

ETHYLENE: A Gaseous Signal Molecule in Plants

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■ **Abstract** Ethylene regulates a multitude of plant processes, ranging from seed germination to organ senescence. Of particular economic importance is the role of ethylene as an inducer of fruit ripening. Ethylene is synthesized from *S*-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC). The enzymes catalyzing the two reactions in this pathway are ACC synthase and ACC oxidase. Environmental and endogenous signals regulate ethylene biosynthesis primarily through differential expression of ACC synthase genes. Components of the ethylene signal transduction pathway have been identified by characterization of ethylene-response mutants in *Arabidopsis thaliana*. One class of mutations, exemplified by *etr1*, led to the identification of the ethylene receptors, which turned out to be related to bacterial two-component signaling systems. Mutations that eliminate ethylene binding to the receptor yield a dominant, ethylene-insensitive phenotype. *CTR1* encodes a Raf-like Ser/Thr protein kinase that acts downstream from the ethylene receptor and may be part of a MAP kinase cascade. Mutants in *CTR1* exhibit a constitutive ethylene-response phenotype. Both the ethylene receptors and *CTR1* are negative regulators of ethylene responses. *EIN2* and *EIN3* are epistatic to *CTR1*, and mutations in either gene lead to ethylene insensitivity. Whereas the function of *EIN2* in ethylene transduction is not known, *EIN3* is a putative transcription factor involved in regulating expression of ethylene-responsive genes. Biotechnological modifications of ethylene synthesis and of sensitivity to ethylene are promising methods to prevent spoilage of agricultural products such as fruits, whose ripening is induced by ethylene.

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INTRODUCTION

Ethylene, the simplest unsaturated hydrocarbon, regulates many diverse metabolic and developmental processes in plants. The history of its discovery as a signal molecule or plant hormone was described by Abeles et al (1992). In the nineteenth century, several articles appeared reporting that leaks in illuminating gas caused premature senescence and defoliation of plants in greenhouses and of trees near gas lines. The Russian plant physiologist Neljubov (1879–1926) (Abeles et al 1992) observed that etiolated pea seedlings grew horizontally in his laboratory but upright in outside air and showed that the abnormal growth habit was caused by contaminating illuminating gas. In 1901, he proved that the active principle in illuminating gas was ethylene. Thus Neljubov is credited with the discovery that ethylene is a biologically active gas. Chemical proof that plants produce ethylene was provided by Gane (1934), who analyzed the gases released by 60 lbs of ripening apples. Thus the stage was set to investigate the function of ethylene as an endogenous signal molecule in plants. Much of the early work on ethylene action was performed at the Boyce Thompson Institute, then located in Yonkers, New York (Crocker 1932). Mainly, the group at this institute provided a bioassay for ethylene. This test was based on the so-called triple response of etiolated pea seedlings, which consists of agravitropic (horizontal) growth, inhibition of stem elongation, and thickening of the stem. The triple response proved to be quite insensitive for measuring physiological levels of ethylene, but a modification of it has served well for the identification of *Arabidopsis* mutants with altered ethylene biosynthesis or sensitivity (Bleecker et al 1988, Guzmán & Ecker 1990). Modern research on ethylene production by plant tissues and on the biological activity of ethylene began with the introduction of gas chromatography (Burg & Stolwijk 1959). This new analytical technique permitted accurate and rapid determination of trace amounts of ethylene.

PHYSIOLOGY OF ETHYLENE ACTION

The range of physiological responses regulated by ethylene is surprisingly wide and has been described by Abeles et al (1992) and Mattoo & Suttle (1991). The following is a synopsis of ethylene-controlled plant processes. In the broadest of terms, ethylene triggers senescence of plant organs, it influences plant growth, it acts as a stress hormone during biotic and abiotic stress conditions, and it exhibits

various morphogenetic effects. Among the senescence processes, ethylene-induced ripening of so-called climacteric fruits has been investigated most intensively because of its great agronomic importance. Climacteric fruits, which include many of our common fruits such as apples, bananas, cantaloupe, and tomatoes, are characterized by a steep increase in ethylene synthesis at the mature green stage and by a concomitant rise in respiration. Fruit ripening is a sequence of biochemical events resulting in loss of chlorophyll, formation of pigments, flavors, and aromas, softening of the flesh, and eventual abscission of the fruit. Whereas in nature these processes ensure dispersal of seeds by animals that eat the fruits, in agriculture the rate at which ripening occurs must be controlled to prevent spoilage of fruits on the way to the consumer. Before the advent of biotechnology, this was accomplished, for example, by removal of ethylene in hypobaric storage compartments. Today, fruit ripening can be controlled by manipulating ethylene synthesis or sensitivity using molecular techniques (see BIOTECHNOLOGICAL APPLICATIONS). Other senescence processes regulated by ethylene are fading of flowers and abscission of leaves and petals. Fading of flowers shares many similarities with ripening of climacteric fruits. It too can now be delayed by reducing synthesis or sensitivity to ethylene with biotechnological methods.

A number of vegetative growth processes are regulated by ethylene, most notably induction of asymmetric stem and petiole growth. One such response, promotion of agravitropic growth, has already been mentioned in the context of the triple response. Another asymmetric growth response to ethylene is the formation of the apical hook, which protects the apex and the young leaves of dicotyledonous plants as they push through the soil. Whereas stem elongation of terrestrial plants is, in general, inhibited by ethylene, growth of semiaquatic plants, such as rice, is strongly promoted.

Almost all biotic and abiotic stress conditions elicit ethylene synthesis in plants. In some instances, ethylene appears to be involved in mediating the response to stress. In other instances, the physiological function of stress ethylene is not understood. The role of ethylene in pathogen-infected plants has been reviewed and further investigated by Hoffman et al (1999), who studied resistance to various pathogens in ethylene-insensitive mutants of soybean. Their results illustrate the complexity of the phenomenon. Depending on the pathogen, disease symptoms seem to be either reduced or enhanced by ethylene, or are not affected. In other words, ethylene appears to mediate defense responses to some pathogens and to suppress them to others. It has been suggested that ethylene helps to restrict the spread of a pathogen by causing leaf abscission in cases where it exacerbates disease symptoms.

Lastly, ethylene exhibits various morphogenetic effects in plant development, two of which are mentioned here. In a number of plants, ethylene promotes adventitious root formation, i.e. development of roots from tissues other than preexisting roots, and may also mediate the effect of rooting factors such as auxin. In the family of the bromeliads, ethylene induces floral development, an activity that

has been exploited to synchronize flowering in pineapple plantations. For such agricultural applications, plants are sprayed with an ethylene-releasing compound, e.g. 2-chloroethanephosphonic acid.

The adaptive value of a gaseous signal molecule for the regulation of developmental processes in plants is documented with three examples. (a) Ripening usually starts in one region of a fruit and spreads to the other regions that lag behind. There are no transport pathways along which a ripening factor could be propagated. Ethylene diffuses freely from cell to cell across membranes and integrates the ripening process throughout the fruit. This coordinating function of ethylene is enhanced by its capacity to stimulate its own synthesis via a positive feedback loop (Kende 1993). (b) In some flowers, pollination induces ethylene synthesis in the stigma and the style (O'Neill 1997). Ethylene, in turn, triggers senescence of the perianth, which has served its purpose once pollination has been achieved. At least some of the ethylene emanating from the stigma and style may diffuse through the air to the perianth to initiate fading of the flower. (c) Semi-aquatic plants have developed an interesting adaptive response to ethylene (Kende et al 1998). When such plants become submerged in rising floodwaters, ethylene accumulates in their submerged stems for two reasons. Ethylene synthesis is promoted at the low oxygen tensions prevailing in submerged tissues, and it becomes physically trapped because its diffusion through water is 10,000 times slower than through air. Ethylene promotes rapid elongation of submerged semiaquatic plants, permitting them to keep part of their foliage above the water.

ETHYLENE BIOSYNTHESIS

Ethylene is formed from methionine via *S*-adenosyl-L-methionine (AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams & Yang 1979). The enzymes catalyzing the conversion of AdoMet to ACC and of ACC to ethylene are ACC synthase and ACC oxidase, respectively (Figure 1) (Kende 1993). ACC synthase produces, besides ACC, 5'-methylthioadenosine, which is utilized for the synthesis of new methionine via a modified methionine cycle (Figure 1) (Miyazaki & Yang 1987). This salvage pathway preserves the methylthio group through every revolution of the cycle at the cost of one molecule of ATP. Thus high rates of ethylene biosynthesis can be maintained even when the pool of free methionine is small.

ACC synthase was first identified in homogenates of ripening tomatoes and shown to require pyridoxal phosphate as cofactor (Boller et al 1979, Yu et al 1979). Its activity was found to limit ethylene synthesis and to be enhanced by factors that promote ethylene formation, e.g. by the plant hormone auxin and by different stress conditions (Yang & Hoffman 1984). Purification of the enzyme proved to be a formidable task because of its lability and low abundance (Bleecker et al 1986). The active site of ACC synthase was identified by reducing the double bond between pyridoxal phosphate and the enzyme using NaB^3H_4 and

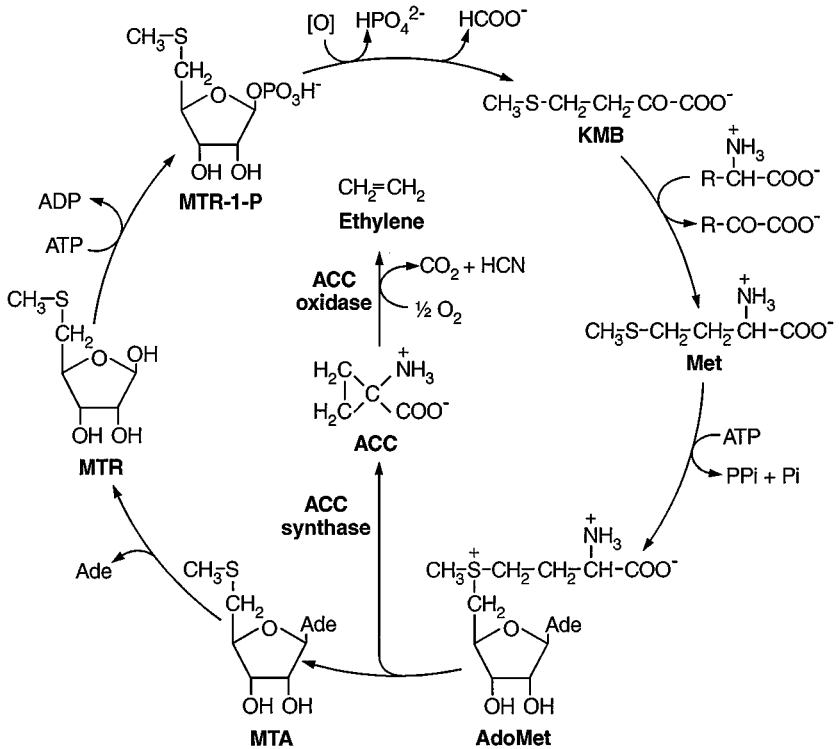


Figure 1 The ethylene biosynthetic pathway and the methionine cycle. ACC, 1-aminocyclopropane-1-carboxylic acid; Ade, adenine; AdoMet, *S*-adenosyl-L-methionine; KMB, 2-keto-4-methylthiobutyric acid; Met, L-methionine; MTA, 5'-methylthioadenosine; MTR, 5'-methylthioribose; MTR-1-P, 5'-methylthioribose-1-phosphate. Modified from Miyazaki & Yang (1987).

by labeling it with [^{14}C]AdoMet (Yip et al 1990). Both approaches led to the conclusion that the cofactor and the substrate bind to the ϵ -amino group of the same lysine moiety and that the amino acid sequence of the active site is similar to that of other pyridoxal phosphate-requiring enzymes such as aminotransferases. The amino acid sequence deduced from ACC synthase cDNAs showed that 11 of 12 amino acid residues conserved in aminotransferases are also present in all ACC synthases (Zarembinski & Theologis 1994). When two different, catalytically inactive mutant forms of ACC synthase were either mixed or expressed together in *Escherichia coli*, the enzyme activity was partially restored, indicating that ACC synthase functions as a dimer (Li et al 1997, Tarun & Theologis 1998). These results, as well as the recently obtained crystal structure of ACC synthase (Capitani et al 1999), established the structural similarity between ACC synthase and aminotransferases.

In all plants investigated thus far, ACC synthase is encoded by medium-size gene families, and expression of ACC synthase genes is differentially regulated by various developmental, environmental, and hormonal signals (Kende 1993, Zarembinski & Theologis 1994). Probably because of the redundancy of genes encoding ACC synthase and also ACC oxidase, genetic studies have yielded relatively few insights into ethylene synthesis. Screens for ethylene-related mutants in *Arabidopsis* yielded plants with a constitutive triple response (Guzmán & Ecker 1990). Some of these were found to overproduce ethylene. Three ethylene overproducer—*eto1*, *eto2*, and *eto3*—have elevated ACC synthase activities (Vogel et al 1998, Woeste et al 1999). In *eto2*, increased ethylene biosynthesis is based on a single nucleotide insertion leading to a change in the 12 terminal amino acids of the ACC synthase isoform ACS5 (Vogel et al 1998).

ACC oxidase could not be isolated by conventional biochemical methods but was identified by functional expression of a ripening-related cDNA in yeast (Hamilton et al 1991). Because the deduced amino acid sequence of the protein encoded by this cDNA exhibited similarity to flavanone 3-hydroxylase, Ververidis & John (1991) extracted melon fruits under conditions that preserved the activity of this dioxygenase and found that full ACC oxidase activity could be recovered when Fe^{2+} and ascorbate were added to the assay mixture. Interestingly, the activity of ACC oxidase is completely dependent on the presence of CO_2 (Dong et al 1992), but the mechanistic role of CO_2 has not been explained. ACC oxidase is encoded by small gene families, and there is some evidence for differential regulation of ACC oxidase gene expression (Prescott & John 1996). Whereas ACC synthase activity has been recognized early on as the limiting and regulated enzyme in ethylene biosynthesis, it has been found only more recently that ACC oxidase transcript levels and enzyme activities also increase in some plant tissues that are induced to form ethylene (Kende 1993).

ETHYLENE PERCEPTION AND SIGNAL TRANSDUCTION

The utility of ethylene as a signal molecule depends on the ability of cells to monitor the changing concentrations of ethylene and to transduce this information into physiological responses appropriate to the cell type. Until recently, our understanding of ethylene signaling was limited to basic phenomenology. Physiological studies revealed that ethylene was effective for many biological responses at nanomolar concentrations (Abeles et al 1992), indicating a requirement for high-affinity receptors. Dose-response curves were generally consistent with Michaelis-Menten kinetics (Chen & Bleecker 1995). Lag times for ethylene responses ranged from 10 to 15 min (inhibition of seedling growth) to hours (enhancement of enzyme activities), and even to days (promotion of leaf senescence). Analysis of mRNA accumulation in tomato fruits indicated a 30-min lag from the time of ethylene application to the time of measurable increases in mRNA abundance (reviewed in Abeles et al 1992).

Differential gene expression has been implicated in many ethylene-response systems (Deikman 1997). Genes that code for proteins associated with cell-wall softening during fruit ripening, for hydrolytic enzymes that dissolve the cell wall during abscission (organ shedding), for proteins during wound-induced defense to pathogens, and for proteinases during leaf senescence all contain promoter elements that are subject to regulation by ethylene (Bleecker & Patterson 1997, Deikman 1997). The challenge of current research efforts is to elucidate the connection between these processes at the end of the response pathway and the primary biochemical events that provide the mechanism for perception and early transduction of the ethylene signal.

A particularly intriguing question has centered on how a proteinaceous receptor, which generally relies on a complex pattern of contact points between the binding site and the ligand to achieve a high-affinity interaction, could perceive a gaseous signal consisting of two hydrogenated carbons that share a double bond. In the 1960s it was suggested that a transition metal cofactor could provide the necessary chemistry for high-affinity interaction based on the known ability of olefins to form stable complexes with transition metals (Burg & Burg 1967). The test of this interesting hypothesis had to await the biochemical characterization of the ethylene receptor. The discovery of saturable binding sites for ethylene in plant tissues by two groups in the late 1970s (Jerie et al 1979, Sisler 1979) provided a basis for pursuing this goal. However, difficulties in the purification of these binding sites (Sisler 1991) and questions about their physiological relevance hampered the direct biochemical approach to the problem of ethylene sensing.

It is only in the last decade and only through the development of molecular genetic approaches in the model plant *Arabidopsis* that many of the biochemical components of ethylene perception and signal transduction are revealing their secrets. These studies have provided strong evidence that ethylene signaling is mediated by a family of copper-containing receptors that signal through a pathway that likely includes a MAP kinase cascade, a metal transporter intermediate, and a transcriptional cascade.

Genetics of Ethylene Signal Transduction

The genetic approach to identify the biochemical components involved in ethylene signal transduction is deceptively simple and illustrates the power of the method. Ethylene inhibits the elongation growth of dark-grown seedlings, induces swelling of the stem, and mediates tight closure of the apical hook (triple response). Ethylene-insensitive mutants were readily identified by screening mutagenized populations of *Arabidopsis* seedlings growing in the dark and in the presence of applied ethylene (Bleecker et al 1988). Reciprocal screens yielded mutants at the *CTR1* locus that showed a constitutive ethylene-response phenotype in the absence of ethylene (Kieber et al 1993).

Genetic analysis of these mutants provided a framework on which to construct a linear model for the ethylene signal transduction pathway, as indicated in Figure 2

(see color insert). Genetically dominant mutations in *ETR1* rendered plants insensitive to applied ethylene (Bleecker et al 1988). Subsequent studies showed that the mutated *ETR1* gene, along with *ETR2*, *ERS1*, *ERS2*, and *EIN4* (Hua et al 1995, 1998; Sakai et al 1998), codes for members of the ethylene-receptor gene family (see below). Loss-of-function mutations in the *CTR1* gene lead to a constitutive ethylene-response phenotype, indicating that the *CTR* gene product acts as a negative regulator of response pathways (Kieber et al 1993). Mutations at *CTR1* are epistatic to the dominant mutations in *ETR* family members because double mutants show the constitutive response phenotype (Kieber et al 1993, Hua et al 1998, Sakai et al 1998).

With the exception of *ERS1*, loss-of-function mutations have been isolated for all of the *ETR* family members (Hua & Meyerowitz 1998). Single-gene mutants do not show an ethylene-response phenotype, which leads to the conclusion that there is functional redundancy between family members. On the other hand, when loss-of-function mutations in three or more *ETR* family members were recombined in a single plant, a constitutive response phenotype was observed. Taken together, these results lead to the conclusion that the *ETR* gene products work in concert with *CTR1* to negatively regulate ethylene-response pathways. According to this model, ethylene would repress the activity of *ETR/CTR1* gene products and derepress response pathways. This genetic model is illustrated in Figure 2.

Recessive mutations in several additional loci result in complete or partial insensitivity to ethylene in Arabidopsis. Of particular significance are the *EIN2* and *EIN3* loci (Guzmán & Ecker 1990, Roman et al 1995). Loss-of-function mutations in *EIN2* render plants completely insensitive to ethylene. Mutations in *EIN2* are epistatic to mutations in *CTR1* because the double mutant shows no response phenotype. Mutations at the *EIN3* locus exhibit reduced sensitivity to ethylene, but in no case complete insensitivity. This lack of complete insensitivity is very likely due to the fact that *EIN3* represents a small family of genes with some functional redundancy. Overexpression of *EIN3* or the related *EIL1* or *EIL2* results in plants displaying a constitutive ethylene-response phenotype (Chao et al 1997). This result was obtained even in *ein2* mutant backgrounds, hence the placement of the *EIN3* family downstream of *EIN2* in the proposed signal transduction pathway.

Biochemistry of Ethylene Signal Transduction

The purely genetic analysis of ethylene signaling described above is rapidly being converted into a more complete biochemical understanding of many components of the transduction system. This conversion has been driven by the increasing facility with which Arabidopsis genes can be cloned based on mutant phenotype. The derived amino acid sequences of genes involved in ethylene signaling have, in many cases, provided clues to biochemical function through homology to signaling components in other biological systems. Functional analysis has been aided by the development of equally facile methods for the genetic transfer of the genes of interest back into plants and into heterologous systems, such as yeast and insect

cells. The biochemical nature of components in the ethylene signal transduction pathway are depicted in Figure 2.

The *ETR1* gene was the first member of the receptor family of genes to be cloned from *Arabidopsis* (Chang et al 1993). The derived amino acid sequence indicated that ETR1 was related to the superfamily of catalytic receptors in bacteria referred to as two-component regulators (Wurgler-Murphy & Saito 1997). Two-component regulators are typically composed of a sensor protein with an input domain that receives signals and a catalytic transmitter domain that autophosphorylates on an internal histidine residue. The second component, a response regulator protein, is composed of a receiver domain that receives phosphate from the transmitter on an aspartate residue and an output domain that mediates responses depending on the phosphorylation state of the receiver. The ETR1 protein has a receiver domain fused to the C terminus of the histidine kinase domain. This modular arrangement is found in a number of bacterial sensors and in the osmosensing Sln1 protein from yeast (Maeda et al 1994). The transmitter domain of ETR1 contains all the conserved residues essential for histidine kinase activity in the homologous bacterial transmitter domains and is capable of autophosphorylating on the conserved histidine when expressed in yeast (Gamble et al 1998). In some bacterial systems, the fused receiver domain may function as a competing substrate for phosphotransfer or as a relay station in a series of phosphotransfer steps (Wurgler-Murphy & Saito 1997). Recent crystallographic data for the ETR1 receiver domain indicate a structure similar to that of bacterial receiver domains that are capable of forming homodimers (Muller-Dieckmann et al 1999).

The sensor component of many two-component regulators contains an input domain that interacts directly with a signaling ligand. Whereas the N-terminal hydrophobic domain of the ETR1 protein showed no homology to any functional domain in the protein databases, the importance of this domain for ethylene signaling was suggested by the fact that all point mutations conferring dominant insensitivity to ethylene in plants were located in this domain (Chang et al 1993). Compelling evidence that the hydrophobic domain of ETR1 was capable of directly sensing ethylene was obtained when expression of the ETR1 gene in yeast resulted in the generation of high-affinity binding sites for ethylene (Schaller & Bleecker 1995). The observed binding activity showed many of the characteristics of ethylene-binding sites that had been measured in plant tissues since the late 1970s (Sisler 1991), including a K_D for binding in the nanomolar range, a half-time release of several hours, and inhibition of binding by a variety of competitive inhibitors of ethylene responses in plant tissues. Further experiments demonstrated that the first 128 amino acids of ETR1, representing the hydrophobic region of the protein, were necessary and sufficient for ethylene-binding activity (Rodriguez et al 1999). Biochemical work on ETR1 expressed in yeast indicated that the protein formed membrane-associated, disulfide-linked homodimers (Schaller et al 1995).

The original hypothesis of Burg & Burg (1967), namely that ethylene binding is mediated by a transition metal cofactor, was confirmed when it became evident that

addition of copper ions was required for the recovery of ETR1 ethylene-binding activity in yeast extracts. Subsequently, it was shown that copper copurified in stoichiometric amounts with the ethylene-binding domain extracted from membranes of yeast overexpressing the ETR1 binding domain (Rodriguez et al 1999). Both ethylene-binding activity and copurification of copper were eliminated when the *etr1-1* mutation, a conversion of Cys65 to a Tyr, was introduced into the protein. Of several transition metals tested, only silver ions mimicked the effect of copper. This is consistent with the close chemical similarities of these two ions and also provides a possible explanation for the inhibitory effect of silver ions on ethylene responses *in vivo* (Abeles et al 1992). Silver appears to be capable of replacing copper and interacting with ethylene, but not in transducing the signal to downstream effectors.

Further evidence for a role for copper in ethylene signaling was recently obtained with the cloning of the *RANI* gene (Hirayama et al 1999). Weak alleles of *RANI* showed an altered response to an ethylene antagonist, *trans*-cyclooctene. More importantly, antisense suppression of the *RANI* gene led to a constitutive ethylene-response phenotype, consistent with a loss of receptor function (see below). *RANI* is homologous to, and can complement mutations in, the *CCC2* gene from yeast. The *CCC2* protein is a copper transporter located in the post-Golgi vesicles of the yeast cell, where it is required for delivery of copper cofactor to yeast membrane proteins. This finding implies that the copper transport system characterized in yeast is conserved in plants and is responsible for the biogenesis of the ethylene holoreceptors.

A model for the structure of the ethylene-binding domain has been suggested according to which an electron-rich hydrophobic pocket formed by membrane-spanning helices coordinates a copper (I) cofactor that interacts directly with ethylene (Bleecker 1999). Binding of ethylene is presumed to alter the coordination chemistry of the copper. This initiates a conformational change in the binding site that is propagated to the transmitter domains of the ETR1 dimer pair.

In addition to *ETR1*, four related genes have been identified in Arabidopsis (Hua et al 1995, 1998; Sakai et al 1998). The five members of the ETR family are related because of common structural elements in the protein and because specific amino acid substitutions in the ethylene-binding domain of any family member confer dominant insensitivity to ethylene throughout the plant. The receptor family can be divided into two subfamilies based on structural similarities. The ETR1-like subfamily, consisting of ETR1 and ERS1, is characterized by three hydrophobic subdomains at the N terminus and a conserved histidine kinase domain. The ETR2-like subfamily, which includes ETR2, EIN4, and ERS2, have an additional hydrophobic extension at the N terminus and possess degenerate histidine kinase domains that lack one or more elements considered necessary for catalytic activity. In Arabidopsis, one member of each subfamily, ERS1 and ERS2, lacks the C-terminal receiver domain that is characteristic of the other members.

ETR1-like genes have also been reported from other plant species, the best characterized being those of the tomato family where both subfamilies discussed

above are represented (Lashbrook et al 1998, Tieman & Klee 1999). The first of these tomato homologs to be discovered was the *never-ripe* (*NR*) gene, so-named because fruits of the *Nr* mutants do not ripen. The *Nr* mutants also show other symptoms of ethylene insensitivity (Lanahan et al 1994, Wilkinson et al 1995). *NR* encodes an ERS homolog, and the *Nr* mutant contains a single amino acid change in the sensor domain, similar to those found in the mutant Arabidopsis genes (Wilkinson et al 1995).

The cloning of the *CTR1* gene was facilitated by the isolation of a mutant generated by insertional mutagenesis (Kieber et al 1993). Sequence analysis of *CTR1* showed that the gene encodes a protein related to the mammalian Raf kinases that initiate MAP kinase cascades. The kinase domain of *CTR1* contains all the conserved subdomains considered necessary for kinase activity (Kieber et al 1993). The homology of *CTR1* to known MAPKKs implies that ethylene signaling may operate through a MAP-kinase cascade. Whereas there are genes with homology to MAPKKs and MAPKs in Arabidopsis, none to date has been associated with ethylene signaling.

The recruitment of a presumptive MAP kinase cascade in ethylene signaling represents the coupling of a primarily prokaryotic signaling system with an exclusively eukaryotic signal processing system. An interesting parallel is the two component-based osmosensing system in yeast where a histidine-kinase-initiated phosphorelay regulates a MAP kinase cascade through the interaction of a response regulator with a Raf-like kinase (Posas et al 1996). However, no intermediate components have been identified genetically or biochemically between the ethylene receptors and the *CTR1* kinase. Rather, biochemical and yeast two-hybrid studies indicate that *CTR1* interacts directly with the transmitter domains of the ethylene receptors (Clark et al 1998). This raises the possibility that signaling between receptors and *CTR1* might not directly involve phosphotransfer. The observed differences in component interactions point to an independent origin for the coupling of the two-component sensor and MAP-kinase cascade signaling systems for osmosensing in yeast and ethylene sensing in plants.

The observations that loss-of-function mutations in *CTR1* and/or multiple receptor genes lead to constitutive activation of ethylene-response pathways are consistent with a model according to which receptors form a complex with *CTR1* that negatively regulates responses in the absence of ethylene. It follows that ethylene binding to receptors would downregulate the activity of the ETR/*CTR1* complex and result in derepression of response pathways. This model also provides an explanation for the dominant ethylene insensitivity caused by specific point mutations in any one receptor gene. Dominance could be due to a gain-of-function mechanism in which mutant receptors fail to turn off in response to ethylene. In support of this possibility, ethylene binding experiments with ETR1 mutant proteins showed that most mutations causing dominant insensitivity to ethylene in planta also eliminate ethylene-binding activity in the yeast-expressed protein (Hall et al 1999). Thus, a gain-of-function mechanism for dominance is supported by both genetic and biochemical data. The idea that ethylene acts as an inverse

agonist in triggering response pathways is unusual but not without precedent. Some bacterial two-component sensor proteins exhibit inhibition of kinase activity in response to ligand binding (Parkinson 1993).

An implication of the presented model is that all receptor isoforms signal through a single CTR1 protein, indicating that each isoform might contribute quantitatively but not qualitatively to the output signal from the primary response pathway. A second implication is that all members of the family participate to some degree in the examined range of ethylene responses throughout the plant because the dominant mutations in any one member, driven by its own promoter, causes overall ethylene insensitivity. However, analysis of mRNA levels for individual receptor isoforms indicates that individual receptor family members are expressed at different levels in different tissues (Hua et al 1998, Lashbrook et al 1998, Tieman & Klee 1999). It is conceivable that the ratios of receptor family members in a particular cell type influence the dose-response relationships, which can vary considerably for different tissues and responses (Chen & Bleecker 1995).

The ethylene signal transduction pathway downstream of CTR1 presents something of an enigma. Whereas MAP kinase cascades often regulate transcription directly by phosphorylating transcription factors, the product of the *EIN2* gene, which is related to the eukaryotic Nramp family of 12-membrane-pass, metal-ion-transporters (Alonso et al 1999), is required for ethylene signaling and acts genetically between *CTR1* and the *EIN3* family of transcriptional regulators (Chao et al 1997). Whether the *EIN2* gene product acts indirectly in ethylene signaling by affecting metal homeostasis, as other Nramp family members are thought to do in some animal systems, or whether the *EIN2* protein is a family member that has been recruited to function directly in ethylene signaling, e.g. by regulating a second messenger, remains an open and intriguing question.

Evidence for a novel function for *EIN2* was obtained when the large cytoplasmic C-terminal domain, which shows no homology to Nramp proteins, was overexpressed in *Arabidopsis* (Alonso et al 1999). Overexpression in an *EIN2* null background resulted in constitutive activation of some but not all ethylene-response pathways. Curiously, the seedling triple response was not activated. Although this result implies that the novel C-terminal domain of *EIN2* acts in the transmission of the ethylene signal to some downstream effectors, the mechanism by which *EIN2* receives signal from the receptor/CTR1 complex remains unknown.

It has long been recognized that many ethylene responses involve changes in gene expression. The cloning of *EIN3* provided the first direct evidence for nuclear regulation in the early signal transduction pathway. Loss-of-function mutation in *EIN3* result in reduced responsiveness to ethylene. The *EIN3* gene was shown to encode a nuclear protein (Chao et al 1997). Whereas overexpression of *EIN3* and the related genes *EIL1* and *EIL2* caused constitutive activation of response pathways, message levels for these genes did not change in response to ethylene. This leads to the conclusion that their biochemical activity or protein stability is regulated by upstream components of the signaling pathway.

A search for target promoters for the EIN3 family of proteins led to the identification of the *ERF1* gene (Solano et al 1998). *ERF1* is a member of a large family of plant-specific transcription factors referred to as ethylene-response-element-binding-proteins (EREBPs). EREBPs were originally identified as *trans*-acting DNA binding proteins that bound to specific promoter elements in ethylene-inducible genes (Ohme-Takagi & Shinshi 1995). Expression of the *ERF1* gene is rapidly induced by ethylene treatment in *Arabidopsis* (Solano et al 1998). More importantly, EIN3 homodimers were shown to interact *in vitro* with a promoter element in the *ERF1* gene. When the *ERF1* gene is constitutively expressed in an EIN3 mutant background, a subset of ethylene responses is activated. These results place ERF1 in the primary signal transduction chain downstream of the previously identified components and clearly indicate that a transcriptional cascade is operating in ethylene signaling (Figure 2, see color insert). Given that ERF1 is also a transcription factor, production of effector proteins that actually mediate different biochemical responses must be at least one step downstream from ERF.

BIOTECHNOLOGICAL APPLICATIONS

Controlling the onset and the rate of fruit ripening and flower fading have been major goals of postharvest physiologists. The ability to manipulate the time of fruit ripening would reduce spoilage that results in major agricultural losses, and a delay in flower senescence would extend the vase life of flowers. Two strategies of genetic engineering have been employed to reach this aim, namely inhibiting ethylene synthesis and reducing sensitivity to ethylene. Hamilton et al (1990) were the first to reduce ethylene formation in tomatoes by expressing an antisense construct of a cDNA encoding ACC oxidase. Ripening of the transgenic fruits was retarded, and their storage life was extended. A similar approach was followed by Oeller et al (1991), who transformed tomato plants with the antisense construct of a cDNA coding for ACC synthase. Ethylene production in transgenic fruits was inhibited by 99.5%, and ripening was suppressed. Applied ethylene restored normal ripening. Ethylene synthesis and ripening could also be inhibited by removing ACC from the precursor pool. This was achieved by expressing a bacterial ACC deaminase gene in tomatoes (Klee et al 1991). As described above, the dominant *etr1* mutation confers ethylene insensitivity upon *Arabidopsis* plants. When expressed in tomato and petunia, *etr1* causes significant delays in fruit ripening, flower fading, and flower abscission (Wilkinson et al 1997).

OPEN QUESTIONS AND FUTURE PROSPECTS

In most instances, ethylene-regulated processes are controlled by environmental and endogenous factors via induction and, in some rare cases, repression of ethylene biosynthesis. The pathway of ethylene biosynthesis has been elucidated in

detail, both with respect to the modulation of ACC synthase and of ACC oxidase activities. Some structural and mechanistic questions are still open, such as the crystal structure of ACC oxidase and the mode of action of CO₂ in the activation of this enzyme. Also, very little is known about the signal transduction pathways that lead to an increase in ethylene synthesis. ACC synthase, the primary point of control, is encoded by medium-size gene families, and it is known that ACC synthase genes are differentially regulated (Kende 1993, Zarembinski & Theologis 1994). What signals mediate the effect of environmental and endogenous conditions, such as stress or the onset of fruit ripening, on ethylene synthesis? Thus two signal transduction pathways need to be understood with respect to ethylene-regulated processes, one that triggers ethylene biosynthesis and one that leads to ethylene responses.

Although the basic framework for the primary events in the ethylene-response pathway is emerging, there are many issues that remain to be resolved. The significance of different receptor isoforms is still not clear. Phenotypic analysis of plants with combinations of receptor-null mutations will help to define the roles that different members of the receptor family play in signal transduction (Hua & Meyerowitz 1998). Additional ethylene-insensitive mutants that have been identified (Johnson & Ecker 1998) need to be placed in the signaling pathway. Screens for second-site mutations using existing mutants may also yield new components of the pathway. Several gaps in our understanding of the biochemistry of signaling between the known components of the transduction pathway also need to be filled in. The nature of the protein complex that forms between the receptors and the Raf-like kinase CTR1 has yet to be determined. The role of the histidine kinase activity, which is associated with two members of the receptor family, requires clarification with respect to signal output. Progress in this area would be facilitated by the development of an *in vitro* assay for receptor/CTR1 signal output. The mechanism by which the receptor/CTR1 complex transmits the signal to the EIN2 protein is still a complete mystery. Likewise, information is lacking on the mechanism by which the cytoplasmic transmitter domain of EIN2 activates the EIN3 family of transcriptional regulators (Chao et al 1997). Identifying specific targets for the ERF1 transcription factor and learning how the products of those genes mediate downstream responses are additional goals for the future.

The biochemical properties of the known signaling components need to be reconciled with the behavior of the ethylene signal transduction system in planta. For example, the model shown in Figure 2 presumes that at least two rounds of transcriptional activation occur for the *de novo* production of proteins that mediate ethylene responses. Yet, the lag time for ethylene effects on seedling growth is reported to be as short as 10 min (Abeles et al 1992). How is it possible that a protein produced after two rounds of transcription/translation can also produce a measurable influence on the rheological properties of the plant cell wall in such a short time? Identification of the response-mediating proteins involved, coupled to detailed kinetic analysis of the growth response, are needed to answer this question. Another question to be resolved is the discrepancy between

the short lag time (<30 min) for the recovery of seedling growth when ethylene is removed (Abeles et al 1992) and the very slow rate of ethylene release (half time of 11 h) from the yeast-expressed ETR1 receptor (Schaller & Bleecker 1995). If ethylene remains bound to saturated receptors, how does the system sense the removal of free ethylene? It is possible that the behavior of receptors in the native plant differs from that in the yeast cell or that other receptor isoforms have faster release kinetics. An alternative mechanism relates to the inverse agonist model for receptor function. Recovery time from responses may be governed by the rate of new receptor synthesis; receptors synthesized after applied ethylene is withdrawn would be in the unoccupied, that is active, state and would thus suppress response pathways. The latter possibility provides an explanation for the observations that expression of some receptor genes is induced by ethylene (Hua et al 1998, Lashbrook et al 1998). Unquestionably, there are many open research directions that will, eventually, lead to a full understanding of how environmental and endogenous factors are transduced to yield ethylene-mediated responses in plants.

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Figure 2 The genetic interactions and biochemical identities of components of the ethylene signal transduction pathway. The ordering of components into a hypothetical linear pathway is based on both genetic (epistasis) analysis, ectopic gene expression studies, and biochemical interactions (see text). Progressing from left to right, ethylene is thought to regulate negatively a family of membrane-associated receptors that related to the bacterial two-component superfamily of catalytic receptors. The histidine-kinase transmitter domains of members of the receptor family interact with the regulatory domain of the Raf-like kinase CTR1. This receptor/CTR1 complex negatively regulates a membrane protein (EIN2) related to a superfamily of metal transporters. The cytoplasmic C-terminal domain of EIN2 positively signals downstream to the EIN3 family of transcription factors located in the nucleus. A target of the EIN3 transcription factors is the promoter of the ERF1 gene, a member of a second family of transcription factors. ERF1 is rapidly induced in response to ethylene and is capable of activating a subset of ethylene responses when ectopically expressed.