyamines (Fluhr and Mattoo, 1996; Mattoo and Suttle, 1991).

Because of its profound effects on plant growth and development, ethylene's biosynthesis, action, and the control of its action by chemical, physical, and biotechnological means have been intensively investigated (Giovannoni, 2001; Kanellis et al., 1997; 1999). Thanks to new tools available in biochemistry and molecular genetics, parts of the ethylene biosynthesis, perception, and signal transduction reactions have been elucidated (Bleecker, 1997; Chang et al., 1993; Ecker, 1995; Giovannoni, 2001; Kanellis et al., 1997; 1999; Kende, 1993; Stepanova and Ecker, 2000; Zarembinski and Theologis, 1994). This knowledge has been applied to enhance the quality of a number of agronomically important crops (see Chap. 17).

This chapter will cover the recent advancements in the field of ethylene research and it is divided into the following topics: biochemical and molecular mechanisms of ethylene synthesis, ethylene action, perception and signal transduction pathways, ethylene and fruit ripening, ethylene and senescence of plant organs, stress ethylene, biochemical and molecular approaches, ethylene involvement in pathogenesis and disease resistance, and control of ethylene biosynthesis and action by chemical means.

II. BIOCHEMICAL AND MOLECULAR MECHANISMS OF ETHYLENE SYNTHESIS

The discoveries that methionine (Lieberman and Mapson, 1964) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979; Lürssen et al., 1979) were ethylene precursors have been the key milestones in the elucidation of the ethylene biosynthetic pathway. Today the whole route of ethylene biosynthesis is well established. The first step consists in the conversion of methionine (Met) into S-adenosyl methionine (SAM) by incorporation of ATP (Adams and Yang, 1977). Then, SAM is converted into ACC and methylthioadenosine (MTA) while methionine is recycled within the so-called Yang's cycle. Ethylene is then generated from ACC via oxidation of this ethylene precursor (Fig. 1).

The key enzymes involved in the ethylene biosynthetic pathway are ACC synthase (ACS) and ACC oxidase (ACO). However, ACC can be also converted into conjugated derivatives by ACC N-malonyltransferase and ACC glutamyltransferase. Other enzymes are also involved either upstream (SAM synthase) or downstream (β-cyanolamine synthase) the ethylene biosynthetic pathway.

A. ACC Synthase Enzyme and Encoding Genes

Soon after the discovery of ACC (Boller et al., 1979; Yu et al., 1979), ACS activity was identified in tomato fruit first and then in variety of other fruits and vegetables (Kende, 1993). Because of its very low abundance in plant tissues, purification of the protein has proved to be difficult. Nevertheless, partial purification of the enzyme was performed and

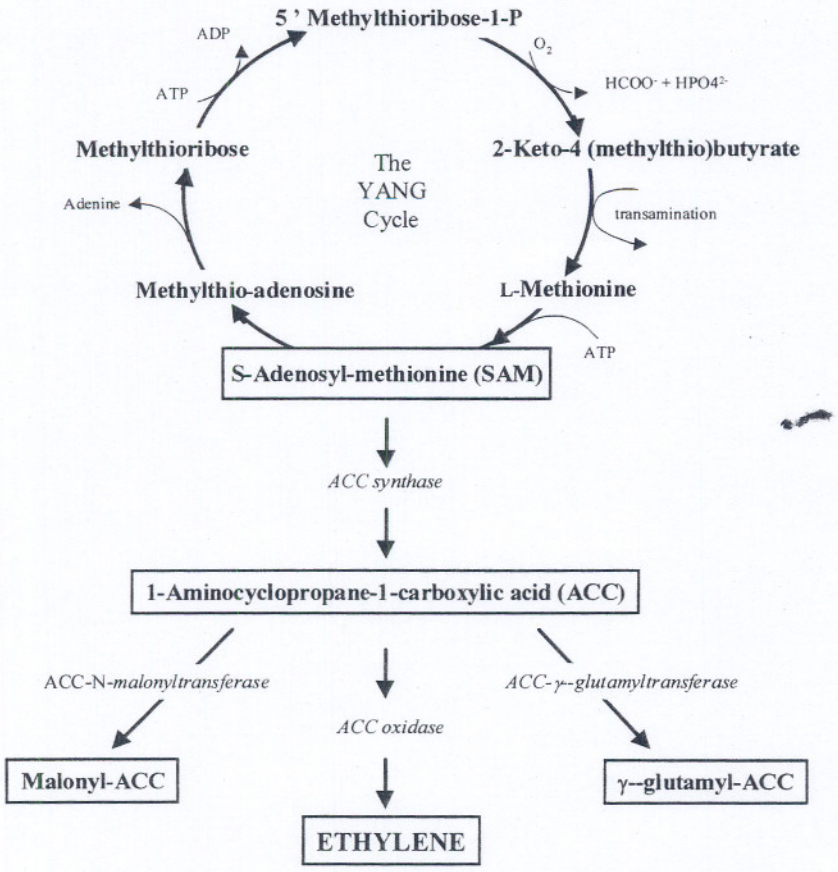

Figure 1 The ethylene biosynthetic pathway according to Yang and Hoffman (1984), with modifications.

antibodies were obtained from wounded tissues of tomato fruit (Bleecker et al., 1986; Mehta et al., 1988) and winter squash (Nakajima and Imaseki, 1986; Nakajima et al., 1988). Finally, following total purification from zucchini (Sato and Theologis 1989) and tomato (Van der Straeten et al, 1990), aminopeptide sequencing was achieved. The purified ACS protein has a molecular weight ranging from 48 to 58 kDa, depending on the plant species (Van der Straeten et al., 1990; Nakajima et al. 1990; Dong et al., 1991; Sato et al., 1991). The enzyme is active in its monomeric form (Li and Mattoo, 1994) but is present as an homodimer in plant tissues (Sato et al., 1991) and when expressed in *Escherichia coli* as a recombinant protein (Hohenester et al., 1994; White et al., 1994).

ACS is a pyridoxal phosphate-dependent enzyme that converts SAM into ACC and MTA via α, γ-elimination (Ramalingam et al., 1985; Wiesendanger et al., 1986a and 1986b). It is also capable, by a β-γ elimination process (Satoh and Yang, 1989a) of releasing vinylglycine that binds irreversibly to the enzymatic site thus causing suicidal inhibition of the enzyme (Satoh and Yang, 1989b). This suicidal reaction has allowed the labeling of the protein with its substrate and the determination of the active site (Yip et al., 1990). The C-terminal of ACS plays an important role in the catalytic activity and dimer-

ization of the protein. The deletion of 46 to 52 amino acids from the C-terminal results in a hyperactive monomeric enzyme that has 9 times higher affinity for SAM than the wild-type enzyme (Li and Mattoo, 1994).

The pioneering work on cloning the first ACS cDNA (Sato and Theologis, 1989; Van der Straeten et al., 1990) provided probes and primers for the isolation of homologous genes from a variety of fruits and vegetables, including tomato (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993), potato (Destefano-Beltran et al., 1995), mung bean (Botella et al., 1992), tobacco (Bailey et al., 1993), mustard (Wen et al., 1993), rice (Zarembiski and Theologis, 1993), melon (Miki et al., 1995) and pepper (Harpster et al., 1996). ACS is encoded by a multigene family of at least nine members in the tomato (Zarembinski and Theologis, 1994). They can be classified into three classes by phylogenetic analysis (Lincoln et al., 1993) or into four classes on the basis of the pI values (Flurh and Mattoo, 1996). The members of the multigene family are differentially expressed during ripening, wounding and auxin treatment (Zarembinski and Theologis, 1994). For instance, in tomato, *LE-ACS2* and *LE-ACS4* genes are expressed during fruit ripening (Van der Straeten et al., 1990; Olson et al., 1991; Rottman et al., 1991), induced in mature green fruits upon treatment with exogenous ethylene (Olson et al., 1991; Lincoln et al., 1993) and superinduced upon wounding of pericarp tissues (Van der Straeten et al., 1990; Yip et al., 1992; Lincoln et al., 1993). In addition, *LE-ACS2* is highly induced in auxin-treated vegetative tissues (Yip et al., 1992). Posttranslational regulation has been also reported (Spanu et al., 1994).

B. ACC Oxidase Enzyme and Encoding Genes

Until recently, all attempts to obtain genuine ACO activity into a cellular fraction failed. Activity was only measured on entire tissues in the presence of saturating concentrations of exogenous ACC and was referred to as ethylene-forming enzyme (EFE). This feature led to the assumption that ACO is a membrane bound enzyme (Apelbaum et al., 1981a). The mystery of this enzyme was fathomed only once the gene encoding the ACO protein has been isolated, giving a brilliant example of "reverse biochemistry." That is, among the ripening related cDNAs isolated from tomato fruit (Slater et al., 1985), the pTOM13 clone was selected as a putative ethylene biosynthetic gene based on its expression during ripening and upon wounding (Holdsworth et al. 1987; Davies and Grierson, 1989). The expression in tomato plants of an antisense construct of the pTOM13 cDNA resulted in reduced capacity to produce ethylene and significant delay in fruit ripening suggesting that this clone might encode the ACO protein (Hamilton et al., 1990). The ultimate identification of the ACO gene was given by functional expression in *Saccharomyces cerevisiae* (Hamilton et al., 1991) and in *Xenopus* oocytes (Spanu et al., 1991). Based on the sequence homology between *pTOM13* and flavanone-3-hydroxylase genes (Hamilton et al., 1990) and the demonstration that iron is an essential cofactor of EFE in vivo (Bouzayen et al., 1991), a soluble ACO activity could be obtained for the first time from melon fruit (Ververidis and John, 1991). Subsequent studies showed that beside iron, the enzyme requires ascorbate and CO_2 (Dong et al., 1992; Smith and John, 1993; Poneleit and Dilley, 1993). The ACO was thereafter purified to homogeneity and antibodies were obtained against the recombinant protein overproduced in *E. coli* (Dupille et al., 1993; Dong et al., 1992, Rombaldi et al., 1994). Biochemical studies showed that ACO is a 36 kDa monomer with an apparent Km for ACC varying from 20 to 60 μM, an optimum pH around 7.4 and an optimum temperature of 28°C (Prescott and John, 1996). Like genuine ethylene-

forming enzyme activity in vivo, both purified and recombinant ACOs discriminate between the enantiomers of ACC analogue, 2-ethyl-ACC (Ververdis and John 1991; Hamilton et al., 1991), the (1R, 2S) enantiomers being preferentially transformed (Hoffman et al., 1982). Even though the protein sequence lacks a signal peptide classically required for crossing the plasma membrane, radiochemical and immunocytolocalization studies showed that, in tomato and apple fruit, ACO is predominantly located in the apoplasm (Bouzayen et al., 1990; Rombaldi et al., 1994; Ramassamy et al., 1998).

The conversion of ACC into ethylene by ACO proceeds via the opening of the cyclopropane ring, carbons 2 and 3 giving ethylene and carbon 1 HCN. The intimate mechanism of the reaction is still a matter of debate. The formation of N-hydroxyl ACC as an intermediate (Dong et al., 1992) has not received confirmation (Stella et al., 1996). Nethertheless, it is well established that for each mole of ACC consumed, one mole of oxygen is utilized. Also, the reaction involves ascorbate as a cofactor, which is transformed mole by mole into dehydroascorbate (Dong et al., 1992). ACO displays an absolute requirement for iron in vivo (Bouzayen et al., 1991) but has been shown to be a nonheme enzyme (Dupille et al., 1992). Three histidine residues serving as putative Fe(II)-binding sites has been identified (Shaw et al., 1996). Carbon dioxide is an essential activator of ACO (Dong et al., 1992; Smith and John, 1993,) as the enzyme activity is 10 times higher in presence of 4% CO_2 than in air (0.03% CO_2). The role of CO_2 in controlling ethylene synthesis has been reviewed by Mathooko (1996). It has been hypothesized that carbon dioxide activation of ACO operates through carbamation of an ε-amino residue of lysine. Catalytic inactivation of ACO occurs in vitro when the enzyme is preincubated in the presence of its substrates. This phenomenon was prevented by omitting any of the reactants in the preincubation medium (Pirrung et al., 1993; Smith et al., 1994). This inactivation could be partially prevented by the addition of catalase, implying the involvement of H_2O_2 generated from the autooxidation of ascorbate by O_2 (Smith et al., 1994). Inactivation of recombinant ACO from tomato by ferrous iron and ascorbate is particularly fast and is accompanied by partial proteolysis (Barlow et al., 1997). Recent research has revealed that in the ACO reaction ascorbate binds to the active site after ACC (Rocklin et al., 1999).

In tomato, ACO is encoded by a small multigene family comprising three members (*ACO1, ACO2*, and *ACO3*) that has been shown to be transcriptionally active (Bouzayen et al., 1993) and differentially expressed (Barry et al., 1996; Lasserre et al., 1996). The heterologous expression in yeast demonstrated that all three genes encode functional proteins, though the ACO isoforms displayed different levels of activity (Bidonde et al., 1998). The lower activity level shown by the ACO2 isoform may be related to its higher content in positively charged groups, resulting in a higher isoelectric point value. In melon, three *ACO* genes have also been isolated and *CMe-ACO1* was shown to be strongly expressed during ripening and in response to exogenous ethylene treatment (Lasserre et al., 1996).

C. ACC Conjugation Activities

1. N-Malonylation of ACC

ACC can be converted into malonyl ACC (MACC), which may participate in regulating ethylene production by diverting ACC from its route to ethylene. This has been suggested in the case of autoinhibition of ethylene production in citrus albedo (Liu et al., 1985a) and during the opening of the hypocotyl hook in etiolated seedlings upon illumination

(Jiao et al., 1987; Vansgronsveld et al., 1988). In some tissues, like preclimacteric apples, more than 40% of the ACC synthesized in the skin and 5% in the flesh are diverted to MACC (Mansour et al., 1986). MACC is synthesized in the cytosol and then transported into the vacuole by an ATP-dependent transtonoplastic carrier (Bouzayen et al., 1988, 1989). MACC sequestration into the vacuole and its release from this compartment are dependent on the protonation of this molecule, which is dictated by the vacuolar pH (Pedreno et al., 1991). Under physiological conditions MACC cannot be metabolized back into ACC. However, some conversion of MACC into ACC has been described in conditions where high concentrations of exogenous MACC were provided to the plant tissue (Jiao et al., 1986).

In comparison with the other enzymes of the ethylene biosynthetic pathway, little is known about N-malonyltransferase. This enzyme is also capable of malonylating D-amino acids, suggesting a possible role in the cell detoxification process by preventing the incorporation of abnormal aminoacids into nascent proteins. The production of D-amino acids has been observed in plants under stress conditions (Rekoslavskaya et al., 1988). ACC *N*-malonyltransferase activity is strongly stimulated by ethylene, which is known to be induced in stress conditions (Liu et al., 1985a, b). Guo et al. (1992) and then Benichou et al. (1995) have purified the ACC *N*-malonyltransferase from etiolated mung bean and characterized 55- and 36-kDa proteins, respectively. A 40-kDa ACC *N*-malonyltransferase was also characterized in tomato (Martin and Saftner, 1995) and mung bean seedling hypocotyls (Chick and Leung, 1997). It has been assumed that various isoforms of the enzyme exist in plant tissues (Benichou et al., 1995).

2. ACC γ-Glutamyltranspeptidase

It has been reported that crude protein extracts of tomato are capable of conjugating ACC into a 1-(γ-L-glutamylamino) derivative (GACC) in the presence of glutathione (Martin et al., 1995). In the pericarp tissues of tomato fruit, GACC-forming activity increased gradually through fruit development to a plateau in orange to fully ripe fruit. The amount and changes of GACC during physiological processes like fruit ripening are still unknown, but the ACC γ-glutamyltranspeptidase involved in GACC biosynthesis deserves further characterization at the biochemical and molecular levels.

D. Other Enzymes Related to the Ethylene Biosynthetic Pathway

SAM synthase catalyzes the conversion of Met to SAM in the presence of ATP and Mg^{2+}, which corresponds to the first step in ethylene biosynthesis. SAM also serves as a propylamine group donor in polyamine biosynthesis and as a methyl group donor in the transmethylation of lipids, nucleic acids, and polysaccharides (Tabor and Tabor, 1984). A possible competition for SAM of these various pathways, especially the biosynthesis of polyamines (Mattoo and White, 1991) has been suggested, and overexpression of SAM hydrolase, an enzyme capable of degrading SAM, results in a strong inhibition of ethylene production during tomato ripening (Good et al., 1994). Several genes encoding SAM synthase have been cloned in plant tissues, including tomato, parsley, and mustard (Flurh and Mattoo, 1996).

The cyanide formed during the conversion of ACC into ethylene (Peiser et al., 1984) can be metabolized by β-cyanoalanine synthase, which catalyzes the formation of β-cyanoalanine and H_2S from cysteine and CN^- (Blumenthal et al., 1968). β-Cyanoalanine can then be hydrated to asparagine (Castric et al., 1972). Interestingly, β-cyanoalanine

synthase activity closely follows ethylene evolution (Manning, 1986) and is stimulated by exogenous ethylene treatments (Goudey et al., 1989).

III. ETHYLENE ACTION: PERCEPTION AND SIGNAL TRANSDUCTION PATHWAY

A. Biochemical Approaches

As in the case of other hormones, ethylene exerts its physiological action by ultimately modifying gene expression. The first step in ethylene perception and transduction pathways consists of binding to a receptor. Based on the observation that the structural requirement for biological activity was similar to the stability constants of olefine-silver complexes, Burg and Burg (1967) were the first to propose that ethylene could bind to a metal-containing receptor. Ethylene binding has been studied in a variety of plant tissues using a radioisotope technique (Sisler, 1979). Using this method, it has been reported that the number of binding sites undergoes very little changes or even decline during ripening of apple fruit and senescence of morning glory flowers (Blankenship and Sisler, 1989), while the concentration of ethylene required to saturate the sites increases (Blankenship and Sisler, 1993). According to these data, the increased sensitivity of apples with maturation would not be ascribed to increased number or affinity of the binding sites. However, uncertainties exist on the physiological significance of ethylene binding on whole tissues. So far, it has proved impossible to isolate and characterize any ethylene-binding protein after radiolabeling and protein purification (Sisler, 1980; Dupille and Sisler, 1995; Harpham et al., 1996).

B. Molecular Genetic Approaches

Our current knowledge of ethylene perception and signal transduction has arisen from molecular genetic approaches using *Arabidopsis* mutants. The mutants were initially isolated by screening for those exhibiting an altered triple-response phenotype. That is, dark-grown *Arabidopsis* seedlings treated with ethylene show inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl, and accentuation of the apical hook. The mutants isolated by this method fall into two classes, those lacking a triple response upon ethylene treatment (ethylene-insensitive) and those exhibiting a triple response even in the absence of ethylene (constitutive triple response). Following the isolation of the first ethylene insensitive mutant (Bleecker et al. 1988), a serial of other mutants has been selected including: *ein1* and *ein2* (Guzman and Ecker, 1990; Chang et al., 1993), *ein3* (Kieber et al., 1993), *ain1* (Van der Straeten et al., 1993) and *ein4* to *ein7* (Roman et al., 1995). Among the constitutive triple-response mutants, some are ethylene overproducers, while the *ctr1* mutant is defective in signal transduction (Kieber et al., 1993). The epistatic relationship between these mutations have provided the following order of the component in the ethylene transduction pathway:

$$C_2H_4 \rightarrow ETR1 \rightarrow CTR1 \rightarrow \rightarrow \rightarrow EIN2 \rightarrow EIN3 \rightarrow \rightarrow \rightarrow \text{responses}$$

1. Isolation and Characterization of Ethylene Receptors

The *ETR1* gene encodes a protein showing homology to the histidine kinase of the bacterial two-component system involved in response to a broad range of environmental stimuli (Chang et al., 1993). When expressed in yeast, *ETR1* gene confers saturable ethylene-

binding sites that can be antagonized by competitors of ethylene action (Schaller and Bleecker, 1995). Moreover, the mutant *etr1* protein expressed in yeast lacked detectable ethylene-binding activity. These data provide strong evidence that *ETR1* actually encodes the ethylene receptor. The ETR1 protein is membrane-bound disulfide-linked dimer (Schaller et al., 1995) with an N-terminal sensor domain adjacent to the histidine kinase domain, with a putative receiver domain in the C-terminal region (Chang et al., 1993). ETR1 is a metalloprotein with a Cu(I) ion in its ethylene binding site (Rodriguez et al., 1999). An *Arabidopsis ETR1* homolog named *ERS* lacking the receiver domain has been cloned (Hua et al., 1995). A mutated version of *ERS* is capable of conferring ethylene insensitivity when introduced in wild-type *Arabidopsis*, indicating that several ethylene receptors may exist in plants. A homolog to *ETR1*, called *Nr*, has been cloned from the ripening-impaired tomato mutant *Never-ripe* (Wilkinson et al., 1995). Interestingly, *Nr* mRNA is positively regulated by ethylene in mature-green tomato fruit, but not in immature green fruit (Wilkinson et al., 1995). On the contrary an *ETR1* homolog of tomato, *eTAE1*, is constitutively expressed in fruit and other vegetative or reproductive tissues (Zhou et al., 1996). Several loci have been found on the RFLP map of tomato chromosomes that are capable to hybridize to *ETR1* (Yen et al., 1995). These findings do not support the suggestion that the number of ethylene-binding sites would not change during ripening (Blankenship and Sisler, 1989). Several other members of the tomato ethylene receptor family have been isolated and characterized (Tieman and Klee, 1999). Genetic studies have demonstrated that the ethylene receptors of *Arabidopsis thaliana* (Hua and Meyerowitz, 1998) and tomato (Tieman et al., 2000) are negative regulators of ethylene response and that the family members are at least partially redundant.

2. Isolation of Other Components of the Ethylene Transduction Pathway

The CTR1 gene is acting downstream of *ETR1* and upstream of *EIN3*. It encodes a protein that resembles the Raf family of serine/threonine kinases (Kieber et al., 1993). Raf is part of a mitogen-activated protein (MAP) kinase cascade known to regulate cell growth and development in mammals, worms, and flies (Chang et al., 1993). The *ctr1* is a recessive mutation that mimics an ethylene response in the absence of the hormone, suggesting that CTR is a negative regulator. The concept of negative regulation means that there is a constant signal flow through the pathway, which is repressed by CTR1, presumably through phosphorylation, and a loss of function of CTR1 protein results in a constitutive ethylene response phenotype. The nature of the CTR1 interacting protein remains unknown.

Further downstream of CTR1, several components of the ethylene signal transduction pathway have been identified and characterized in *Arabidopsis thaliana*. These include EIN2, EIN3, and ERF1. The exact function of EIN2 is still unclear, but the N-terminal transmembrane domain shows similarity to metal-ion transporters (Alonso et al., 1999). *EIN3* and *ERF1* act downstream of *EIN2* and encode *trans*-acting transcription factors that confer ethylene responsiveness to a number of ethylene-regulated genes.

C. Ethylene-Responsive Genes

Ethylene activates the transcription of a number of specific genes during various phases of plant development or under the action of various stimuli. The involvement of ethylene in the regulation of gene expression has been demonstrated by the following means: (a) treating tissues with exogenous ethylene, (b) blocking ethylene action using the hormone antagonists, (c) analyzing mutants impaired in ethylene perception, and finally (d) charac-

terizing transgenic plants with reduced ethylene production. Using these methods, a number of genes have been found to be ethylene-regulated (see reviews by Broglie and Broglie, 1991; Deikmann, 1997). They belong to the following:

Ripening or senescence-related genes: *E4*, *E8*, *E17*, *J49* (Lincoln et al., 1987; Lincoln and Fischer, 1988), proteinase inhibitor (Margossian et al., 1988), cellulase (Lashbrook et al., 1994), polygalacturonase (Theologis et al., 1993), glutathion-S-transferase (Itzhaki and Woodson, 1993), *ACO* (Lasserre et al., 1997)

Pathogenesis-related genes: chitinase (Broglie et al., 1986), β-1,3-glucanase (Felix and Meins, 1987), hydroxyproline-rich glycoproteins (Ecker and Davis, 1987)

Wound-induced genes: phenyalanine ammonia lyase, 4-coumarate-CoA ligase, chalcone synthase (Ecker and Davis, 1987)

Novel early ethylene-regulated genes (*ER*) have been isolated from tomato fruit by differential display (Zegzouti et al., 1999). Among the ER clones, several displayed homology to regulatory genes that may participate in the ethylene response at the level of signal transduction, transcription and translation.

Mechanisms by which ethylene regulates gene expression are diverse. Treatment of early mature-green tomatoes with exogenous ethylene induces a rapid accumulation of mRNAs corresponding to ethylene-responsive genes (Lincoln and Fischer, 1988; Zegzouti et al., 1999). Posttranscriptional processes also play an important role in the regulation of gene expression by ethylene. Analysis of ACS-antisense tomato fruit indicated that during ripening, accumulation of polygalacturonase (PG) transcripts is developmentally regulated. Moreover, PG mRNA but not PG polypeptide accumulates in this transgenic fruit with reduced ethylene production. Synthesis of PG protein occurs when fruit are treated with ethylene or propylene (Theologis et al., 1993) indicating that PG gene expression is regulated by ethylene at the posttranscriptional level. The molecular mechanisms underlying the ethylene-dependent regulation of gene transcription is now becoming clearer. EIN3 and related proteins belong to a subfamily of transcription factors that include E4/E8 binding proteins and that bind to primary ethylene response elements (Solano et al., 1998; Coupe and Deikman, 1997). They are involved in triggering the primary ethylene response by inducing the transcription of a second class of ethylene transcription factors. These latter, called ethylene-responsive element–binding proteins (EREBP), interact with an ethylene responsive element (ERE), the GCC box, present in the promoter of a number of ethylene-regulated genes (Ohme-Takagi and Shinshi, 1995; Chao et al., 1997).

D. Molecular Basis of the Control of Gene Expression by Ethylene

1. Characterization of Ethylene-Responsive Elements

Several consensus DNA sequences located upstream of the transcribed regions have been characterized in ethylene-responsive genes by using promoter fusion to reporter genes. Ethylene-responsive elements have been identified in the promoter of chitinase (Broglie et al., 1989; Shinshi et al., 1995), E8 (Deikman and Fischer, 1988), glutathione S-transferase (Itzhaki et al. 1994) and a basic PR protein (Meller et al., 1993). A GCC motif is found in many of the pathogenesis-related promoters which has been shown to be necessary for ethylene-responsive transcription (Shinshi et al., 1995) and a 47bp fragment containing two copies of this motif has been shown to be sufficient to confer ethylene responsiveness (Ohme-Takagi and Shinshi, 1995). A GCC motif is also present in the promoter of melon *ACO* gene, *CMe-ACO1* (Lasserre et al., 1997). However GCC motifs are present neither

in the promoters of *E4*, *E8* genes (Xu et al., 1996) nor in the promoter of the glutathione S-transferase gene (Itzhaki et al., 1994). But a nearly conserved octanucleotide sequence (ATTTCAAA) is present in both chitinase, and *E4* and *E8* gene promoters (Maxson and Woodson, 1996). Also, TACCAAC or TACCACC motifs are present in both the *E4* (Xu et al., 1996) and glutathione S-transferase (Itzhaki et al, 1994) gene promoters respectively. In the *E4* promoter, at least two *cis*-elements are required for ethylene-responsive transcription. These data indicate that both similar and diverse transcription factors may be involved in ethylene activation of genes.

2. Isolation of Ethylene-Related *Trans*-Acting Factors

More recently, trans-factors corresponding to DNA-binding proteins that interact with promoter of ethylene-responsive genes have been isolated. By using gel-retardation methods, nuclear factors have been shown to specifically interact with some of these regulatory regions. The nature of these proteins starts to be elucidated. Four different cDNAs encoding ethylene-responsive element–binding proteins (EREBPs), capable of interacting with with the GCC box of the chitinase gene promoter were cloned (Ohme-Tagaki and Shinshi, 1995). The DNA-binding domain of the EREBPs does not share homology with known transcription factors or DNA binding proteins. In carnation, a cDNA-binding protein that interacts with the ethylene-responsive element of the glutathione S-reductase gene has been cloned (Maxson and Woodson, 1996). The predicted protein encoded by this cDNA has a molecular weight of 32kDa and shares homology with other plant nucleic acid binding proteins from maize and *Arabidopsis*.

IV. ETHYLENE AND FRUIT RIPENING

The ripening of fleshy fruits corresponds to a series of biochemical, physiological, and structural changes that make the fruit attractive to the consumer. With the development of molecular biology approaches, ripening is now considered as a genetically programmed event involving the regulated expression of specific genes (Grierson, 1987). Recent reviews related to the role of ethylene in fruit ripening (Giovannoni, 2001; Jiang and Fu, 2000; Lelièvre et al., 1997b) and fruit quality (Salveit, 1999) are available.

A. Role of Ethylene in Ripening of Climacteric and Nonclimacteric Fruits

Fruits were initially divided into two groups, known as climacteric and nonclimacteric, depending upon whether or not they developed increased respiratory activity and ethylene production during ripening (Biale, 1964). Recent findings, however, have fueled a debate on the coupling of respiration climacteric and increased ethylene production. A series of experiments in tomato and melon revealed that while harvested fruit exhibited a clear coincidence between an increase in respiration and ethylene production, fruit ripened on the vine failed to develop any rise in respiration despite a marked peak of ethylene production (Miccolis and Salveit, 1991; Shellie and Salveit, 1993; Salveit, 1993). Several groups subsequently challenged these findings (Knee 1995; Andrew, 1995; Hadfield et al., 1995), but this question is still a matter of controversy. In any case, however, the sharp increase in ethylene production at the onset of the climacteric phase is considered as controlling the initiation of changes in color, aromas, texture, flavor, and other biochemical or physio-

logical attributes. By contrast, the ripening of nonclimacteric fruits is generally considered as an ethylene-independent process and little is known of the regulatory mechanisms underlying the biochemical changes.

One of the most striking characteristics of climacteric as compared with nonclimacteric fruits is their capacity to produce autocatalytic ethylene (McMurchie et al., 1972). It has been speculated that two regulatory systems of ethylene production exist. System I, operating in both climacteric and nonclimacteric fruits as well as in vegetative tissues, would be responsible for basal and wound-induced ethylene production, while system II would be responsible for the upsurge of ethylene production during ripening of climacteric fruit. The essential role of ethylene in the ripening of climacteric fruits is demonstrated by studies showing that inhibitors of ethylene action completely block ethylene production and ripening (Saltveit et al., 1978; Hobson et al., 1984; Sisler and Lallu 1994; Dupille and Sisler, 1995). However, some ripening pathways of nonclimacteric fruits are also regulated by ethylene as they can also be inhibited by inhibitors of ethylene action (Goldschmidt et al., 1993).

B. Expression of Genes Involved in Ethylene Biosynthesis and Perception During Ripening

The competence of climacteric fruits to synthesize autocatalytic ethylene is developmentally regulated and requires as a primary step the stimulation of *ACS* and *ACO* gene expression by nonethylene regulatory factors. Then, autocatalytic ethylene production proceeds via the upregulation by ethylene of its biosynthetic genes, namely *ACS* and *ACO* (Lelièvre et al., 1997b). Since both ACO and ACS are encoded by multigene families it can be speculated that this transition to autocatalytic ethylene production may be related to a cascade of expression of different members of the gene families. In tomato and melon fruit, differential expression of the *ACO* gene family has been demonstrated (Barry et al., 1996; Lasserre et al., 1996). In tomato fruit, both *ACO1* and, at a lower level, *ACO3* are expressed at the onset of ripening. However, while ACO1 transcripts continue to accumulate throughout the ripening phase (Barry et al., 1996) *ACO3* shows a transient increase in expression at the breaker stage with subsequent sharp decrease concomitant with the rise in ethylene production.

At least three *ACS* genes are expressed in the fruit, with the most abundant mRNA species corresponding to *LEACS2*, and to a lesser extent to *LEACS4*. A third gene hybridizing to LEACS6 probe (Lincoln et al, 1993) was shown to be expressed in fruit. Differential expression of *ACS* genes has also been demonstrated in melon (Diallinas et al., 1997; Yamamoto et al., 1995) and winter squash (Nakajima et al., 1990; Nakagawa et al., 1991). While the data reported above clearly indicate that genes encoding *ACO* and *ACS* are differentially regulated during fruit ripening, it is still premature to conclude that specific members of these gene families are linked to the proposed system I and system II of ethylene production or rather that the same member of the gene family is regulated by different factors.

One gene, designated *E8*, related to *ACO* and a member of FeII dioxygenases, is strongly expressed during fruit ripening. An antisense construct of this gene stimulates ethylene production during ripening of tomato fruit detached from the plant at, or well prior to, the onset of ripening, indicating that *E8* could be involved in the negative feedback regulation of ethylene biosynthesis (Penarrubia et al., 1992). However, the effects of antisense *E8* on ripening and quality have not been reported.

Ethylene acts through a receptor and a transduction pathway. Tomato contains a family of ethylene receptors named *LeETR* 1 to 5 and *NR*. A semidominant mutation of the *NR* gene is responsible for the inability to ripen of the *Never-ripe (Nr)* natural mutant of tomato. Interestingly, *NR* mRNA accumulation starts at the breaker stage, is positively regulated by ethylene in mature-green tomato fruit and not in immature green fruit (Wilkinson et al., 1995). On the contrary, an *ETR1* homolog of tomato, *eTAE1*, is constitutively expressed in fruit and other vegetative or reproductive tissues (Zhou et al., 1996). Transgenic tomatoes expressing the *etr1* mutant cDNA exhibited altered ethylene response, including ripening, indistinguishable from the *Nr* mutant (Wilkinson et al., 1997). Slowing down the expression of *LeETR4*, a gene highly expressed in fruit but weakly in vegetative tissues, results in increased sensitivity to ethylene, with early ripening (up to 11 days earlier than wild-type) and more rapid development of color, indicating that LeETR4 is a negative regulator of the ethylene transduction pathway (Tieman et al., 2000). Antisense *NR*, on the contrary, had a normal phenotype, but in this case reduction in *NR* expression resulted in a functional compensation consisting in an increased expression of *LeETR4*.

C. Molecular Genetic Approaches to Understanding and Control of Fruit Ripening

In recent years, control of fruit ripening has also been achieved using genetic engineering methods. Transgenic fruits harboring antisense ACO and ACS constructs displayed strong reduction of ethylene production and significant inhibition of ripening (Hamilton et al., 1990; Oeller et al., 1991; Ayub et al., 1996; Bolitho et al., 1997). The same goal has been achieved by over expression of a bacterial ACC deaminase (Klee, 1993) and a viral S-adenosylmethionine hydrolase (Good et al., 1994), two genes that reduce the availability of the ethylene precursors. In all these transgenic plants, the wild-type ripening phenotype can be restored by the application of exogenous ethylene or its analog propylene. More recently successful control of fruit ripening has also been obtained through genetic alteration of ethylene sensitivity. These experiments demonstrated that expression of the mutated *etr1* gene of *Arabidopsis* in tomato causes significant delays in fruit ripening (Wilkinson et al., 1997). The ability of *etr1* to function in heterologous plants suggests that this pathway of ethylene recognition and response is highly conserved.

The availability of transgenic and naturally occurring mutant lines provided new tools for discriminating ethylene-dependent and ethylene-independent ripening pathways in climacteric fruits. Behind the altered phenotype of transgenic fruit lies the differential expression of ripening and ethylene-regulated genes. Many aspects of ripening are initiated similarly in control and mutant fruits suggesting that they are regulated by factors others than ethylene. However, ACS or ACO antisense fruit still make some residual ethylene that may be sufficient to stimulate expression of genes that are sensitive to very low amounts of ethylene. For example, accumulation of transcripts encoding endo-polygalacturonase, a cell degrading enzyme, was thought to be unaffected in both ACS and ACO-antisense fruit compared to the wild type (Oeller et al., 1991, Picton et al., 1993), while a reexamination of PG mRNA accumulation indicated that it was in fact ethylene-regulated (Sitrit and Bennett, 1998). It is accepted that the triggering of ripening of nonclimacteric fruits does not require ethylene. However, it is known that ethylene is involved in the regulation of some aspects of ripening, such as chlorophyll degradation and carotenoid synthesis in citrus (Goldsmith et al., 1993). An ethylene-inducible chlorophyllase has been

characterized in citrus, a classic nonclimacteric fruit (Jacob-Wilk et al., 1999). The emerging picture is one where both ethylene-dependent and independent pathways coexist in both climacteric and nonclimacteric fruits.

V. ETHYLENE AND LEAF SENESCENCE

Leaf senescence is a developmentally regulated process characterized by a progressive loss of chlorophyll and photosynthetic capacity, a decline in protein and nucleic acid content, and a drop in starch and lipids (Thomas and Stoddart, 1980). Changes in the activity of hydrolytic enzymes are in line with these catabolic changes (Laurière, 1983; Mattoo and Aharoni, 1988). The senescence process is of great practical value because during leaf senescence, nutrients are recycled to other parts of the plant. However, premature leaf senescence may limit yield of field crops and is a major cause of postharvest deterioration of leafy vegetables (Kader, 1985).

In some species, leaf senescence is accompanied by a rise in ethylene production and a role for ethylene in senescence is clearly demonstrated by the inhibitory effect on chlorophyll breakdown of inhibitors of ethylene synthesis and action (Aharoni et al., 1979).

Recently, the generation of ethylene-insensitive mutants of *Arabidopsis* (Ecker, 1995) has provided interesting experimental systems to determine the role of ethylene in leaf senescence. Ethylene-insensitive mutants loose their chlorophyll at a lower rate than wild-type leaves, and the life span of the leaf is prolonged (Zacarias and Reid, 1990; Grbic and Bleecker, 1995). Delayed leaf senescence coincides with delayed induction of senescence-associated genes and higher expression of photosynthesis-associated genes (Grbic and Bleecker, 1995). Leaves of tomato transgenic plants in which ethylene synthesis is reduced by inhibiting the *ACO* expression via RNA antisense technology exhibit a delay of 10 to 14 days in leaf senescence, but once the senescence begins, it progresses normally (Picton et al., 1993; John et al., 1995). It can therefore be concluded that ethylene regulates the timing of leaf senescence rather than being necessary for the occurrence of the senescence syndroms. A review on the molecular genetic regulation and manipulation of leaf senescence has appeared (Gan and Amasino, 1997).

VI. ETHYLENE AND FLOWER SENESCENCE

The senescence of flowers includes petal wilting or abscission and floret abscission. For the horticulturist, the senescence of flowers also includes the yellowing of leaves associated with the flower stalk. Similarly to the senescence of other plant organs, petal senescence is mediated by a series of coordinated physiological and biochemical changes (increased activity of hydrolytic enzymes, protein and chlorophyll breakdown, loss of cellular compartmentation, and so on) requiring gene expression and protein synthesis. In some flower species (carnation, petunia, and others), the senescence process is associated with an increased synthesis of ethylene, while in some other species (many bulb flowers) ethylene is not the trigger for petal senescence. For most flowers, the importance of ethylene in their senescence was estimated by testing the effects of exogenous ethylene, and an extensive classification of ethylene sensitive and insensitive flowers is available (Woltering, 1987; Woltering and Van Doorn, 1988). However in some ethylene-sensitive species, like the carnation, ethylene-resistant mutants exist. They differ from normal cultivars for polyamine metabolism (Serrano et al., 1991). From a practical point of view, inhibitors

of ethylene biosynthesis and action are very efficient in preventing the senescence of ethylene-sensitive flowers.

Most of our knowledge concerning the process of ethylene-mediated flower senescence is derived from studies of a limited number of model flowers (carnation, petunia, and cymbidium). Among these models, carnation has by far been used most extensively. Reviews specific to the senescence of carnation flowers are available (Cook and van Staden, 1988; Van Altvorst and Bovy, 1995). Many similarities exist between ethylene-sensitive flowers and climacteric fruits in terms of regulation of ethylene biosynthesis and action. In particular, positive (autocatalysis) and negative (autoinhibition) feedback regulation has been described and ethylene biosynthetic genes, *ACO* and *ACS* have been isolated (Woodson et al., 1992; Park et al., 1992). The *ACO* gene family of petunia is composed of three transcriptionally active members (Tang et al., 1993). *ACO1* expression is responsible for the increase in ACO activity during petal senescence, while *ACO3* and *ACO4* are specifically expressed in the pistil during flower development (Tang et al. 1993). Two *ACS* genes are expressed in carnation flowers (Henskens et al., 1994; Have and Woltering, 1997). Both ageing and ethylene stimulated the occurrence of these transcripts in an organ-specific way. *CARACC3* was expressed mainly in petals, while *CARAS1* was preferentially expressed in the styles.

The major specificity of flower senescence is that pollination greatly affects ethylene metabolism and flower longevity. One of the earlier events following pollination is an increased ethylene synthesis in the pistil associated with a strong stimulation of ACS activity (Pech et al., 1987). The nature of the signal leading to increased ACS activity following pollination remains to be elucidated. However, since ACS in the stigma is synthesized from pre-existing mRNA (Pech et al., 1987), there might be a role for posttranscriptionnal events (phosphorylation) in pollination-induced ethylene synthesis. The rapid stimulation of ethylene production in the stigma is followed by a wave of increased ethylene production and expression of ethylene biosynthetic genes in other organs (O'Neil et al. 1993; O'Neil and Nadeau, 1996). This suggests that a transmissible signal is involved in postpollination interorgan communication. The physical growth of the pollen tube through the style does not seem absolutely required (Hoekstra and Weges, 1986). It has been suggested that some short-chain saturated fatty acids may be postpollination signals (Halevy et al., 1996), but their role remains unclear.

With the isolation of genes involved in ethylene synthesis and action, it has become possible to control flower senescence using genetic engineering methods. Transgenic carnation mutants in which ethylene synthesis is reduced by antisense ACO RNA have been generated (Savin et al., 1995). These mutants exhibit extended vase life but they remain sensitive to exogenous ethylene. The recent discovery of the ethylene receptor has opened new perspectives for the manipulation of ethylene sensitivity. The expression of an *NR* homolog (*tETR*) has been shown to be strongly stimulated during the senescence of tomato flowers, indicating that changes in ethylene sensitivity are mediated by modulation of receptor levels (Payton et al., 1996). The generation of ethylene-insensitive petunia flowers expressing an *etr1* mutated ethylene receptor of *Arabidopsis* under the control of the constitutive 35S promoter exhibited extended survival, both on and off the plant (Wilkinson et al., 1997). However, reproduction and horticultural performance was altered, such as lower fruit set and reduction of commercially available rooted cuttings (Gubrium et al., 2000). Use of tissue-specific promoters to drive expression of the transgene may allow the development of plants with longer-lasting flowers but with otherwise unaltered horticultural performance.

VII. STRESS ETHYLENE: PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR APPROACHES

Plants, unlike most eukaryotic organisms, cannot escape environmental changes and have instead adapted specific systems to tolerate them and survive. Wherever they grow, plants are subjected to a great variety of stresses that tend to restrict their chances of development and reduce their potential growth. Stress is a significant deviation from the conditions optimal for life, eliciting changes and responses at all functional levels of the organism, which may become permanent (Larcher, 1995). The term *stress* is also used for an external factor capable of inducing a potentially injurious strain in living organisms (Levitt, 1972). As a stress is imposed, plants usually exhibit a cascade of responses, occurring on different time scales, that involve molecular, biochemical, physiological, and morphological adjustments leading to stress tolerance or avoidance (Mooney et al., 1991).

Plant tissues produce ethylene when they are under unfavorable conditions or environmental factors. Stress ethylene can be caused by abiotic (mechanical, wounding, chilling, drought, flooding, and chemicals) and biotic stimuli (insect attack, and viral, bacterial, and fungal diseases) (Abeles et al., 1992; Morgan and Drew, 1997). Stress ethylene is responsible for alterations of growth, development, and differentiation in plants (Hyodo, 1991). It produces inhibition of growth, promotion of internode growth in aquatic plants, stem thickening, epinasty, abscission, acceleration of senescence and ripening, and development of disease symptoms (Abeles et al., 1992). Ethylene increases respiration, induces certain genes, and modifies certain metabolic activities (Ecker and Davis, 1987; Mattoo and Suttle, 1991).

Stress ethylene is synthesized via the methionine and ACC pathway and generally it involves effects on both ACS and ACO activities and their transcripts. One of the key reactions of the pathway is the conversion of SAM to ACC, which controls the ethylene production and is catalyzed by ACS (Kende, 1993, Yang and Hoffman, 1984). This view was firstly supported by the observation that application of aminoethoxyvinylglycine (AVG), a potent inhibitor of ACS, effectively eliminates the increase in ACC formation and the production of stress ethylene (Yang and Hoffman, 1984). Furthermore, pretreatment with cycloheximide, an inhibitor of protein synthesis, eliminates stress-induced ACC accumulation and ethylene production (Yang and Hoffman, 1984). Stress induces the synthesis of ACS, which in turn causes rapid accumulation of ACC and a marked increase in the production of stress ethylene (Yang and Hoffman, 1984). Recent evidence indicates that ACO is also a key step controlling ethylene biosynthesis during stress conditions (Barry et al., 1996; Blume and Grierson, 1997; English et al., 1995; Lasserre et al., 1996). The stimulation of ethylene production by stress typically occurs with a lag of 10 to 30 min and subsides later after reaching a peak within several hours (Yang and Hoffman, 1984). In recent decades, the study of stress has gained increasing importance and attempts have been made to clarify the role of stress ethylene as a signal or mediator in the stress response (Abeles et al., 1992; Morgan and Drew, 1997).

A. Mechanical and Wounding Stress

Fruits and vegetables are subjected to wounding and mechanical stress during harvesting, sorting, packing, and transportation, which may adversely affect ripening and senescence processes. Wounding refers to stress caused by cutting, gashing, abrasion, stabbing, bruising or intruding, which may cause injury on the surface or to the inner tissues (Hyodo, 1991). Mechanical stress is encountered when growth of plant tissues and organs is re-

stricted by certain physical barriers (mechanical impedance) (Morgan and Drew, 1997). Wounding has relevance to the development of crop resistance to plant pathogens that require a wound lesion for infection. Wounded plant tissues and organs release ethylene and show an increase in the activity of ACS and ACO. ACS induction kinetics vary depending upon the conditions used (Kende and Boller 1981; Mattoo and Anderson, 1984). Genes encoding ACS and ACO are not expressed in unripe, preclimacteric fruits but their expression can be induced by wounding (Diallinas and Kanellis, 1994; Mattoo et al., 1993). It seems that preclimacteric fruits are potentially capable of expressing *ACS* and *ACO* genes, but their expression is suppressed by an unidentified mechanism that is relieved by wounding.

Wound-induced ethylene production has been observed, among others, in tomato fruit, sweet potato (*Ipomoea batatas* L. Poir.) roots, bean (*Phaseolus vulgaris* L.) leaves, winter squash fruit, cucumber (*Cucumis sativus* L.) fruit, and cantaloupe melons (Abeles et al., 1992) as well as in sunflower hypocotyls (Liu et al., 1997). It is widely accepted that stress ethylene biosynthesis is regulated by de novo synthesis of ACS (Abeles et al., 1992). ACS activity in wounded tomato pericarp tissues reached a peak by 10 h (Li et al., 1992). The level of ACS transcript increased approximately fivefold at 8 h after wounding. Wounded tomato fruit tissue contained at least three ACS isoenzymes with pI values of 5.3, 7, and 9 (Mehta et al., 1988). Wounding of preclimacteric cantaloupe fruit also increased the ACS and ACO activities (Hoffman and Yang, 1982).

The *ACS* multigene family is independently regulated by different kinds of stresses (Fluhr and Mattoo, 1996). Tomato *LE-ACS2* and *LE-ACS4* genes are induced in response to wounding and ripening, while *LE-ACS3* is only induced by wounding in vegetative tissues but not in ripening fruit (Yip et al., 1992). Potato *ACS* genes, *ST-ACS1A* and *ST-ACS1B*, are transiently down regulated upon wounding, while *ST-ACS2* is upregulated after wounding in potato tuber (Destefano-Beltran et al., 1995). Wound-inducible genes (*CM-ACS1*, formerly called *WSACS2, CM-ACS3*, and *CM-ACS4*) are also found in winter squash; however, the pattern of induction is different (Watanabe et al., 2001). *CM-ACS1* and *CM-ACS3* transcripts increase progressively while *CM-ACS4* is induced within the first 3 h and decreases later (Watanabe et al., 2001). Soybean (*Glycine max* L.) *GM-ACS1* was shown to be a wound-specific transcript that is rapidly and transiently induced upon wounding (Liu et al., 1993). Similarly, a transient and rapid induction of mung bean ACS occurs within 10 min after mechanical strain (Botella et al., 1995). Tomato, squash, and zucchini wound-inducible ACS transcripts are also rapidly induced (within 20 min) upon wounding, but their levels remained stable for more than 8 h (Huang et al., 1991; Li et al., 1992; Nakajima et al., 1990; Olson et al., 1991). The difference in wound induction kinetics between fruit and soybean ACS transcripts suggests a higher turnover rate of the soybean gene product (Liu et al., 1993). It has been suggested that some or all the *ACS* genes are regulated by either a short-lived repressor protein (Liang et al., 1992) or by a rapid-turnover RNase (Franco et al., 1990) responsible for the degradation of some transcripts.

Although it has frequently been proposed that ACS activity is the sole regulatory step in ethylene biosynthesis (Theologis, 1992), ACO may also play an important role in the regulation of ethylene biosynthesis (Barry et al., 1996; Dunlap and Robacker, 1994; Lasserre et al., 1996). It has been suggested that *ACO* genes are all weakly and constitutively expressed and that some are specifically induced during particular developmental stages or in response to stimuli (Lasserre et al., 1996). ACO transcript levels increase greatly following mechanical wounding of tomato leaves, melon leaves and fruits, and

mung bean hypocotyls (Diallinas and Kanellis, 1994; Hamilton et al., 1990; Holdsworth et al., 1987; Kim and Yang, 1994; Lasserre et al., 1996; Smith et al., 1986). *ACO* is also encoded by a multigene family and is differentially regulated in response to wounding, development, and tissue specificity. In tomato, only ACO1 transcript accumulates in wounded leaves, and in a transient way. ACO1 transcript was rapidly induced within 30 min, peaked in abundance after 2 h, showing an 11-fold increase over the level in unwounded leaves, and then declined thereafter (Barry et al., 1996). ACO activity also increases almost fivefold in response to wounding (Barry et al., 1996). The increase in enzyme activity is due to de novo synthesis of protein, which in turn is the result of activation of transcription. In melon, only one member of the *ACO* gene family, (*CMe-ACO1*), is expressed in wounded leaves (Lasserre et al., 1996). This gene is rapidly induced after wounding and ethylene treatment in leaves. Studies on the promoter region suggest that this induction occurs via two different signal transduction pathways (Bouquin et al. 1997).

Ethylene can cause an increase in the expression of many defense-related genes and may play a central role in wound repair and defense responses (Ecker and Davis, 1987). Wounding of tomato fruit tissue provokes differential gene expression (Mehta et al., 1991) as reflected by differential changes in the abundance of specific proteins, translatable mRNAs, and specific gene transcripts. Few mRNAs appear to be markedly downregulated by ethylene in the wounded tissue (Henstrand and Handa, 1989). Parsons and Mattoo (1991) have shown that the expression of pT53, a cDNA clone from tomato fruit, is weakly repressed by wounding in the early red and red stage. Also, Lincoln et al. (1993) have reported that the expression of *LE-ACS2* is transiently repressed by wounding during the first 2 h and then increases after 4 to 6 h. In melon fruits, ascorbate oxidase (*AO*) expression was rapidly and dramatically repressed following wounding (Diallinas et al., 1997). These authors suggested that endogenous ethylene produced in response to wounding might be the cause of *AO* repression. On the contrary, wound ethylene fails to induce detectable levels of PG mRNA, a ripening-specific gene. This suggests the existence of at least two distinct signals that can affect the expression of plant defense-response genes (Ecker and Davis, 1987). Other messenger molecules that have been reported to transduce wound-related gene expression include systemin (Pearce et al., 1991), abscisic acid (Pena-Cortes et al., 1989), and jasmonic acid (JA) (Farmer et al., 1992; Farmer and Ryan, 1992). Diallinas and Kanellis (1994) have suggested that, in melon fruit, the regulation of defense gene expression is a coordinated process in response to both ethylene and an ethylene-independent wound signal. In this context, it has been shown that ethylene is an absolute requirement for the wound response in tomato (O'Donnell et al., 1996). These authors suggested a model in which ethylene and JA act together to regulate the gene expression of a wound-responsive gene (proteinase inhibitor proteins) by influencing each other's level in plant response to wounding. Moreover, it was suggested that at least one site of ethylene action in the wound response is the regulation of JA level in the plant. Jasmonate levels increase following wounding and the importance of JA to wound-responsive gene expression has been confirmed (Pena Cortes et al., 1993). The role of JA on wound signaling and its relation with other components has been recently reviewed (Leon et al., 2001).

B. Temperature Stress

Plant tissues exposed to changes in temperature show altered gene expression in response to either high or low temperature (Guy, 1990; Sachs and Ho, 1986). Upon exposure to

chilling stress, sensitive species show different physiological and biochemical responses (Lyons, 1973; Wang, 1982). The degree of dysfunction or modification is related to the duration and reduction of temperature as well as the tissue developmental stage and specificity. Chilling injury occurs in low temperatures, generally between zero and 12°C. This range of temperature is of special importance in the storage of different fruits and vegetables as it affects the ability to ripen and reduces their storage life. Chilling injury is a common physiological disorder observed when sensitive species like tomato, squash, cucumber, eggplant (*Solanum melongena* L.), okra [*Abelmoschus esculentus* (L.) Moench.], melon, and others are exposed to this temperature range. A common consequence of chilling stress is the stimulation of the production of both ethylene and carbon dioxide, especially upon transfer of stressed tissues to warmer temperatures (Wang, 1989). It has been suggested that, the level of stress ethylene production is a good indicator of chilling sensitivity (Morgan and Drew, 1997). However, it is not known whether this chilling-induced ethylene production is a consequence of the injury or whether it participates in the mechanism of adaptation to chilling stress. This ethylene production is generally related to an accumulation of ACC and an increase in ACS transcript and activity (Wang and Adams, 1982) after chilling. However, prolonged chilling resulted in marked reduction in ethylene production and ACO activity in various chilling-sensitive tissues (Chen and Patterson, 1985; Etani and Yoshida, 1987). This was thought to be related to membrane alteration, as in vivo ACO activity is known to depend on membrane integrity (John et al., 1985). Chilling-induced ethylene production was observed in cucumber fruit, and ACC level and activity increased rapidly when the fruit were transferred to warmer temperature following chilling. It was postulated that mRNA coding for ACS was stimulated by the chilling treatment but that translation occurred only when the tissue was transferred to warm temperatures (Wang and Adams, 1982). In tomato, a chilling-sensitive species, fruit ethylene production was inhibited at 4°C but increased as in untreated fruit upon transfer to 24°C (Watkins et al., 1990). Further studies showed that *ACO* expression was stimulated upon chilling and RNA accumulated at 4°C but levels declined rapidly upon transfer back to 24°C (Hobson and Grierson, 1993). This pattern is in contrast with other ripening related RNAs, which declined steadily to low levels at 4°C (Watkins et al., 1990). In mung bean hypocotyls, the impaired conversion of ACC to ethylene during chilling was due to both deterioration of membrane properties and decrease in the ACO activity (Corbineau et al., 1997). Therefore it seems that induction of ethylene biosynthetic enzymes ACS and ACO participates in the regulation of ethylene synthesis during chilling stress. Water deficit has also been implicated in the severity of the appearance of chilling injury symptoms. Generally, it is believed that chilling interacts with leaf water deficit to induce ACC synthesis (Morgan and Drew, 1997).

In some winter pears, a cold treatment is required for the accumulation of ACO and ACS transcripts and ACO protein that allow autocatalytic ethylene production upon rewarming (Lelièvre et al., 1997a). In other types of fruit that have no chilling requirement, like apple fruits, low temperatures hasten the induction of ethylene synthesizing competency and provoke homogeneous ripening (Knee et al., 1983; Jobling et al., 1991).

Transfer of plants to high temperature produces stress that depends on the temperature level, the duration of exposure, and the plant growing conditions (Lurie, 1998). Still, postharvest heat treatments are normally used to control diseases and to maintain fruit quality during storage (Couey, 1989; Klein and Lurie 1990; McDonald et al., 1999). Recently, the relation of heat treatment and fruit ripening has been reviewed (Paull and Chen, 2000).

Fruits exposed to heat stress ripen more slowly than control fruit (Lurie and Klein, 1991). This is of great importance for the postharvest life of fruits and vegetables. When plant tissues are exposed to high temperature, a stimulation of ethylene production is observed, up to a limit (Abeles et al., 1992). Above 35 to 36°C, inhibition of ethylene production was observed when plants were incubated at constant temperature (Field, 1981, 1985; Saltveit and Dilley, 1978). This inhibition occurs at the step converting ACC to ethylene, as ACC accumulation was detected in heat-treated tissues (Yu et al., 1980; Field, 1981, 1985) and a rapid loss of ACO was shown in different fruits (Klein and Lurie, 1990; Paull and Chen 2000). Ethylene inhibition is not only due to disruption of enzyme activities (Ogura et al., 1975) but also by inhibition of gene expression (Picton and Grierson, 1988). For example, a heat shock reduced the level of ACO transcripts as well as ACO activity in tomato (Lurie et al., 1996) and pea (Steed and Harrison, 1993).

In tomatoes, elevated temperatures also inhibit ethylene synthesis and cause an accumulation in ACC content (Biggs et al., 1988). However, if fruits were maintained at higher temperature, ACC accumulation was not observed. Therefore it seems that ACO is more sensitive to loss than ACS during exposure to heat stress (Atta Aly, 1992).

In addition, heat stress provokes flower abscission in pepper (Huberman et al., 1997), and a possible role for ethylene was proposed (Morgan, 1984; Osborne, 1989). However there is no clear relationship between heat-stress induced ethylene increase and abscission in pepper (Aloni et al., 1994) and bean (Gross, 1992). Temperature treatments most active in provoking flower or fruitlet abscission were not associated with an ethylene increase (Huberman et al., 1997).

Exposure to high temperatures causes a dramatic change in protein synthesis, with accumulation of heat-shock proteins at the expense of normal proteins (Matters and Scandalios, 1986). Heat-shock proteins may protect cells from deleterious effects of extreme temperatures by enhancing the range of thermotolerance (Vierling, 1991) as appearance of heat-shock proteins coincided with increased chilling resistance. It is important to consider that heat stress applied before chilling treatment might slow ethylene production, delaying ripening and preventing chilling injury. This is possible by a modulation of gene expression with an induction of heat-shock gene expression and an inhibition of ripening-related genes (Lurie et al., 1996).

C. Hypoxia and Flooding

Conditions of O_2 deficiency occur naturally during submergence of the roots or whole plant in water, a phenomenon known as flooding (Kennedy et al., 1992). Generally, plants respond to this stress with a decrease in photosynthesis as a result of the stomata closure, inhibition of stem and root growth, aerenchyma formation, hypocotyl swelling, leaf epinasty and senescence, development of adventitious roots, chlorosis, leaf abscission, and premature fruit drop (Grichko and Glick, 2001b, Jackson, 1985). Flooding induces the production of ethylene, which is implicated in the initiation of the above processes (Abeles et al., 1992). Tomato plants, a sensitive species, respond to flooding with reduction in stem growth, wilting, chlorosis, epinasty, abscission, adventitious root formation on stems at or above the water level (Jackson, 1997, Vartapetian and Jackson, 1997).

During flooding of tomato plants, there is an increase of ethylene concentration in the aerial parts. This ethylene is due to de novo synthesis of ACC in flooded roots, which is transported to shoots where it is oxidized to ethylene via the action of ACO (Bradford et al., 1980; Drew, 1997; Olson et al., 1995). The new production of ACC is ought to the

induction of ACS. Activity of ACS in tomato roots under low O_2 conditions reached a peak after 12 h, which was followed by an increase in ACC content. Low levels of ACS activity were also induced in the leaves of tomato plants when their roots were subjected to low O_2 conditions (Wang and Arteca, 1992). The isolation of the *ACS* genes was an important step toward elucidating the molecular mechanism of flooding- or hypoxia-induced ethylene synthesis. Olson et al. (1995) reported the induction of *LE-ACS3* and *LE-ACS2* genes in tomato roots after 1 and 10 h of flooding, respectively. The DNA sequence of the genomic clone revealed an anaerobic response element in the promoter region of the *LE-ACS3* gene that was identical to the reverse core consensus element from maize alcohol dehydrogenase (*adh1*) gene (Olive et al., 1991). Later, it was found that *LE-ACS7* an early flooding-induced *ACS* gene preceded the expression of *LE-ACS3* (Shiu et al., 1998). *LE-ACS7* is believed to be the primary gene in the root-to-shoot signaling in flooded plants (Shiu et al., 1998). Interestingly, although *LE-ACS3* is induced early, however, a large portion of its polyadenylated pre-mRNA remains unspliced during the low O_2 stress (Shiu et al., 1998).

While ACS generally plays the main role in regulating ethylene synthesis (Theologis, 1992; Yip et al., 1988), recent evidence indicates that ACO may also be involved in the upregulation of ethylene production in flooded tissues (English et al., 1995, He et al., 1996). ACC enhances expression of *ACO* genes in aerial parts of flooded tomato plants, stabilizes ACO mRNA and increases the activity of pre-existing ACO (English et al., 1995). Control of ACC levels and consequently ethylene production in transgenic tomato plants may ameliorate the adverse effects of flooding on plants. Transgenic tomato plants harboring a bacterial ACC deaminase gene under the control of root and hypoxia promoters showed some increased tolerance to flooding (Grichko and Glick, 2001a).

In addition to the induction of ethylene biosynthesis, it seems that flooding also induces an *NR*-like ethylene gene similar to ethylene receptors *ETR2*, *ERS1*, and *ERS2*, which are induced by ethylene in *Arabidopsis* (Shiu et al., 1998, Theologis, 1998).

During controlled atmosphere (CA) storage of fruits and vegetables, prevention and/or retardation of senescence and ripening through control of ethylene production is achieved by applying hypoxic conditions. During CA or modified atmosphere (MA) storage, there are no aerated tissues where the hypoxic conditions can be overcome and ethylene biosynthesis restored. Storage of detached horticultural crops under low O_2 greatly extends their commercial life. There is compelling experimental evidence indicating that the retarding effects of hypoxia also involve inhibition of ethylene biosynthesis and action, because the inclusion of relatively high levels of the gas in low O_2 treatments fails to substantially overcome the retarding effects of low O_2 on fruit ripening (Kanellis et al., 1989a, b).

Included in the effects of low O_2 on fruits and vegetables are reduction of respiration, delay in the onset of the climacteric rise in ethylene evolution in climacteric fruits, and decrease in the rates of fruit ripening and tissue senescence (Solomos and Kanellis, 1997). It appears that hypoxia induces a metabolic depression, thereby decreasing the demand for biological energy. That depression may not be ascribed to the inhibition of ethylene action alone but rather to the suppression of developmentally regulated genes that precede the induction of ethylene biosynthesis, and whose expression is necessary for the induction of *ACS* and *ACO*, hence ripening (Sachs and Ho, 1986; Solomos and Kanellis, 1997). In addition, since O_2 is a cosubstrate for ACO (Yip et al., 1988), it is suggested that the effect of low O_2 on ethylene production is due to the reduced ACO activity. Low oxygen levels associated with low temperature, delays the stimulation of ACS and ACO activities

and the accumulation of the corresponding transcripts (Gorny and Kader, 1996) thus delaying the autocatalytic process. Low O_2 also reduces the expression of genes involved in fruit ripening that are either ethylene-regulated or not, indicating that all low oxygen-induced effects are not due to the reduction in ethylene biosynthesis (Kanellis et al., 1989a,b,c; Solomos and Kanellis 1997). At high concentration, CO_2 is a competive inhibitor of ethylene action (Burg and Burg, 1967) that limits the induction of ACS and ACO activities (Bufler, 1984, 1986; Blanke, 1991) and transcripts (Gorny and Kader, 1996). The effects of CO_2 on ethylene biosynthesis and action are extensively reviewed by Mathooko (1996).

Recent studies in the physiology, biochemistry, and molecular biology of hypoxic and anoxic responses of higher plants have shown that, depending upon the species, the beneficial effects of low O_2 in maintaining the quality of fruits and vegetables may involve much more than control of ethylene biosynthesis and action (Aggelis et al., 1997; Kanellis, 1994; Kanellis et al., 1991, 1993; Mathooko, 1996; Solomos and Kanellis, 1997).

VIII. ETHYLENE INVOLVEMENT IN PATHOGENESIS AND DISEASE RESISTANCE

Increased ethylene synthesis has been shown to be associated with pathogen attack (Boller, 1995; Elad, 1990). The precise role that ethylene plays in the activation of gene expression during pathogen attack or elicitor treatment is not clear. The dominant view is that ethylene can be considered an indicator rather than an inducer of phytoalexin synthesis (Paradies et al., 1980) and some pathogenesis-related (PR) proteins (Mauch et al., 1984). Ethylene formation has been shown to correlate well with the synthesis of PAL and hence phytoalexin synthesis in host plants. However, Hughes and Dickerson (1989) demonstrated that, in leaves of French bean treated with fungal elicitors and ethylene inhibitors, ethylene was not the signal for the PR response but could amplify the tissue response by induction of PAL. Ethylene has been implicated in the induction of defense-related gene arrays (Ecker and Davis, 1987) and both acidic and basic-type PR proteins. In spite of supporting data, a causal role for ethylene has not been established (Boller, 1991).

The use of inhibitors of ethylene biosynthesis and action, complemented in recent years by ethylene-insensitive mutants and transgenic plants, has indicated that elicitors and pathogens utilize at least two different pathways for PR gene activation. One pathway that is ethylene-independent (Lawton et al, 1995) can be represented by the elicitor endoxylanase, an enzyme of fungal origin that can degrade β-1-4-xylan linkages in the plant cell wall and is a potent inducer of chitinase accumulation (Lotan and Fluhr, 1990). The other pathway is ethylene-dependent and is exemplified by ethylene itself (Ecker and Davis, 1987; Penmetsa and Cook, 1997). The ethylene action seems to involve a phosphorylation step that is necessary for signal transduction and the induction of PR gene expression and microlesion formation in tobacco leaves (Raz and Fluhr, 1993).

It has also been suggested that ethylene plays a role in disease resistance (Dixon and Lamb, 1990). However, when plants are exposed to gaseous ethylene, they do not show enhanced disease resistance but instead sometimes show increased susceptibility. Furthermore, certain ethylene-insensitive *Arabidopsis* mutants show reduced disease symptoms following inoculation with an avirulent bacterial pathogen (Bent et al., 1992). Some pathogens have coevolved to develop a mechanism to use the host plant's ripening hormone as a signal to differentiate and start the infection process. As a result, the ripe fruit are more readily infected than green, developing fruit. Pathogens that attach to the

fruit during the growing season may remain latent until the fruit ripen, when the pathogen penetrates and causes extensive damage (Flaishman and Kolattukudy, 1994; Kepczynska, 1989). Ethylene may also be involved in the strengthening of the cell wall and induction of enzymes that follow a pathogen or pest attack and are usually restricted to the immediate vicinity of the infection (Enyedi et al., 1992).

IX. CONTROL OF ETHYLENE BIOSYNTHESIS AND ACTION BY CHEMICAL MEANS

A number of chemicals capable of either generating ethylene or specifically inhibiting ethylene biosynthesis and action are available.

A. Ethylene-Releasing Compounds

A number of molecules capable of generating ethylene have been described. Most of them include a chloroethyl residue bound to phosphate, silicium, or sulfur (Beaudry and Kays, 1988). The first major commercial ethylene-releasing compound is (2-chloroethyl)phosphonic acid (ethrel). Beaudry and Kays (1988) have described practical uses in detail. They range from the stimulation of leaf abscission in cotton to induction of flowering in bromeliads and stimulation of ripening and/or color development in a variety of fruits.

B. Inhibitors of ACC Synthase

As already mentioned above, ACS is a pyridoxal phosphate enzyme. Two types of inhibitors have proved to be very effective in inhibiting its activity. The first type corresponds to analogs of L-vinylglycine, a competitive inhibitor of the enzyme (Satoh and Yang, 1989a). It includes rhizobitoxine and aminoethoxy vinylglycine (AVG). The second type comprises analogs of hydroxylamine that interact directly with pyridoxal phosphate like amino oxyacetic acid (AOA). Applications of AVG by infiltration in pears (Ness and Romani, 1980) or by spraying on apples (Bangerth, 1978) result in a slowing down of ethylene synthesis and in a delay of ripening. A commercial development program is currently being developed for the use of AVG in reducing preharvest fruit drop and loss of firmness in apples (Schaffer et al., 1996). AVG and AOA are also effective in increasing the vase life of cut flowers (Wang and Baker, 1980; Broun and Mayak, 1981).

C. Inhibitors of ACC Oxidase

ACO activity is strongly inhibited by Co^{2+} and Ni^{2+}. These ions are probably acting by competing with iron, an essential cofactor of ACO (Bouzayen et al., 1991). Co^{2+} have been shown to delay flower senescence (Chandra et al., 1980) and stimulate the growth of stems and leaves (Miller, 1951; Thimann, 1956). Free radical scavengers, like *n*-propyl gallate, acetyl salycilate, and Na benzoate (Apelbaum et al., 1981b; Baker et al., 1978; Leslie and Romani, 1986) also inhibit the conversion of ACC into ethylene. They may act by scavenging either putative free radical forms of ACC or iron. Uncouplers like DNP (dinitrophenol) and CCCP (carbonylcyanide *m*-chloro-phenylhydrazone) also inhibit ACO (Yu et al., 1980; Apelbaum et al., 1981a). Finally, α-aminoisobutyric, a competive inhibitor of ACO is capable of inhibiting ethylene synthesis in plant tissues (Satoh and Esashi, 1982).

D. Inhibitors of Ethylene Action

Several molecules are known to interfere with ethylene perception. It has long been observed that CO_2 is a competitor of ethylene action (Burg and Burg, 1967). But CO_2 is also a cofactor of ACO. Its mode of action is therefore complex, but CO_2 is largely being used in controlled and modified atmosphere storage for extending the storage life of many fruits and vegetables (Kader et al., 1994). One of the most powerful inhibitors of ethylene action is Ag^+ (Beyer, 1976). When applied as a complex with thiosulphate, it rapidly migrates into plant tissues and can prevent senescence and abscission (Reid, 1980; Veen, 1983). It is being used commercially for extending the vase life of cut flowers. However, being a heavy metal, its use may be banned because of the risks of toxicity to the environment. Cyclic olefines like norbornadiene and diazocyclopentadiene (DACP) are strong inhibitors of ethylene action that are capable to inhibit senescence and fruit ripening (Sisler and Yang, 1984; Sisler and Blankenship, 1993). However, the most powerful inhibitor of ethylene action is 1-methyl-cyclopropene (1-MCP). It seems to irreversibly bind to the ethylene receptor (Serek et al., 1995; Sisler et al., 1996) and is active at very low levels (few ppbs). This compound, applied as a gas, has potential commercial applications for preventing the senescence of cut or potted flowers. A number of experiments have been carried for controlling fruit ripening (Abdi et al., 1998; Fan et al., 2000; Feng et al., 2000) and for reducing scald on apples (Fan et al., 1999; Watkins et al., 2000), but commercial applications are still awaited. The latest developments of inhibitors of ethylene responses in plants at the receptor level have been reviewed by Sisler and Serek (1997).

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