Ethylene signaling in *Arabidopsis*: Events from the membrane to the nucleus

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Abstract
Ethylene is a plant hormone that regulates a diverse set of developmental and physiological processes including seed germination, abscission, and senescence. Molecular genetic analysis of ethylene signaling in *Arabidopsis thaliana*, has led to the identification of a number of genetic loci that are required for normal ethylene responses. Cloning of several of the mutant loci has provided significant insight into the nature of the ethylene signaling pathway. The *Raf* kinase homolog CTR1 is predicted to negatively regulate the ethylene response pathway through a MAP kinase cascade. ETR1 has been demonstrated to bind ethylene, and a family of homologous genes encode products that may function in a receptor complex. EIN3 and three homologous gene products contain several protein motifs that are reminiscent of transcription factors, and transduce the ethylene signal downstream. Additional loci are defined by the *ein2, ein3ein1, ein6*, and *ein7* mutations, and a number of mutations defining tissue-specific mediators of ethylene responses have also been identified. In this review, the different ethylene-related mutations and their gene products are considered in detail, and possible models of the manner in which the pathway functions are proposed. © Elsevier, Paris.

Key words
Ethylene signaling, histidine kinase, MAP kinase, N-acetyltransferase, *Raf* kinase, transcription factor, *Arabidopsis thaliana*.

Abbreviations
ctr, constitutive triple response; ein, ethylene insensitive; etr, ethylene resistant; MAPK, mitogen activated protein kinase; MEK, MAP kinase kinase.

Introduction

In addition to its well-known role as the plant hormone responsible for fruit ripening, ethylene has been demonstrated to play a role in diverse physiological processes like seed germination, abscission, senescence, and responses to pathogen attack (Abeles *et al.*, 1992). Each of the aforementioned processes is a complicated response, integrating a number of signals, environmental and hormonal, to control the proper series of events that are necessary to produce the desired response. It is therefore of great interest to determine the manner in which ethylene influences these processes, causing profound changes at the gross morphological level, and finely regulating gene expression to achieve its effects. Understanding the steps in the signal transduction chain regulated by ethylene will be critical to our understanding of how different signal transduction pathways interact to control the responses of a plant to its environment.

To that end, a number of groups have endeavored to dissect the ethylene signal transduction pathway of *Arabidopsis thaliana* using a molecular genetic approach. Plants deficient in their responses to ethylene were identified, and these mutants were analyzed to identify the nature of their mutations. Classical genetic techniques like epistasis testing have revealed a linear signaling pathway, remarkably simple compared to other signaling pathways (Ecker, 1995; Roman *et al.*, 1995; Chao *et al.*, 1997), such as those regulated by the red/far-red light receptor phytochrome (Millar *et al.*, 1994). Cloning of some of the mutant loci has identified key components of the ethylene signaling pathway, and evidence of a conserved mechanism of signaling that is common to prokary-
otes and eukaryotes has emerged. In this review, we will discuss the phenotypes of plants identified in screens for ethylene mutants, the genes found to be disrupted in some of them, and the manner in which the signaling pathway may function.

**Mutants and phenotypes**

Neljubow first demonstrated the triple response of etiolated (dark-grown) seedlings to exogenously applied ethylene (Neljubow, 1901). Untreated etiolated seedlings typically possess long slender hypocotyls, elongated roots and closed apical hooks. Seedlings treated with ethylene, however, exhibit shortening and thickening of the hypocotyls and roots, and exaggeration of the apical hook (Bleecker et al., 1988; Guzman and Ecker, 1990). This response mimics that observed under natural conditions, in which seedlings penetrating the soil are subjected to physical resistance, a stress that has been demonstrated to stimulate ethylene production (Goeschl et al., 1966). It has been proposed that the ethylene-induced exaggeration of the apical hook may protect the delicate apical structures from physical damage as the seedling penetrates the soil (Darwin and Darwin, 1881). Mutant screens exploiting the triple response allowed the identification of three classes of mutants (Ecker, 1995).

**Ethylene insensitive mutants**

Mutants that are insensitive to the effects of ethylene may be easily selected as seedlings with exaggerated apical hooks and elongated hypocotyls and roots among wild type plants displaying the triple response. Because they do not display the proper response to ethylene, insensitive mutants are proposed to be deficient in the perception or transmission of the ethylene signal.

Plants possessing mutations in the etr1 gene were the first mutants isolated by this screen (Bleecker et al., 1988). etr1 Mutations were found to segregate as dominant traits, and conferred resistance to ethylene for a wide range of responses. In ethylene, etr1 mutant seedlings display a complete loss of the triple response, possessing elongated hypocotyls and roots (Bleecker et al., 1988; Guzman and Ecker, 1990). In addition, the curvature of the apical hook of etr1 seedlings in air-grown plants is significantly reduced compared to wild type (Roman et al., 1995), whereas adult etr1 plants are significantly larger than their wild type counterparts (Guzman and Ecker, 1990). These two observations suggest that etr1 mutants are insensitive to the ethylene that is endogenously produced, because the effects of ethylene include promotion of hook formation in etiolated seedlings, and inhibition of cell expansion (Guzman and Ecker, 1990). etr1 Mutants are also deficient in ethylene-promoted leaf senescence, seed germination, gene expression, and feedback inhibition of ethylene biosynthesis (Bleecker et al., 1988; Lawton et al., 1994; Chen and Bleecker, 1995). Assays of ethylene binding in etr1-1 leaves demonstrated that the mutant bound 80% less ethylene than wild type seedlings, leading to the proposal that the ETR1 gene encodes an ethylene receptor (Bleecker et al., 1988). Subsequent cloning and characterization of the ETR1 locus has suggested that this may indeed be the case (see below), (Chang et al., 1993). Mutation of the ein4 locus also results in a dominant ethylene insensitive phenotype, displaying insensitivity to ethylene that is comparable to etr1 (Roman et al., 1995).

Like etr1 and ein4, mutations of the EIN2 locus cause virtually complete insensitivity to the effects of ethylene (Guzman and Ecker, 1990; Roman et al., 1995). Unlike the aforementioned mutants, however, ein2 mutations segregate as recessive alleles (Guzman and Ecker, 1990; Roman et al., 1995). Nevertheless, ein2 mutants display complete insensitivity to ethylene as seedlings, possessing elongated hypocotyls and roots, and a significantly reduced apical hook. Much like etr1 and ein4, the apical hook of ein2 seedlings is significantly reduced in air as well (Roman et al., 1995). As adult plants, ein2 mutants are significantly larger than wild type plants, again suggesting that the mutant is resistant to the endogenous ethylene that it produces, levels of which are significantly higher than their wild type counterparts (Guzman and Ecker, 1990). This observation suggests that ein2 mutants are deficient in the negative feedback mechanism that regulates ethylene biosynthesis. Similarly, strong ein2 mutants are completely deficient in ethylene-inducible gene expression (Lawton et al., 1994).

etr1, ein4, and ein2 are mutations that appear to disrupt virtually all ethylene-dependent responses in the plant (Bleecker et al., 1988; Guzman and Ecker, 1990; Roman et al., 1995). Additional ethylene insensitive mutants that have been isolated appear to affect ethylene signaling to a lesser degree. Mutations at the ein3 locus are inherited as single-gene recessive mutations and cause reduced sensitivity to ethylene (Roman et al., 1995). Hypocotyls and roots of etiolated seedlings possessing ein3 mutations are significantly longer than...
wild-type plants when grown in ethylene, yet the degree of insensitivity is considerably less than that observed in ein2 plants (Roman et al., 1995). Moreover, the apical hooks of ein3 seedlings are less prominent in air than those of wild type seedlings, although they still retain significant sensitivity to ethylene (Roman et al., 1995). In addition, ethylene-responsive gene expression is still present in ein3 seedlings, but is reduced compared to wild type (Lawton et al., 1994; Chao et al., 1997). Adult ein3 plants are also larger than wild type (Kieber et al., 1993). Cloning of the ein3 locus (see below) indicates that one allele would be predicted to encode a significantly truncated protein that may severely affect function, suggesting that the weak ethylene insensitive phenotype observed in ein3 seedlings is not merely the result of leaky expression (Chao et al., 1997). Rather, it suggests that ein3 mutations incompletely impair all ethylene responses that have been tested.

The ein5/ain1, ein6, and ein7 loci were also selected as weak ethylene insensitive mutants (van der Straeten et al., 1993; Roman et al., 1995). ein5/ain1 Mutants display reduced sensitivity to ethylene when measured by the triple response, but clearly display some ethylene responsiveness (van der Straeten et al., 1993; Roman et al., 1995). Much like the mutants described above, ein5/ain1 plants possess larger rosettes than wild type plants, owing to their resistance to endogenous levels of ethylene (van der Straeten et al., 1993). Moreover, ein5/ain1 mutants display delayed senescence compared to wild-type plants, a phenotype it shares in common with stronger ethylene insensitive mutants (van der Straeten et al., 1993). ein6 and ein7 possess weak ethylene insensitive phenotypes, and are less well-characterized than the other mutants (Roman et al., 1995). The ethylene insensitive phenotype of ein6 appears to primarily affect the hypocotyl and apical hook, as roots possess similar ethylene sensitivity as wild type seedlings (Roman et al., 1995). In addition, ein6 mutants have been found to be hypersensitive to the microtubule-stabilizing agent taxol (Roman and Ecker, 1995).

**Constitutive response mutants**

Just as ethylene insensitive mutations suggest the loss of the ability to transduce the ethylene signal, constitutive response mutations would be proposed to cause constitutive activation of the ethylene signaling pathway. Mutants such as this are selected as seedlings displaying the triple response when grown in the absence of exogenous ethylene. Plants displaying the constitutive triple response may result from constitutive activation of the ethylene signal transduction pathway or from overproduction of ethylene. Because the ethylene biosynthetic pathway has been well-characterized, and the enzymes involved in biosynthesis have been identified (Yang and Hoffman, 1984), it is possible to use inhibitors of these biosynthetic steps to distinguish between the two possibilities. Indeed, use of inhibitors of ethylene biosynthesis like aminooxyacetic acid and silver ion has allowed two types of constitutive response mutants to be identified (Kieber et al., 1993; Ecker, 1995).

The first class – mutants that overproduce ethylene – have been termed eto mutants (ethylene overproducer). Three eto loci (eto1, eto2, and eto3) have been identified, and their constitutive triple response phenotype is reverted by treatment with inhibitors of ethylene biosynthesis, or by treatment with ethylene receptor antagonists like silver ion (Guzman and Ecker, 1990; Kieber et al., 1993). These observations suggest that eto mutations affect ethylene production. eto1 is inherited as a recessive trait, whereas eto2 and eto3 are inherited as completely dominant traits (Guzman and Ecker, 1990; Kieber et al., 1993). All three eto mutants produce significantly higher levels of ethylene as seedlings, producing the constitutive triple response phenotype (Guzman and Ecker, 1990; Kieber et al., 1993). eto1 and eto3 also produce higher levels of ethylene as adult plants, whereas the levels of ethylene in adult eto2 mutants are similar to those observed in wild type plants (Kieber et al., 1993).

The second class of constitutive response mutants could not be reverted by inhibitors of ethylene biosynthesis or action, and were designated ctr mutants (constitutive triple response) (Kieber et al., 1993). The ctr class of mutants is represented by one locus, termed ctr1, that is inherited as a recessive trait (Kieber et al., 1993). Much like eto mutants, ctr1 seedlings display a constitutive triple response in the absence of exogenous ethylene. As adult plants, ctr1 mutants are severely stunted, and measurement of cell density in the leaf epidermis indicates that the smaller growth habit is due, at least in part, to reduced cell expansion (Kieber et al., 1993). In addition, leaves of ctr1 mutants displayed increased rates of senescence following excision from the plant. ctr1 plants flower much earlier than wild type plants and have reduced fertility (Kieber et al., 1993). Examination of ctr1
flowers indicates that the gynoecium may develop earlier than in wild type plants. Alternatively, development of other floral organs such as stamens may be retarded relative to that of the gynoecium (Kieber and Ecker, 1993). Moreover, the reduced transmission of ctr1 alleles results from a defect in the female gametophyte (Kieber and Ecker, 1994). The recessive nature of ctr1 mutations indicates that the wild-type gene product acts to negatively regulate activity of the ethylene signaling pathway (Kieber et al., 1993). Cloning of the ctr1 locus has provided significant insights into the nature of the ethylene signaling pathway (see below; Kieber et al., 1993).

**Tissue-specific ethylene insensitivity**

In addition to isolating mutants that display insensitivity to ethylene in the whole plant, it is equally useful to identify mutants that are deficient in ethylene responses in particular tissues. Such mutants may provide insight into the manner in which ethylene controls specific developmental processes in different parts of the plant. Using the triple response as a reference, mutants that were insensitive to ethylene in the apical hook or the root were isolated. No mutants that specifically confer ethylene insensitivity in the hypocotyl have yet been isolated.

Mutants in the **HOOKLESS1** (hls1) gene were selected based on the absence of an apical hook in etiolated, air-grown seedlings (Guzman and Ecker, 1990). The roots and hypocotyls of hls1 seedlings are completely sensitive to the inhibitory effects of ethylene, displaying similar responses as the hypocotyls and roots of ethylene-treated wild type seedlings (Roman et al., 1995). In contrast, the apical hook of hls1 seedlings is completely insensitive to the hook promoting effects of ethylene (Roman et al., 1995). The effects of hls1 mutations are not confined to the hook, as the cotyledons and petioles of air-grown, etiolated hls1 seedlings are larger and longer, respectively, than their wild type counterparts (Lehman et al., 1996). In contrast, the hypocotyls of air-grown hls1 seedlings are shorter than those of wild-type plants. Thus, HLS1 is required to produce opposite growth effects in different tissues. An additional phenotype of hls1 plants is that they flower earlier than wild type. This effect has been attributed to the increased rate of leaf initiation in hls1 mutants early in development (Lehman et al., 1996).

Growth of etiolated seedlings on auxin polar transport inhibitors produces a phenotype remarkably similar to that of hls1 seedlings, causing a hookless phenotype (Lehman et al., 1996). This suggests that HLS1 plays a role in regulating the effects of auxin, perhaps by controlling its distribution or the sensitivity of cells to it in regions that undergo differential growth, such as the apical hook. Indeed, the expression patterns of auxin-regulated genes are perturbed in hls1 seedlings, further underscoring a role for HLS1 in regulating auxin responses (Lehman et al., 1996). The cloning and identification of the HLS1 locus (see below) may provide important information about how HLS functions in the context of ethylene- and auxin-dependent growth (Lehman et al., 1996).

Two loci that affect the ethylene sensitivity of roots have been isolated and designated eir1 (for ethylene insensitive root) and aux1. Both mutants were identified as plants that possessed elongated roots when grown in ethylene or on ACC, its immediate precursor (Pickett et al., 1990; Roman et al., 1995). aux1 has previously been identified in screens for mutants displaying auxin-resistant root growth (Maher and Martindale, 1980). The hypocotyls and apical hooks of eir1 and aux1 mutants displayed normal ethylene sensitivity (Roman et al., 1995). An additional phenotype possessed by these two mutants is that their roots display agravitropic growth (Pickett et al., 1990; Roman et al., 1995). When eir1/aux1 double mutants were tested for ethylene sensitivity, they did not display an additive response, indicating that the gene products function in the same pathway (Roman et al., 1995). The identity of the AUX1 gene product suggests that it may play a role in auxin transport or signaling (Bennett et al., 1996), and for that reason, its molecular function will not be considered further in this review.

**Ordering gene products in a pathway**

The existence of mutations that result in constitutive activation of the ethylene signaling pathway, and those that result in its loss of function, allows the use of epistasis analysis to determine the order in which the gene products act. The phenotype of double mutants possessing a constitutive response mutation and an ethylene insensitive mutation will indicate the order in which the two genes act. For instance, if the double mutant has the phenotype of the constitutive response mutation, it may be concluded that it acts downstream of the ethylene insensitive mutation in the pathway. Conversely, if the double mutant appears like the ethylene insensitive mutation, then it is likely to act downstream of the constitutive response mutation in

the signaling cascade. The specificity of the ein and constitutive response mutations for the ethylene signaling pathway has yielded very reliable information about the order in which the affected gene products act to transduce the ethylene signal (Ecker, 1995).

F2 progeny of crosses between etr1 or ein2 and etol displayed segregation ratios that indicated epistasis of these two ein mutations over etol (Roman et al., 1995). These results predict that the gene products affected by ein mutations normally function downstream of ethylene biosynthesis. Quite different results were obtained in crosses between ctrl and ein or etr mutants. Double mutants of ctrl and etr1 or ein4 all displayed the phenotype of ctrl1, indicating that CTRL1 acts downstream of these two gene products (Roman et al., 1995). In contrast, double mutants between ctrl1 and ein2, ein3, ein5/ain1, ein6, or ein7 all displayed the phenotypes of the ein mutants, indicating that these EIN gene products act downstream of CTRL1 in the ethylene signaling cascade (Roman et al., 1995). From these results, a simple signal transduction cascade may be proposed, in which ETO gene products regulate ethylene production and ETR1 and EIN4 act far upstream in the pathway, perceiving the ethylene signal (see below). CTRL1 functions upstream in the pathway as well, after ETR1 and EIN4, negatively regulating the activity of the ethylene signaling pathway. EIN2, EIN3, EIN5/AIN1, EIN6, and EIN7 act downstream of CTRL1, and transduce the ethylene signal (fig. 1 A and B; Ecker, 1995; Roman et al., 1995; Chao et al., 1997).

Transgression analysis, a technique in which mutations possessing similar phenotypes are combined to assess whether the two mutations confer an additive phenotype, has also been used to determine whether the ethylene signaling pathway is linear or branched. Double mutants between etr1 and ein2 do not display an additive phenotype, indicating that the two mutations function in the same pathway (Roman et al., 1995). Similarly, double mutants between ein2 and some of the weaker ein mutants all display the same phenotype as ein2, again suggesting that they function in a linear pathway (Roman et al., 1995). Interestingly, double mutants of weaker eins do not display an additive phenotype either, indicating that these gene products do not control specific downstream branches of the ethylene signaling pathway (Roman et al., 1995).

Double mutant studies using the tissue-specific ethylene insensitive mutants hls1, aux1, and etr1 have also been performed and yielded interesting results.
All three tissue specific mutations were epistatic to ctrl and eto1 (Roman et al., 1995). That is, the root-specific aux1 and eir1 mutations conferred long roots in double mutant combinations with ctrl or eto1. hsl/ctrl and hsl/eto1 double mutants lacked an apical hook like hsl mutants, but possessed the short hypocotyls and roots that are characteristic of ctrl and eto1 mutants. These results indicate that hsl1, aux1, and eir1 all act downstream of ctrl and ethylene biosynthesis in the ethylene signaling cascade (Roman et al., 1995).

In transgression tests, double mutants possessing ein2 and aux1 or eir1 mutations did not possess roots that were significantly longer than ein2 mutants alone (Roman et al., 1995). Similarly, aux1/eir1 double mutants did not possess longer roots in ethylene than either single mutant. These data indicate that EIN2, AUX1, and EIR1 all function in the same pathway (Roman et al., 1995). In contrast, double mutants between eir1 and ein3 or ein5/ait1 produced an additive phenotype with regard to root length, suggesting that EIR1 functions in a pathway that is independent from EIN3 and EIN5/AIN1 (Roman et al., 1995). The level of root insensitivity to ethylene in the eir1/ein3 and eir1/ein5/ait1 double mutants was similar to that observed in ein2 single mutants, suggesting that EIN2 m.y function upstream of all three gene products (Roman et al., 1995). In double mutant combinations with ein mutations, hsl1 plants all lacked an apical hook, consistent with its single mutant phenotype (Roman et al., 1995).

Cloned genes

The CTR1 gene was isolated by T-DNA tagging, and was the first ethylene signaling gene to be cloned (Kieber et al., 1993). RNA blot analysis indicated that CTR1 is constitutively expressed, and its expression is not altered by ethylene treatment. Sequence analysis indicated that the CTR1 gene bore significant homology to the Raf family of mammalian serine/threonine protein kinases (Kieber et al., 1993). In mammalian systems, Raf kinases have been demonstrated to be activated by the small GTP binding protein Ras, and to regulate the mitogen-activated protein kinase (MAPK) cascade. MAP kinases are activated by phosphorylation by MAP kinase kinases (MEK). MEKs are activated by MAP kinase kinase kinases (MEK kinase), a class of proteins of which Raf kinases are members (reviewed in Cobb et al., 1994). Thus, CTR1 is predicted to negatively regulate activity of the ethylene signal transduction pathway through the activation of a MAP kinase cascade (Kieber et al., 1993; Roman et al., 1995).

Mutants possessing the eir1 mutation were found to bind significantly less ethylene than their wild type counterparts (Bleecker et al., 1988). From these data, it was proposed that the ETR1 locus may encode an ethylene receptor. Epistasis analysis indicates that ETR1 functions early in the ethylene signaling pathway, and these data are also consistent with the idea that ETR1 is an ethylene receptor (Roman et al., 1995). Identification of the ETR1 gene indeed suggests that ETR1 may be an ethylene receptor. The sequence of ETR1 shares significant homology with proteins from prokaryotes that are members of the two component regulator class of receptors (Chang et al., 1993).

Three short stretches of ETR1 in the amino terminal region are predicted to encode transmembrane domains (Chang et al., 1993). After this transmembrane region, two short stretches of ETR1 and ERS (see below) bear homology to the cyanobacterial RcaE protein, a light quality sensor that also possesses a putative histidine kinase domain (Kehoe and Grossman, 1996). Following the transmembrane domains, a region bearing significant homology to histidine kinase domains of bacterial sensor proteins is present, possessing all of the conserved regions and residues that are found in other histidine kinases (Chang et al., 1993). Towards the carboxyl terminus of the protein, a region of ETR1 that is homologous to the prokaryotic response regulator proteins is present (Chang et al., 1993). The response regulator domain contains the conserved aspartate residue to which a phosphate is transferred from the conserved histidine of the histidine kinase domain (Parkinson, 1993). The structure of ETR1, containing both a histidine kinase and a response regulator domain within the same protein, is unusual, but not unique.

Protein studies in transgenic yeast have provided insight into aspects of the structure of ETR1. Using antibodies against ETR1, it was determined that the molecule exists as a homodimer in transgenic yeast as well as Arabidopsis (Schaller et al., 1995). Moreover, ETR1 is found associated with membranes in yeast (Schaller et al., 1995). Dimerization is mediated through the amino terminal domain of ETR1, and requires at least one of two cysteines located within the first ten amino acids, suggesting a disulphide linkage. Interestingly, Cys^65, which produces a strong dominant ethylene insensitive phenotype when
mutated, does not affect dimerization (Schaller et al., 1995). Dimerization of bacterial sensor proteins is thought to be important because it allows intermolecular histidine phosphorylation (Parkinson, 1993). The disulphide linkage that is proposed to mediate dimerization is a structure that appears to be unique to ETR1 (Schaller et al., 1995).

It was also found that yeast expressing ETR1 bound ethylene at nanomolar levels (Schaller and Bleecker, 1995). Ethylene binding was conferred by the amino terminal region of ETR1, consistent with observations in prokaryotic systems in which ligand binding is typically conferred by the amino termini of bacterial sensor proteins (Parkinson, 1993). Binding of ETR1 was saturable, and the dose-dependent binding curve that is observed is similar in shape to that observed when measuring ethylene-dependent growth inhibition in Arabidopsis (Schaller and Bleecker, 1995). Moreover, treatment of yeast cells with antagonists of ethylene binding, like trans-cyclooctene, significantly interfered with ethylene binding. Despite having no effect on ETR1 dimerization, mutation of Cys68 virtually eliminated ethylene binding. This residue may be important for coordination of the metal ion that is thought to mediate ethylene binding (Schaller and Bleecker, 1995). In addition, mutation of the cysteines that are required for proper dimerization reduces binding by half, suggesting that dimerization significantly enhances the competence of ETR1 to bind ethylene (Schaller and Bleecker, 1995).

Low stringency hybridization of genomic Southern blots with ETR1 indicated that homologs are present in the Arabidopsis genome (Chang et al., 1993). One homolog, termed ERS, bears strong homology to ETR1 in both the amino terminal and histidine kinase domains (Hua et al., 1995). Unlike ETR1, however, ERS lacks a response regulator domain. When Ile62 is converted to Phe in ERS, mimicking a strong mutation of ETR1, expression in transgenic Arabidopsis results in a dominant ethylene insensitive phenotype that affects both the seedling and adult plant (Hua et al., 1995). Epistasis analysis of ers mutants with ctrl indicate that CTR1 functions downstream of ERS. These data suggest that like ETR1, ERS functions in the perception of the ethylene signal and the transmission of that signal to activate the ethylene response pathway (Hua et al., 1995). This is likely to be the case, as the ethylene-insensitive never ripe mutation of tomato was found to result from the mutation of an ERS homolog (Wilkinson et al., 1995). Cloning of the EIN4 and ETR2 genes demonstrated that they encoded ETR1 paralogs as well (Hua et al., 1997). EIN4 and ETR2 both possess histidine kinase and response regulator domains, although the putative His autophosphorylation site is not present in ETR2 (Hua et al., 1997). In addition, a fourth gene, homologous to ETR1, was identified during the sequencing of chromosome 1 (BAC F19P19; Accession number AC000104). Detailed biochemical analysis will clearly be required to understand how these five molecules interact to regulate ethylene responses.

Cloning of the weak ethylene insensitive ein3 mutant gene revealed that the EIN3 protein bore homology to transcription factors, possessing several basic domains that may be involved in DNA binding, as well as acidic and proline-rich regions, motifs that may be found in transcriptional activators (Chao et al., 1997). In addition, one segment of the EIN3 protein is predicted to form coiled coil structures, indicating that EIN3 may interact with other proteins to perform its function. Consistent with its proposed role as a transcription factor, EIN3:β-glucuronidase fusions were found to be nuclear-localized (Chao et al., 1997). Low stringency hybridization of genomic Southern blots with EIN3 identified several additional genes that bore homology to EIN3. Cloning of three of these genes, termed EIL1-3 (for EIN3-Like), determined that the genes possessed significant sequence similarity to EIN3, and retained a similar domain structure in their predicted protein products (Chao et al., 1997).

The weak nature of ein3 mutations may reflect the possibility that homologous EIL proteins partially compensate for ein3 deficiencies. The inability of the EIL genes to completely suppress an EIN3 deficiency may reflect tissue-specific EIL functions, or the presence of insufficient levels of EIL proteins to fully compensate for EIN3 deficiency. Indeed, expression of EIL1 or EIL2 using a strong promoter, efficiently complements an ein3 mutation, much like expression of a wild type EIN3 cDNA does (Chao et al., 1997). Moreover, overexpression of EIN3 or EIL1 in wild type or ein2 mutants produces a constitutive response phenotype in air-grown seedlings, that is similar to that observed in ctrl mutants (Chao et al., 1997). This observation indicates that when produced in sufficient quantities, EIN3 and EIL1 are capable of constitutively activating the ethylene response pathway in the absence of an ethylene signal, reinforcing the notion that EIN3 and EIL1 are downstream mediators of the ethylene response pathway.
Cloning of the HLS1 gene revealed that it bears homology to N-acetyltransferases (Lehman et al., 1996; Neuwald and Lands, 1997). Given the observation that the expression patterns of auxin-responsive genes in the apical hook region are disrupted in hls1 mutants, a candidate target molecule for modification by HLS1 is the auxin indole-3-acetic acid (IAA) itself, or a precursor thereof (Lehman et al., 1996). In situ hybridization measurements of HLS1 mRNA indicate that the gene is expressed uniformly in cells of the cotyledon, apical hook, hypocotyl, and root. No differential expression of HLS1 in regions undergoing differential elongation was observed (Lehman et al., 1996). Moreover, HLS1 expression was found to be stimulated by ethylene itself, suggesting that the exaggeration of the apical hook that is observed in ethylene-treated seedlings is a direct result of HLS1 activity. Indeed, overexpression of HLS1 produced seedlings with exaggerated apical hooks in the absence of ethylene (Lehman et al., 1996). Studies of HLS1 expression in ein2 mutants demonstrate that HLS1 mRNA levels do not significantly accumulate. The reduced levels of HLS1 protein that would be predicted to be present in ein2 seedlings may explain the reduced apical hook observed in ein2 and other ein mutants (Lehman et al., 1996). From these data, it may be concluded that HLS1 is a downstream effector of the ethylene response pathway, perhaps achieving its effects by differentially regulating auxin levels in a cell-specific manner.

Biochemistry of ethylene signaling

Knowing the identities of a number of components in the ethylene signaling pathway as well as their general order of activity, based on epistasis and transgression analysis, it is possible to propose a general model for ethylene signaling (fig. 1 A and B). Production of ethylene is regulated by gene products affected by eto mutations. The ethylene that is produced is perceived by a receptor consisting of at least the putative histidine kinase ETR1, and perhaps ERS, EIN4, and ETR2. Ethylene binding results in the inactivation of a MAP kinase cascade that is regulated by the Raf/MEK kinase homolog, CTR1. Inactivation of this inhibitory MAP kinase cascade allows activation of the ethylene signaling cascade. Epistasis and transgression analyses suggest that EIN2 acts early in the cascade, and the signals are ultimately transmitted by gene products affected by the weak ein mutations ein3, ein5/sain1, ein5, and ein7. As a possible transcriptional activator, EIN3 and EIL proteins are likely to be primary regulators of molecules that effect ethylene-dependent processes in terminal branches of the signaling pathway, such as HLS1.

Significant similarity between the ethylene signaling pathway in plants and the osmosensing pathway of Saccharomyces cerevisiae has been found, with the identification of the yeast two component response regulator SLN1 as the primary osmosensor (Ota and Varshavsky, 1993; Maeda et al., 1994). Much like ETR1, molecular genetic studies of the osmosensing pathway have demonstrated that SLN1 regulates a downstream MAP kinase cascade (Maeda et al., 1994). SLN1 has a similar domain structure as ETR1, possessing a non-homologous amino terminal domain that is thought to function as the osmosensor, and histidine kinase and response regulator domains (Ota and Varshavsky, 1993). Activation of SLN1 by osmotic stress induces histidine autophosphorylation, after which, the phosphate is transferred to the conserved aspartate of the response regulator domain (Posas et al., 1996). This phosphate is transferred to the histidine of YPD1, a molecule that bears homology to a molecule from the thermophlic bacterium Thermotoga maritima, known as CheA, a protein that functions in a two-component chemotactic response pathway. The phosphate that has been transferred to YPD1 is subsequently transferred to the conserved aspartate of the SSK1 response regulator homolog, inactivating the molecule (Posas et al., 1996). This allows the activation of the MEK kinase homologs SSK2 and SSK22, initiating a MAPK cascade that contains the MEK PBS2, and the MAPK HOG1 (Maeda et al., 1995).

Unlike the osmosensing pathway in yeast, in which SLN1 is the sole receptor of this branch of the pathway (SHO1 regulates a second branch of the osmotic response pathway), ETR1 has multiple paralogs, such as ERS, EIN4, and ETR2 (Hua et al., 1997). Thus, the nature and actual structure of the receptor is unclear. It is possible that a complex of ETR1 paralogs is present at the cell surface perceiving ethylene and initiating the signaling pathway. Indeed, one explanation for the dominant nature of ETR1 and ERS mutations is that they poison a receptor complex, effectively inhibiting ethylene binding, as may occur in the C65Y mutation of etr1-1 (Chang et al., 1993). ETR1 has been shown to form homodimers (Schaller et al., 1995), but that does not preclude the formation of heterodimers with
ERS or another paralog, nor the formation of a multimeric complex.

Aside from the nature of the receptor or receptor complex, the manner in which it functions to activate the ethylene response pathway is an open question. As described above, the dominant nature of ETR1 mutations may be the result of a poisoned receptor complex. Such a poisoned complex would be unable to interact with the CTRL kinase, directly or indirectly, and inhibit it. This would result in constitutive pathway repression. Under this model, the ETR receptor is off in the absence of ethylene, and binding of the hormone activates it, allowing it to inhibit CTRL function (fig. 1A). If this model is correct, it may be predicted that deletion of the ETR1 gene will result in an ein phenotype. Alternatively, ctrl mutations may result in a receptor complex that is locked in the "active" conformation (Chang et al., 1993). Under wild type conditions, and in the absence of ethylene, this complex would constitutively stimulate CTRL pathway activity, resulting in receptor-dependent repression of pathway activity. In the presence of ethylene, activity of the receptor complex would be inhibited, and CTRL function would cease in the absence of its stimulus (fig. 1B). In this case, it may be predicted that deletion of the ETR1 gene will result in a ctrl phenotype, as the activator of CTRL activity would not be present, allowing the EIN2-dependent signaling cascade to be constitutively active. Either model is possible, and further testing will determine which is the case.

The manner in which the receptor interacts with CTRL is currently unknown. Regardless of the way in which ETR1 regulates the activity of the ethylene pathway, CTRL activity must be inhibited, either through direct inhibition following ethylene binding, or indirect inhibition by removal of a signal that stimulates CTRL activity. This requires that another molecule interacts with CTRL. The interacting molecule may be a protein such as RAS, a known direct regulator of Raf kinase activity in mammalian systems (Xia et al., 1996). Because of its structure, following transfer of the phosphate from its histidine kinase to its response regulator domain, ETR1 may interact with CTRL directly. Alternatively, ETR1 may initiate a series of phosphate transfer reactions, much like SLN1, in which a terminal response regulator molecule like SSK1 interacts with CTRL (Posas et al., 1996). The nature of this interaction will only become clear through detailed molecular and biochemical analysis of the receptor complex and CTRL.

As described above, as a Raf kinase, CTRL is predicted to mediate its repressive effects on the ethylene signaling pathway through a MAP kinase cascade (figs. 1A and B). In Arabidopsis, multiple homologs of MEKs and MAPks have been cloned (Mizoguchi et al., 1993; 1996). Identification of which MEK or MAPK functions in the ethylene response pathway cannot be determined by simple sequence analysis. It may be predicted that mutants with similar constitutive response phenotypes to ctrl may be recovered and contain defects in a MEK or MAPK, but none have been reported in the literature. The difficulty in isolating such mutations may reflect genetic redundancy, in which loss of one MEK or MAPK may be compensated for by another member of the family. Detailed biochemical analysis of molecules that interact with CTRL may prove useful in identifying downstream components of its signaling pathway.

Ultimately, it is of interest to identify the downstream components of the ethylene signaling cascade. EIN2 is likely to be required for initial steps in the signaling cascade, and molecules like EIN5/AIN1, EIN6, and EIN7 probably function downstream. EIN3 and its homologs EIL1-3 have sequence similarity to transcription factors, and EIN3 has been shown to be nuclear-localized (Chao et al., 1997). Overexpression of EIN3 and EIL1 cause constitutive ethylene responses, and, thus, are positive activators of the ethylene response. As such, they are likely to bind to the promoters of genes whose products function in the terminal parts of the signaling pathway. Candidates for such genes are those encoding EREBs, proteins that bind to an ethylene response element, the GCC box (Ohme-Takagi and Shinshi, 1995). Production of EREBs and their subsequent binding to other promoter elements would allow amplification of an ethylene signal from an apparently linear early signaling mechanism. Indeed, the promoter of HLS1, a target of the ethylene response pathway and downstream effector of apical hook responses, contains a GCC box (Lehman et al., 1996; figs. 1A and B).

Conclusion

Identification of the components of the ethylene response pathway will require the cloning of the genes identified by mutation in the future. In addition, it is likely that new mutants may be isolated from screens based on the triple response. The existence of single
alleles of ein6 and ein7 indicate that this mutant screen is not yet saturated. Other screens that are based on ethylene responses in adult plants may also prove useful in identifying genes required for the primary ethylene response, as well as those that are involved in very specific ethylene-dependent responses, such as senescence or fruit ripening. Powerful techniques like yeast two-hybrid assays will allow the identification of proteins that interact with known components of the response pathway. These in-depth studies will allow a detailed understanding of the mechanism by which ethylene regulates a diverse set of developmental responses.

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References


