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THE ROYAL SOCIETY

The molecular basis of ethylene signalling in *Arabidopsis*

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The simple gas ethylene profoundly influences plants at nearly every stage of growth and development. In the past ten years, the use of a genetic approach, based on the triple response phenotype, has been a powerful tool for investigating the molecular events that underlie these effects. Several fundamental elements of the pathway have been described: a receptor with homology to bacterial two-component histidine kinases (ETR1), elements of a MAP kinase cascade (CTR1) and a putative transcription factor (EIN3). Taken together, these elements can be assembled into a simple, linear model for ethylene signalling that accounts for most of the well-characterized ethylene mediated responses.

Keywords: plant hormones; ethylene; alternative splicing; signal transduction; plant development; *Arabidopsis*

1. INTRODUCTION

Plants respond to a diverse array of endogenous and environmental signals. Ethylene, the simplest olefin, has been shown to play a role in many of these responses, from germination and cell expansion to stress responses and fruit ripening (reviewed by Abeles *et al.* (1992)). The control of ethylene production and perception is thus a critical problem in plant biology. The ethylene biosynthetic pathway has been substantially characterized and the most important regulatory enzymes, aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, have been cloned and characterized in a number of species (Yang & Hoffman 1984; Kende 1989, 1993). These two enzymes are encoded by multigene families in all species that have been examined, including *Arabidopsis thaliana*, and the members of these families appear to be differentially regulated during development, in various tissues and in response to wounding, hormones, and a variety of stresses (Zarembinski & Theologis 1994; Morgan & Drew 1997).

In the presence of ethylene and the absence of light, the seedlings of many dicotyledonous plants develop a morphology known as the triple response. First demonstrated in pea (Neljubow 1901), the triple response of *Arabidopsis* consists of a shortened root and hypocotyl, a radially expanded hypocotyl and an exaggerated apical hook (see figure 1). It has been postulated that the triple response is a mechanism for protecting the meristem from the stresses associated with germination (Darwin & Darwin 1881; Goeschl *et al.* 1966). Researchers have used the triple response as the basis of a series of simple but powerful genetic screens for the identification of elements affecting ethylene biosynthesis and ethylene responses (Bleecker *et al.* 1988; Guzman & Ecker 1990; Kieber *et al.* 1993; Roman *et al.* 1995). These screens have been crucial to the characterization of ethylene signalling, and established an important paradigm for plant signalling in general (Kieber 1997*a*,*b*).

Mutants that fail to display the triple response in the presence of exogenous ethylene (ethylene-insensitive) as well as mutants that constitutively display the response have been identified. In addition, there are mutants that act only in a subset of tissues or during specific developmental stages.

2. *ETR1*—THE ETHYLENE RECEPTOR

Mutants that do not display a triple response in the presence of saturating levels of exogenous ethylene have been designated ethylene-insensitive. The first such mutant identified was the genetically dominant *etr1* (ethylene-resistant), which was defective in many ethylene responses including promotion of seed germination and senescence of detached leaves (Bleecker *et al.* 1988). Significantly, ethylene-induced genes are not induced by either exogenous or endogenous ethylene in *etr1*, and *etr1* leaves bind only 20% as much exogenous ethylene as wild-type leaves. A similar mutation, *ein1*, was isolated independently and found to be allelic to *etr1*.

The *etr1* mutant was cloned and characterized by Chang (Chang *et al.* 1993). *ETR1* encodes a protein with similarity to bacterial two-component sensing systems. Two-component systems are the main route by which bacteria sense and respond to external stimuli such as phosphate availability and osmolarity (Stock *et al.* 1990; Parkinson 1993). Bacteria employ many such two-component systems which are characterized by the linking of a sensor or input domain with an associated response regulator. The carboxy terminus of ETR1 is similar to both the histidine kinase sensor component and the response regulator domains of two-component systems. Analysis of genetic epistasis placed ETR1 early in the ethylene signal transduction pathway (Kieber *et al.*

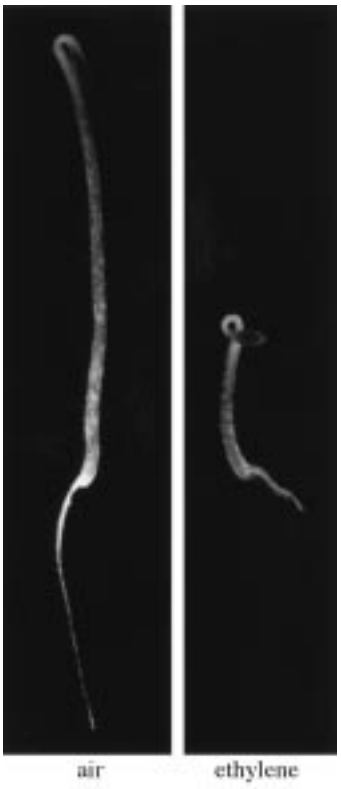

Figure 1. Phenotype of three-day-old etiolated seedlings of *Arabidopsis*, ecotype WS, grown in air (left) or in 10 p.p.m. ethylene (right). The seedling on the right displays the triple response: inhibition of elongation of the root and hypocotyl, radial swelling of the hypocotyl and exaggeration of the curvature of the apical hook. Actual length of wild-type hypocotyls in air is about 6 mm.

1993; Roman *et al.* 1995). A substantial body of evidence indicates that *ETR1* is an ethylene receptor. Perhaps the most compelling evidence comes from the study of yeast cells expressing wild-type and mutant ETR1 protein. Cells expressing wild-type ETR1 bound ethylene with a high affinity, and this binding was saturable, whereas yeast expressing the mutant *etr1-1* protein did not display detectably saturable ethylene binding (Schaller & Bleecker 1995). Furthermore, ethylene binding by yeast expressing *ETR1* could be reduced by competitive inhibitors of ethylene binding such as *trans*-cyclooctene.

ETR1 is present as a small gene family in *Arabidopsis*, and one homologue, *ERS*, has been cloned (Hua *et al.* 1995). *ERS* is 67% identical to *ETR1* at the amino-acid level but lacks the receiver domain of the response regulator. *ERS* is genetically distinct from *ETR1*, but may have some functional redundancy with it. A mutation in *ERS* analogous to the *etr1*-4 allele resulted in dominant ethylene-insensitivity when expressed in *Arabidopsis*. Analysis of genetic epistasis places *ERS* upstream of *CTR1* (see following paragraphs), as is *ETR1*. Thus, both *ETR1* and *ERS* may act as ethylene receptors, perhaps as multimers, which may explain why only dominant mutations of *ETR1* have been identified. It is possible that the *ein4* and *etr2* mutations are also members of the *ETR1* gene family. Both mutations are dominant and both function very early in the ethylene signal transduction pathway (Chang & Meyerowitz 1995; Roman *et al.* 1995).

Ethylene-insensitive mutants have been identified in tomato as well. The *Never-ripe* (*Nr*) mutation is a dominant, ethylene-insensitive mutation that affects both seedlings and adult plants (Lanahan *et al.* 1994). The sequence of the Nr protein is similar to *ETR1* (Yen *et al.* 1995) and, as in *ERS*, Nr lacks a response regulator domain.

3. THE *EIN3* GENE

Apart from *ETR1*, the best-characterized ethylene insensitive mutant is *ein3*, which most probably functions downstream of *EIN2*. *ein3* mutants retain somewhat more ethylene responsiveness than *ein2*, but are clearly affected at all stages of growth (Chao *et al.* 1997). For example, the hypocotyls of *ein3* mutants are partly inhibited from elongating when etiolated seedlings are exposed to ethylene, but their phenotype is less severe than that of *ein2*. Adult *ein3* mutants also have larger rosettes than wild-type plants and their detached leaves senesce much more slowly as measured by chlorophyll degradation. These phenotypes indicate that *EIN3* functions in ethylene-dependent responses at several stages of plant growth. The role of *EIN3* was further elucidated by analysis of Northern blots by using ethylene-regulated genes as probes. The basal and induced level of mRNA accumulation for these genes was reduced in *ein3*-1 and *ein3*-2 mutants when compared with wild-type plants, a result also obtained by Lawton *et al.* (1994).

EIN3 was cloned by using the T-DNA insertion allele *ein3-2* (Chao *et al.* 1997). The *EIN3* genomic sequence contains a single intron in the 5′ untranslated portion of the gene. *EIN3* has no significant similarity to any other sequences in the database. The predicted protein contains 628-amino-acid residues, is generally hydrophilic and has a molecular mass of 69 kDa. There are several significant structural features of the protein: a highly acidic amino-terminus (amino acids 1–52) that overlaps with a predicted coil structure; five small basic domains distributed along the length of the entire gene; a proline-rich domain (amino acids 199–240), and a carboxy-terminus rich in asparagine residues. Basic domains II and III are predicted to contain α-helices. Several of these features indicate that EIN3 may function as a transcriptional regulator. Evidence that EIN3 is localized to the nucleus was provided by a series of experiments in which fusions between *EIN3* and a GUS reporter gene were introduced into *Arabidopsis* leaf mesophyll protoplasts or soybean suspension cell protoplasts (Chao *et al.* 1997). Analysis of the results of these experiments indicated that the EIN3 protein contains signals sufficient to target GUS to the nucleus. Although EIN3 does not contain any known DNA binding domains, it is possible that the DNA coil–basic region motif near the amino terminus mediates DNA-binding in a novel manner.

Analysis of Southern blots of *Arabidopsis* genomic DNA probed with an *EIN3* cDNA clone resulted in the isolation of three *EIN3*-like (*EIL*) clones. These *EIL1*, *EIL2* and *EIL3* clones, together with *EIN3*, identify a family of genes encoding proteins with similarity ranging from 59% to 85%. The most conserved regions are the amino-terminal portions of the proteins. The basic domains, the coil structure and the proline-rich stretch

are also present in each protein and are similarly arranged. Interestingly, the EIL family members appear to be functionally redundant with EIN3. When *ein3*-1 mutants were transformed with CaMV 35S promotor:*EIL* cDNA gene fusions, both the seedling and adult phenotypes were reverted. Genetic mapping places each member of the family of the genes on different chromosomes in positions that do not correspond to any other well-characterized ethylene mutation.

4. ADDITIONAL ETHYLENE MUTANTS

In addition to *ETR1* and *EIN3,* a number of other ethylene-insensitive mutations have been identified, although only a few are well characterized (Guzman & Ecker 1990; Roman *et al.* 1995). *EIN2*, *EIN3*, *EIN5*, *EIN6* and *EIN7* all show a reduced ability to respond to exogenous or endogenous ethylene and all appear to act downstream of the ethylene receptor. EIN2, EIN3 and EIN5 probably function as part of a single, primarily linear ethylene signal transduction pathway (Roman *et al.* 1995; Kieber 1997*a*,*b*). *ein2* mutants have an extremely strong ethylene-insensitive phenotype (Guzman & Ecker 1990). Unlike *etr1* mutants, *ein2* mutants are also deficient in the development of symptoms in response to infection by virulent pathogens (Bent *et al.* 1992). It is possible that the *EIN2* gene product coordinates several types of ethylene responses.

The *ein6* mutant exists as a single, genetically recessive allele (Roman *et al.* 1995). It is only weakly ethylene-insensitive, but several other aspects of its phenotype are notable. First, *ein6* appears to have a reduced sensitivity to the drug taxol (Roman & Ecker 1996). This is significant because taxol is believed to affect microtubule polymerization and organization (Baskin *et al.* 1994), a process that has been related to the response of cells to ethylene treatment (Heinstein & Chang 1994). If reduced taxol sensitivity proves to be the result of the *ein6* mutation and not a second, linked mutation, these results might indicate that *EIN6* is a downstream component of ethylene signalling, specifically relating ethylene perception to the effects of ethylene on cell shape. When *ein6* is combined with the constitutive ethylene response mutant *ctr1*, seedlings of the double mutant are ethylene-insensitive but the adult phenotype is very similar to that of *ctr1* single mutants. Thus, the epistasis appears to switch, depending on developmental stage. This result is surprising, as *CTR1* acts upstream of all other characterized ethylene-insensitive mutants except *ETR1* (and its *EIN1* allele), *ETR2* and *EIN4*, which, as described here, are most likely to be members of a family of ethylene receptors. If *ein6* is in fact a downstream component of the ethylene signalling pathway that includes *ctr1*, then these results might indicate that EIN6 may function only in seedlings (Roman & Ecker 1996).

A number of other ethylene-insensitive mutants have been identified in *Arabidopsis*, but these are less well characterized. The *ain1* (ACC-insensitive) and *ein5* mutations are recessive, while *ein7* is semi-dominant (Van der Straeten *et al.* 1993; Roman *et al.* 1995). The weak phenotype of these mutants makes genetic complementation and epistasis analysis difficult, but they all map to a similar location on the bottom of chromosome 1.

The mutants described here are affected in a wide range of ethylene-mediated responses and therefore probably disrupt general elements in ethylene signalling, elements that regulate responses in many tissues and at many stages of development. There are two mutants, *ethylene-insensitive roots1* (*eir1*) and *hookless1* (*hls1*), which appear to affect specific ethylene responses of the root and hypocotyl, respectively (Roman *et al.* 1995; Lehman *et al.* 1996). Of these two, only *hls1* is well characterized. The *hls1* mutation disrupts the formation of the apical hook in *Arabidopsis* seedlings grown in both air and ethylene (Guzman & Ecker 1990). The *HLS1* gene has been cloned and is most similar to *N*-acetyltransferases, a diverse group of proteins found in organisms as different as bacteria and mammals (Lehman *et al.* 1996). The expression pattern of *HLS1* indicates that it does not influence hook formation by differential expression across the adaxial and abaxial tissues of the hook. However, the gene is expressed at a higher steady-state level when induced by ethylene, and overexpression of the wild-type *HLS1* gene product resulted in seedlings that displayed a constitutively exaggerated apical hook. The *hls1* mutation also altered the expression of two auxin-related genes, *AtAux2-11* and *SAUR-AC1,* in tissues that are involved in apical hook formation but not in other tissues. Taken together, the evidence indicates that ethylene acts through HLS1 to affect hook curvature and that HLS1 may act by regulating the activity or transport of auxin in the apical hook.

5. CONSTITUTIVE RESPONSE MUTANTS

There are two classes of mutants in *Arabidopsis* that constitutively display the triple response in the absence of exogenous ethylene. The first class produce as much as 100-fold more ethylene than wild-type plants, as three-day-old etiolated seedlings. (Guzman & Ecker 1990; Kieber *et al.* 1993; K. Woeste and J. J. Kieber, unpublished observations). These Eto mutants (ethylene overproducing) can be phenotypically reverted by inhibitors of ethylene biosynthesis or binding, such as aminoethoxyvinylglycine (AVG) (Amrhein & Wenker 1979) and silver ion (Beyer 1976). A total of five Eto loci have been identified: four are genetically dominant and one, *eto1*, is recessive. It is likely that Eto mutations identify important genes in the regulation of ethylene biosynthesis. The dominant mutation *eto2* was recently found to be the result of a single base pair insertion in the carboxy terminus of the *ACS5* gene (Vogel *et al.* 1998). This gene encodes an isoform of ACC synthase which is the limiting step in the ability of *Arabidopsis* to produce ethylene in response to low levels of cytokinin.

The second class of constitutive response mutants do not make more ethylene than wild-type plants and display a triple response phenotype even in the presence of inhibitors of ethylene biosynthesis and binding. These constitutive triple response mutants (Ctr) grow as if they were continuously exposed to ethylene, regardless of the amount of ethylene in their environment. This type of mutant is affected in ethylene signal transduction. The *ctr1* mutation is recessive and has profound effects on the morphology and development of both seedlings and adult plants (Kieber *et al.* 1993). Leaf epidermal cells of *ctr1*

mutants growing in air are one-fifth the size of those of wild-type plants and approximately the same size as those of wild-type plants grown in ethylene. Ethylene inhibits cell elongation in several plant species (Apelbaum & Berg 1971; Steen & Chadwick 1981; Lang *et al.* 1982; Roberts *et al.* 1985; Shibaoka 1994; Yuan *et al.* 1994) and is believed to have an important role in the regulation of plant stature. A deficiency in cell expansion caused by a constitutive ethylene response accounts for many of the other phenotypes of *ctr1*: a shortened hypocotyl, a compact inflorescence, and a reduced root system. Correspondingly, Ein mutants have a larger cell size than wild-type plants and form rosettes that are 25% larger than wild-type rosettes when grown in air (Bleecker *et al.* 1988; Guzman & Ecker 1990).

Ethylene causes the proliferation of root hairs in many plant species, and in some species it can lead to the production of root hairs from ectopic locations (Cormack 1935; De Munk & De Rooy 1971; Abeles *et al.* 1992; Dolan *et al.* 1994). This phenotype is observed in *ctr1* mutant plants as well (Dolan *et al.* 1994), indicating that CTR1 may negatively regulate events that determine hair cell fate. Thus, ethylene may be the diffusible signal that has been proposed to regulate root hair cell differentiation (Bünning 1951). Consistent with this, ethylene-insensitive mutants show a reduction in the number of root hair cells, and the *axr2* mutant, which has roots that are insensitive to ethylene, does not develop root hairs (Wilson *et al.* 1990). Wild-type plants grown in media containing compounds such as silver ions that inhibit ethylene action also have fewer and smaller root hairs.

Other phenotypes of *ctr1* are consistent with the conclusion that *CTR1* functions as a negative regulator of ethylene responses and that it acts in a pathway or pathways that are common to almost all tissues and stages of development. *ctr1* mutant alleles are transmitted at a reduced frequency relative to the wild-type allele, and this is owing to defects in transmission through the female gamete (Kieber & Ecker 1994). Etiolated *ctr1* seedlings open their apical hook more slowly than wild-type plants when they are exposed to light. In addition, adult *ctr1* plants flower later and produce a more compact inflorescence than wild-type plants. At the molecular level, ethylene-regulated genes are constitutively expressed at high levels in *ctr1* mutants. These phenotypes can be copied by growing wild-type plants in ethylene. The role of CTR1 in stress and disease-related responses has not been closely examined.

Analysis of genetic epistasis indicates that ethylene responses in *Arabidopsis* flow through a primarily linear pathway. *CTR1* is completely epistatic to *ETR1*, *EIN4*, *ETR2* and *ERS* and is thus predicted to act downstream of the receptor for ethylene. *EIN2*, *EIN3*, *EIN5*, *EIN6* and *EIN7* all mask the seedling phenotype of *ctr1* mutants and so are predicted to act further downstream. The epistasis of *EIN6* is not complete in adult plants (discussed earlier) which may indicate that some differences in the regulation of ethylene responses may exist between adult and seedling plants, perhaps through an independent signalling pathway. The possibility of a separate, at least partly independent, ethylene signalling pathway is also indicated by the constitutive triple response mutant *ctr2* (K. Woeste and J. J. Kieber, unpublished observations). Adult *ctr2* plants have a severely ethylene-affected phenotype that is lethal at the rosette stage. An ethylene-affected phenotype of this strength is not found in even the predicted complete loss of function mutations of *ctr1* or in plants growing in saturating levels of ethylene.

The *CTR1* gene has been cloned and characterized (Kieber *et al.* 1993). The amino-acid sequence of CTR1 is most similar to the Raf family of protein kinases. Raf is part of a conserved cascade of protein kinases that are central to the signal transduction of a diverse group of external regulatory signals ranging from growth hormones to mitogens and developmental signals (Campbell *et al.* 1995). The various Raf proteins from animal cells share three highly conserved regions (CR1 to CR3 (Heidecker *et al.* 1992)). The CR1 domain consists of a binding domain for the protein Ras that overlaps a cystine-rich zinc finger motif (Ghosh *et al.* 1994). CR2 contains a high proportion of serine and threonine residues that include the targets of Raf-1 autophosphorylation as well as phosphorylation by other serine–threonine protein kinases such as protein kinase C (Kolch *et al.* 1993; Morrison *et al.* 1993). At the carboxy terminus of Raf is the CR3, the kinase catalytic domain. Although Raf and CTR1 have almost no homology in their amino-terminal domains, the homology in the kinase catalytic domain is high. The carboxy terminus of CTR1 has all the hallmark features of a serine–threonine protein kinase, and expression studies have confirmed that CTR1 does have intrinsic Ser–Thr protein kinase activity (Y. Huang, H. Li and J. J. Kieber, unpublished observations).

Receptors generally activate Raf proteins indirectly through the binding of a protein called Ras, a small GTP-binding protein (reviewed in Avruch *et al.* 1994; Daum *et al.* 1994). GTP-bound Ras localizes Raf from the cytoplasm to the plasma membrane where Raf becomes activated, most likely by a phosphorylation in the amino half of the protein. This model of regulation of Raf may not apply to CTR1, as CTR1 lacks homology to the amino half of Raf, and a Ras homologue has not been reported in plants. However, it may be possible that the requirement for Ras *per se* is bypassed in plants, as the ethylene receptor ETR1 has homology to the archetypal bacterial two-component regulator CheY, and CheY has a 3D structure similar to Ras (Lukat *et al.* 1991). Thus, CTR1 may be directly regulated by ETR1. Homologues of both *CTR1* and *ETR1* have been found in other higher plants as well, including tomato and maize, suggesting that these components of ethylene signalling may be conserved among all higher plants (Kieber & Ecker 1994; Wilkinson *et al.* 1995; Zhou *et al.* 1996).

The downstream target of Raf is the dual-specificity protein kinase MEK, which is activated by phosphorylation of two conserved serine residues (Alessi *et al.* 1994). Activated MEK in turn activates MAP kinase (MAPK) by tyrosine and threonine phosphorylation. Activated MAPK phosphorylates various downstream targets including several transcription factors. Because the protein kinase catalytic domain of CTR1 is highly homologous to the CR3 domain of Raf that recognizes MEK (Van Aelst *et al.* 1993), it is possible that one or more of the many MEK homologues in *Arabidopsis* is involved in ethylene signalling.

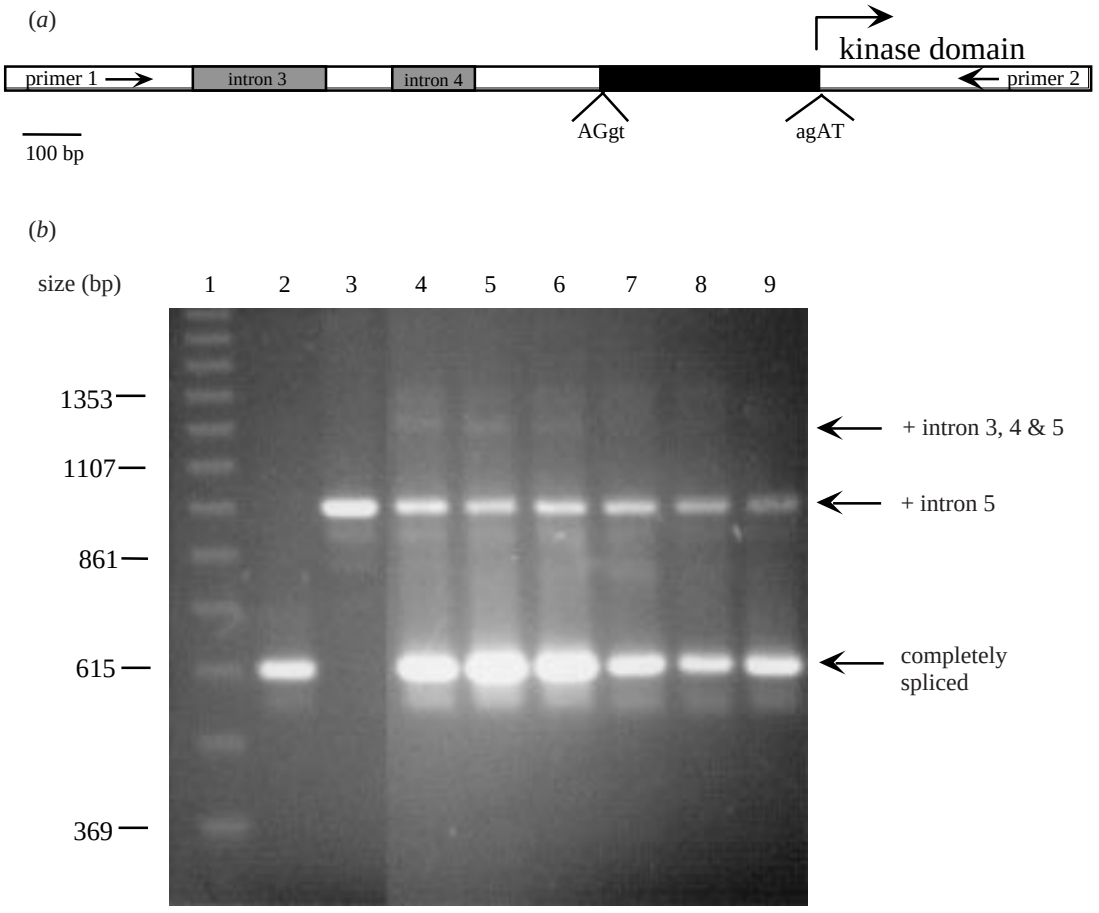

Figure 2. (*a*) Cartoon of the portion of the *CTR1* gene used to evaluate alternative splicing of intron 5. Regions of the gene are shown to scale. Exons or portions of exons are shown with open boxes, introns are shaded. The sequences of the donor and acceptor sites of intron 5 are shown below; nucleotides in the exon are upper case, nucleotides in the intron are lower case. Primer 1 (5′-CCATAGGTAGCCTCTCTGT-3′) and primer 2 (5′- GATCACACCACGGGATGTCC-3′) were used to amplify this region from cDNA. (*b*) Products from amplification of cDNA using primers 1 and 2. Lane 1 is a 123 bp ladder (BRL); lane 2, pCTC1, a cDNA clone of completely spliced *CTR1*; lane 3, pCTCM1-1, a cDNA clone containing intron 5; lanes 4–7, cDNA from three-day-old etiolated *Arabidopsis* seedlings, ecotype WS, treated for 50 h (lane 4), 8 h (lane 5), 4 h (lane 6) or 1 h (lane 7) with 10 p.p.m. ethylene. Lane 8, PCR products from cDNA of untreated, etiolated, three-day-old *Arabidopsis* seedlings grown in air. Lane 9, PCR products from cDNA made from leaves of adult plants grown in air. Arrows indicate products resulting from incompletely spliced mRNA (+ intron 5) or completely spliced product. The upper arrow (+ intron 3, 4 & 5) indicates the size of products predicted if all introns in the region were unspliced. Total RNA (1 µg) from treated or untreated seedlings or adult tissues was reverse-transcribed by using Moloney Murine Leukaemia Virus reverse transcriptase and 1 µl of this product used in subsequent steps. PCR amplification was for 32 cycles of 94 °C (1 min), 52 °C (1.5 min), 72 °C (2 min).

One important effect of ethylene on plants is the alteration of gene expression. Plant genes such as ACC oxidases (K. Woeste and J. J. Kieber, unpublished observation) and other genes related to ethylene biosynthesis, genes for proteins involved in fruit ripening as well as genes related to plant defences to pathogen invasion all show an increased level of transcription in response to ethylene treatment (reviewed by Deikman (1997)). DNA sequences that confer ethylene responsiveness (ethylene response elements (ERE)) to a minimal promotor have been identified from pathogenesis-related (PR) genes, and proteins that can bind to an ERE have been identified in tobacco (Ohme-Takagi & Shinshi 1995). The steady-state level of mRNA for these ERE binding proteins (EREBP) is rapidly increased by ethylene treatment. It is possible that proteins of this type are the primary target of ethylene signalling and that they, in turn, regulate a host of other secondary ethylene responses.

The regulation of *CTR1* expression is not well understood. The steady-state level of *CTR1* transcript is not strongly increased when wild-type plants are treated with ethylene (Kieber & Ecker 1993). When λ cDNA clones containing *CTR1* were isolated, three of 30 clones contained an unspliced intron 5 (Pallin 1996). This alternative splicing created a cDNA 357 bp longer than the most prevalent transcript (see figure 2). To further examine the splicing of this intron, we used RT-PCR with primers flanking introns 3, 4 and 5 of *CTR1* to examine the proportion of message containing these introns. Total RNA was isolated from adult tissues and organs of *Arabidopsis* and from three-day-old seedlings grown in air or treated with ethylene for 1, 4, 8 and 50 h and converted to cDNA by using reverse-transcriptase. The resulting products were

amplified using primers that flank the alternatively spliced intron as well as two adjacent introns. The products of the PCR reaction revealed that about 25% of *CTR1* RNA contains an unspliced intron 5, regardless of tissue source or treatment (figure 2). In contrast, there were almost no detectable unspliced products corresponding to the other introns. The incompletely spliced variant includes the sequence from nucleotide 2027 to 2384 (Kieber & Ecker 1993), which places it precisely midway between the presumed regulatory domain in the amino terminus and the kinase domain at the carboxy terminus. This intron is the largest of 14 introns in the *CTR1* gene; it contains canonical donor and acceptor splice sites and does not appear to contain any unusual secondary structure. Neither is the nucleotide sequence of the intron homologous to any known regulatory sequences. The alternatively spliced product is also present in the purified polyadenylated RNA even though it contains translational stops in all three frames (not shown). The predicted product of the unspliced mRNA would correspond to the full length of the presumed regulatory domain of CTR1. It is not known if the truncated CTR1 protein is expressed, but *Arabidopsis* overexpressing the amino terminus of CTR1 displayed a dominant negative phenotype (H. Li and J. J. Kieber, unpublished observations). It is possible that a truncated CTR1 protein (without a kinase domain) could function to increase ethylene sensitivity by competing with full-length CTR1 for an upstream activating signal.

Alternative splicing can play a critical adjunct to the regulation of promotor activity, and the production of alternative products can be related to separate gene functions (McKeown 1992). The *CTR1* homologue B-Raf is expressed as a series of isoforms which are the result of alternative splicing of two exons upstream of the kinase domain (Papin *et al*. 1995). The unspliced intron of *CTR1* is present as a small but consistent portion of the mRNA pools of both seedling and adult *Arabidopsis* plants, although it is unclear if the incomplete splicing of the *CTR1* transcript is regulated. It does not appear to be regulated by ethylene or tissue type and it is possible the unspliced form has no function apart from indicating that splicing at this site is inefficient.

6. CONCLUSIONS

The genetic approach based on the triple response phenotype has been a powerful tool for investigating ethylene signal transduction. Several fundamental elements of the pathway have been described (a receptor, elements in a kinase cascade, a presumed transcription factor) and taken together can be assembled into a model for ethylene signalling (see figure 3). A plant cell's perception of ethylene begins when the gas binds to a receptor that is almost certainly encoded by a member or members of the *ETR1* gene family. Binding probably occurs in the hydrophobic region of ETR1 and this may induce a conformational change that alters the activity of the ETR1 histidine kinase domain. This change in activity either directly or indirectly results in an inactivation of the kinase activity of CTR1 and the inactivation of the downstream kinases, MEK and MAPK. As a result, EIN2 becomes active and in some way communicates the ethylene signal to EIN3. The various ethylene-regulated responses originate either by transcription or by post-transcriptional events that lead to the phenotypes associated with exposure to ethylene. The elements described in the model provide only a skeleton; most of the details of ethylene signalling, especially the regulation of these elements, remain obscure. The triple response screen will no doubt continue to provide insights into some of these details as it is far from saturated; some genes are represented by only single alleles. Modifications of the triple response screen may be necessary to identify elements with redundant or overlapping functions. In addition, there are tantalizing hints that alternative signal transduction pathways for ethylene may exist in plants. These may be involved in the fine tuning of responses: responses specific for particular elicitors, particular tissues or stages of growth. Biochemical and molecular genetic approaches should prove more fruitful now that key elements in the pathway have been cloned. Elements that act in, or respond to, more than one hormone signalling pathway have already been identified; for example, the *axr* mutants, which are resistant to auxin and ethylene and in some cases other hormones as well (Pickett *et al*. 1990; Wilson *et al*. 1990; Hobbie *et al*. 1994). The further characterization of these genes should provide important insights into how complex growth and development is regulated in plants.

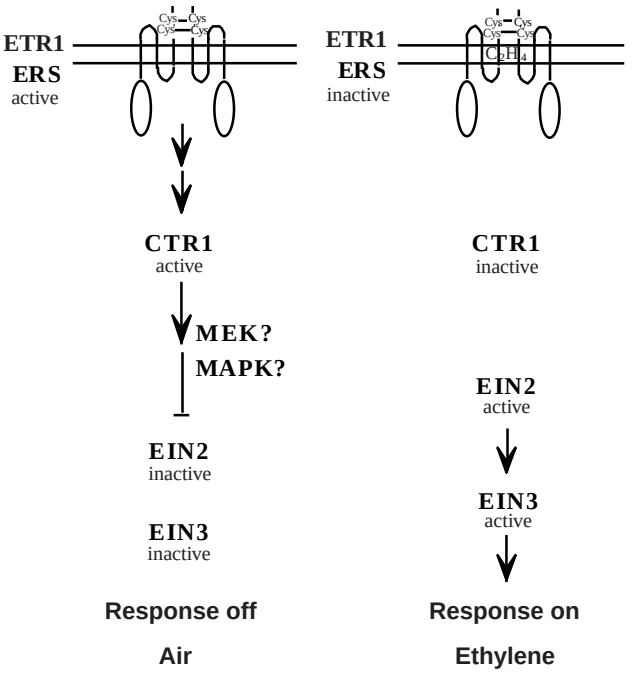

Figure 3. Model for ethylene signal transduction in *Arabidopsis*. Arrows represent positive regulatory steps and the flat symbol represents a negative regulatory step. Each arrow may represent several steps in the transduction pathway because direct interactions have not yet been demonstrated. The components depicted here are discussed in greater detail in the text.

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