Arabidopsis Ethylene-Response Gene *ETR1*: Similarity of Product to Two-Component Regulators

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Ethylene behaves as a hormone in plants, regulating such aspects of growth and development as fruit ripening, flower senescence, and abscission. Ethylene insensitivity is conferred by dominant mutations in the *ETR1* gene early in the ethylene signal transduction pathway of *Arabidopsis thaliana*. The *ETR1* gene was cloned by the method of chromosome walking. Each of the four known *etr1* mutant alleles contains a missense mutation near the amino terminus of the predicted protein. Although the sequence of the amino-terminal half of the deduced ETR1 protein appears to be novel, the carboxyl-terminal half is similar in sequence to both components of the prokaryotic family of signal transducers known as the two-component systems. Thus, an early step in ethylene signal transduction in plants may involve transfer of phosphate as in prokaryotic two-component systems. The dominant *etr1-1* mutant gene conferred ethylene insensitivity to wild-type *Arabidopsis* plants when introduced by transformation.

Gaseous ethylene serves as a hormone in plants to regulate a diverse and complex range of responses throughout plant growth and development (*1*). Ethylene is well-known as the fruit-ripening hormone. Other responses to ethylene include flower and leaf senescence, leaf abscission, flower initiation, and breaking of seed dormancy. Biosynthesis of ethylene in plants is highly regulated and is under both positive and negative feedback control. Environmental stresses such as wounding, chilling, pathogen invasion, and flooding enhance ethylene biosynthesis. Adaptive responses to this stress-induced ethylene include acceleration of senescence, abscission of infected organs, and induction of specific defense proteins. A number of ethylene-induced genes have been isolated and characterized (*1*).

Although the biosynthetic pathway of ethylene is understood (*2*), our knowledge of the molecular mechanisms underlying ethylene perception and signal transduction is limited. Saturable binding sites for ethylene have been demonstrated in plant tissues and extracts (*1*). However, no direct connection between these binding sites and physiological responses to ethylene has been established. Several components involved in ethylene signal transduction have been defined by mutants isolated in *Arabidopsis thaliana* with the use of a simple screen, based on the classic ethylene response in seedlings known as the triple response (*3–7*). In the dark, germinating seedlings show high rates of ethylene production when their growth is mechanically impeded, such as occurs during soil emergence (*8*). In response to this ethylene, dark-grown seedlings display inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl, and retention and accentuation of the apical hook. This change in growth habit is thought to facilitate penetration of the soil while protecting the delicate shoot apex (*7, 8*). *Arabidopsis* mutants that are insensitive to ethylene lack these responses.

Mutations in at least three different genes cause insensitivity to ethylene (*3–5*). One of these is *ETR1*. Four mutant alleles of *ETR1* have been identified, and all four are dominant to the wild-type allele (*9*). A study of one of these mutant alleles, *etr1-1*, revealed an absence of all measured ethylene responses in various parts of the plant (seeds, roots, hypocotyls, leaves, and stems) (*3*). In addition, saturable ethylene binding in *etr1-1* was approximately one-fifth of that in the wild type (*3*). Therefore, ETR1 acts early in the ethylene signal transduction pathway, possibly as an ethylene receptor, or as a regulator of the pathway.

We now describe the cloning and DNA sequence of the *Arabidopsis ETR1* gene. The amino-terminal half of the predicted ETR1 protein shows no clear sequence similarities to the available protein sequence databases, whereas the remaining carboxyl-terminal portion contains a high degree of sequence identity with the large family of prokaryotic signal transducers known as the two-component systems.

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In bacteria, the two protein components, referred to as the sensor and the response regulator, function together to regulate adaptive responses to a broad range of environmental stimuli. Adaptive responses include chemotaxis, host recognition for pathogen invasion, osmoregulation, phosphate regulation, nitrogen regulation, and stress-induced sporulation (*10, 11*).

In general, each component contains a conserved domain and a variable domain. Most sensor proteins consist of a variable amino-terminal domain (typically located in the periplasmic space flanked by two transmembrane domains), and a conserved carboxyl-terminal histidine protein kinase located in the cytoplasm. Signal perception by the amino-terminal domain of the sensor results in autokinase activity by the histidine kinase domain. The phosphate group on the histidine is then transferred to a certain aspartate residue within the conserved amino-terminal domain of the cognate cytoplasmic response regulator. Phosphorylation of the aspartate activates or inhibits the variable carboxyl-terminal domain of the response regulator. This last domain, which in many cases is a transcriptional activator, is absent from some of the bacterial response regulators, and from the predicted ETR1 protein. Although most sensors and response regulators characterized in bacteria are two separate proteins, the predicted ETR1 protein, as well as a subset of the bacterial family, contain both components in a single protein.

ETR1 gene cloning and characterization. The *ETR1* locus was mapped to the lower portion of chromosome 1 between the genetic markers *ap1* and *clv2*. *ETR1* is 6.5 ± 1.0 centimorgans (cM) from *AP1* and 2.8 ± 1.1 cM from *CLV2* (*12*). Of the restriction fragment length polymorphisms (RFLPs) that reside in this region of chromosome 1, one marker, λbAt315, completely cosegregated with the *etr1-1* phenotype in 112 F_2 chromosomes (*13*). We therefore used the λbAt315 clone as a probe to initiate a chromosome walk in the *ETR1* gene region. Various cosmid libraries were used for the walk: an *ETR1* wild-type library, an *etr1-1* mutant library, and yeast genomic DNA libraries containing subclones of two yeast artificial chromosomes (YACs) that hybridized to λbAt315 (*14*). Using restriction analysis and sequential hybridization to these libraries, we obtained a contiguous map (contig) of overlapping cosmids that spanned approximately 230 kb (Fig. 1) (*14*).

We used fine-structure RFLP mapping to orient the walk with respect to the chromosome and to refine the position of the *ETR1* gene to a 47-kb interval of the contig (Fig. 1). To create the fine-structure map, we isolated meiotic recombinants from F_2 self-

progeny of crosses between the *etr1-1* mutant (ecotype Columbia) and *ap1* or *clv2* marker plants (both of ecotype Landsberg) (*15*). Recombination break points were mapped with the use of DNA fragments of the chromosome walk as RFLP probes (Fig. 1) (*15*). The nearest break points flanking the *ETR1* gene defined a 47-kb DNA interval.

To search for potential *ETR1* transcripts, we screened an *Arabidopsis* seedling cDNA library with DNA fragments in the 47-kb region (*16*). The first cDNA clone we obtained, designated λC4, was detected with the 4.25-kb Eco RI fragment (Fig. 1).

We determined the nucleotide sequences of the λC4 cDNA and the corresponding genomic DNA (Fig. 2) (*17*). As λC4 lacked approximately 1000 nucleotides (nt) of the 5′ end (based on RNA blots described below), we extended the cDNA in the 5′ direction by reverse transcription followed by polymerase chain reaction (PCR) (*17*). The longest extension gave a total cDNA length of 2786 bases [not including the poly(A) tail]. A potential TATA box (5′-ATAATAATAA) lies 33 bp upstream of this 5′ end in the genomic sequence. The size of the endogenous transcript, estimated from RNA blots, is about 2800 bases. Based on comparison of the cDNA and the genomic DNA sequences, the gene has six introns, one of which is in the 5′ untranslated leader. The exons encode a single open reading frame of 738 amino acids (Fig. 2). The predicted protein has a calculated molecular size of 82.5 kD.

We established that this gene is, in fact, *ETR1* by comparing the nucleotide sequences of the wild-type allele and the four mutant alleles (*18*). In each mutant, we found a single nucleotide substitution that results in a missense mutation. All four mutations are clustered in the amino-terminal region of the deduced protein sequence (Fig. 2).

The *ETR1* message was examined in RNA blots (*19*). The 2800-nt *ETR1* transcript was present in all *Arabidopsis* organs and tissues examined—leaves, roots, stems, flowers, and seedlings. No differences were observed in size or quantity of *ETR1* transcripts from the wild-type or the *etr1-1* mutant, and treatment with ethylene did not detectably alter the amount of *ETR1* mRNA in dark-grown wild-type seedlings.

When we hybridized the *ETR1* gene to *Arabidopsis* genomic DNA blots at normal stringency, only the expected fragments of the *ETR1* locus were observed (*20*). At reduced stringency, however, a number of fragments were detected, which suggests that a family of similar genes exists in *Arabidopsis* (*21*).

The predicted amino-terminal sequence of ETR1 (residues 1 to 313) has no similarity to sequences in the available protein sequence databases (*22*). The remaining carboxyl-terminal sequence, however, is highly similar to the conserved domains of both the sensor and the response regulator of the prokaryotic two-component system of signal transduction (*22*). In bacteria, the histidine protein kinase domain of the sensor is characterized by five sequence motifs arranged in a specific order with loosely conserved spacing (*10*). The deduced ETR1 sequence contains all five motifs with the same relative order and spacing found in the bacterial proteins (Fig. 3A) (*23*). The deduced sequence is most similar to the sequences of *Escherichia coli* BarA (*24*) and *Pseudomonas syringae* LemA (*25*). Over the entire histidine kinase domain (241 amino acids), 43 and 41 percent of amino acids are identical with BarA and LemA, respectively, and 72 and 71 percent are similar, respectively (*23*). The function of BarA is unknown, although it was cloned on the basis of its ability to complement a deletion in the *E. coli* osmotic sensor protein EnvZ (*24*). LemA is required for pathogenicity of *P. syringae* on bean plants (*25*). Other bacterial proteins with sequences highly similar (*23*) to this domain of the putative ETR1 protein are: *Xanthomonas campestris* RpfC (35 percent identity), which may be involved in host recognition for pathogenicity on cruciferous plants (*26*); *E. coli* RcsC (34 percent identity), which is involved in regulation of capsule synthesis (*27*); and *E. coli* ArcB (25 percent identity), which is responsible for repression of anaerobic enzymes (*28*).

Adjacent to the putative histidine kinase domain, the deduced ETR1 sequence exhibits the characteristic structural features and amino acids of the conserved domain of bacterial response regulators. Predictions of response regulator structure are based on the known three-dimensional structure of the chemotaxis response regulator, CheY, of *Salmonella typhimurium* and *E. coli*. CheY consists of five parallel β strands surrounded by five α helices (*29*). The protein sequences of many bacterial response regulators can be aligned with this secondary structure based on residues that correspond to the hydrophobic core (*11*). The deduced ETR1 sequence can be similarly aligned (*30*). In addition to the characteristic secondary structure, bacterial response regulators contain at specific positions four highly conserved residues—three aspartates and a lysine (*10, 11*). The side chain of the conserved lysine protrudes into an acidic pocket formed by the three aspartates (*11*). The third aspartate is also the recipient of the phosphate from phosphohistidine (*11*). Except for the conservative substitution of glutamate for the second aspartate, these amino acids are found at the corresponding positions in the deduced ETR1 sequence (Fig. 3B) (*31*). In this domain (a stretch of 121 amino acids in ETR1), the deduced ETR1 sequence is most similar (*31*) to the sequences of *Bordetella parapertussis* BvgS (29 percent identity, 60 percent similarity), which controls virulence-associated genes for pathogenicity in humans (*32*), *E. coli* RcsC (29 percent identity, 64 percent similarity), *P. syringae* LemA (26 percent identity), *X. campestris* RpfC (25 percent identity), and *E. coli* BarA (20 percent identity). The deduced ETR1 sequence shares as many identical amino acids with the bacterial response regulator sequences as the bacterial sequences share between themselves [20 to 30 percent identical (*11*)].

All of the bacterial proteins that are similar in sequence to ETR1 are also topologically similar to ETR1 in that they contain both the histidine kinase domain and the conserved domain of the response regulator. These bacterial proteins also contain potential membrane-spanning domains flanking their variable amino-terminal domains. From hydropathy analysis (*33*), the amino-terminal end of the predicted ETR1 protein consists of three closely-spaced hydrophobic regions (residues 26 to 43, 48 to 76, and 83 to 115) (Fig. 2), one or more of which could comprise a membrane-spanning domain (*34*). All four *etr1* mutations lie within these hydrophobic regions, indicating that this amino-terminal

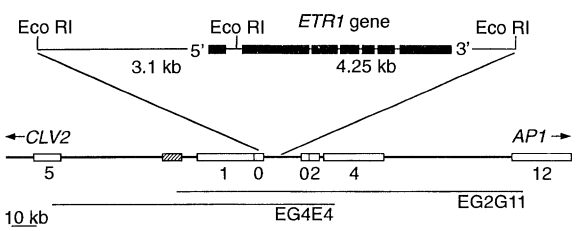

Fig. 1. Map of the *ETR1* gene relative to the chromosome walk contig. The structure of the *ETR1* gene is shown, with solid bars representing exons. The four smallest introns are enlarged to allow visibility. In the contig, the starting position of the chromosome walk (λbAt315) is indicated by a hatched bar. Open bars represent the DNA segments used as probes to detect recombination breakpoints. The maximum number of breakpoints detected by each probe is given below each bar (fewer breakpoints were sometimes detected by different RFLPs); numbers to the right of the *ETR1* gene indicate the number of F_2 recombinants (out of a total of 74) that occurred between *etr1-1* and *ap1*, and the numbers to the left of the *ETR1* gene indicate the number of F_2 recombinants (out of a total of 25) that occurred between *etr1-1* and *clv2*. The nearest breakpoints flanking the *ETR1* gene are at most about 47 kb apart. The relative positions of YACs EG2G11 and EG4E4 are shown with respect to the contig.

region plays an important role in ethylene signal transduction.

Ethylene insensitivity in *etr1-1* transformed wild-type plants. Ethylene insensitivity was conferred to wild-type *Arabidopsis* plants when the dominant *etr1-1* mutant gene was stably introduced by *Agrobacterium*-mediated transformation. The mutant gene was contained on a 7.3-kb genomic DNA fragment (the two Eco RI fragments shown in Fig. 1), which includes approximately 2.7 kb upstream of the putative transcription initiation site, and approximately 1 kb downstream of the polyadenylation site (*35*). The transformation vector carried a gene for kanamycin selection in plants, in addition to the *etr1-1* gene (*35*). Following *Agrobacterium*-mediated transformation of root explants (*35*), we regenerated ten independent kanamycin-resistant plants. Eight of these plants produced ethylene-insensitive self-progeny as evaluated by the dark-grown seedling response to ethylene (Fig. 4). As a control, we transformed wild-type plants with the 7.3-kb DNA fragment of the wild type. From this transformation, we regenerated six independent kanamycin-resistant plants. All six plants gave rise only to ethylene-sensitive self-progeny that did not appear to be different from the wild type in their ethylene response.

Quantitative differences in ethylene insensitivity appeared in the *etr1-1* transformants (Fig. 4). Of the ten kanamycin-resistant lines, six produced individuals showing clear ethylene insensitivity in hypocotyl elongation and complete or almost complete insensitivity in root elongation. Two other lines produced plants showing partial insensitivity in both the hypocotyl and root, and two lines appeared to be wild type. Partial or undetected ethylene insensitivity in the transformants may be due to low expression of *etr1-1*, which can be caused by position effects (*36*), DNA methylation (*37*), or possibly by incomplete transfection (*38*).

Potential ETR1 function. The sequence and structural homology to the prokaryotic two-component systems indicates that ETR1 may be a sensor of ethylene, that is, the amino-terminal domain perceives ethylene and transduces the signal through phosphate transfer reactions by the two carboxyl-terminal domains. Whether ETR1 perceives ethylene by directly binding it, or

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                                  M  E  V  C  N  C  I  E  P  Q  W  P  A  D  E   15
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L  L  M  K  Y  Q  Y  I  S  D  F  F  I  A  I  A  Y  F  S  I  P  L  E  L  I   40
ACTTTGTGAAGAAATCAGCCGTGTTTCCGTATAGATGGGTACTTGTTCAGTTTGGTGCTTTTATCGTTCTTTGTG  975
Y  F  V  K  K  S  A  V  F  P  Y  R  W  V  L  V  Q  F  G  A  F  I  V  L  C   65
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G  A  T  H  L  I  N  L  W  T  F  T  T  H  S  R  T  V  A  L  V  M  T  T  A   90
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K  V  L  T  A  V  V  S  C  A  T  A  L  M  L  V  H  I  I  P  D  L  L  S  V   115
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                                    V  A  V  A  L  S  H  A  A  I  L  E  E   315
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TAGAACaacaacaatggcgttacttagtttctcaatcaacccgatctccaaaac                      3879
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Fig. 2. Genomic DNA sequence and deduced amino acid sequence of *ETR1*. Exons in the nucleotide sequence are shown in uppercase letters, with the putative 5′ end of the cDNA at nucleotide 224. Amino acids shown in boldface have the following replacements in the mutant alleles: Ala[31] to Val in *etr1-3*, Ile[62] to Phe in *etr1-4*, Cys[65] to Tyr in *etr1-1*, and Ala[102] to Thr in *etr1-2*. Three hydrophobic regions are underlined. The nucleotide sequence is on file with GenBank (accession number L24119). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

by indirectly monitoring it through some other components, is not known. For most bacterial sensors, the exact functions of amino-terminal domains are unknown. In the case of the *Rhizobium meliloti* protein FixL, the stimulus is received directly; FixL is a hemoprotein that senses oxygen by binding it directly (*39*). For *E. coli* CheA, the ligand is bound by a separate receptor protein (*40*). If ETR1 binds ethylene, then the binding site is likely to contain a transition metal such as Zn^{2+} or Cu^{2+}, as predicted by the affinity of alkenes for metals, and from observations on the physiological activity of ethylene analogs (*41*).

There are two general explanations for the dominant nature of the mutant alleles—a poisoned complex (*3*), or constitutive suppression of ethylene response. If ETR1 functions as a multimer, mutations in ETR1 could disrupt the formation or activity of the multimer, resulting in dominance, as described for dominant negative mutations (*42*). Bacterial sensors may act as dimers in which one subunit phosphorylates the other subunit (*43*). The other explanation—constitutive suppression of the ethylene response—could suffice if signaling by ETR1 is blocked or locked into a particular state. It has been proposed that in bacteria a conformational change in the amino-terminal domain of the sensor is coupled to a concomitant change in the histidine kinase domain, and vice versa, corresponding to opposing on and off states (*10*). This model is supported by the observation that bacterial histidine kinase domains that are uncoupled from their amino termini become constitutive kinases (*10*). Similarly, amino-terminal mutations in *ETR1* might either prevent ethylene binding (or prevent some other interaction), or alter steric constraints, locking the carboxyl-terminal domain into a conformation that corresponds to the activated state. The response to constitutive activation (signaling the absence of ethylene) would be dominant to wild type. At least two of the *ETR1* mutations could affect steric constraints because they do not alter polarity or charge, only the size of the side chain. For example, in the *etr1-3* allele, the alanine at position 31 is replaced by valine, which is slightly larger. In the *Caenorhabiditis elegans mec-4* protein (which is unrelated to the two-component systems), an alanine-to-valine substitution in a hydrophobic region also produced a dominant mutant (*44*); steric hindrance was suspected to be the cause, in that only amino acids with larger, not smaller, side chains at that position produced the mutant phenotype (*44*).

The target of ETR1 action in ethylene signal transduction remains unknown. Many of the bacterial response regulators contain a variable carboxyl-terminal domain, which is often a DNA-binding transcriptional activator that is activated by aspartate phosphorylation of the response regulator. The predicted ETR1 protein does not contain such a domain. Potential ETR1 targets include components represented by the other ethylene response mutants in *Arabidopsis*. One of these is CTR1, which acts downstream of ETR1 based on epistasis analyses (*6*). CTR1 appears to be a negative regulator of ethylene response, because loss of CTR1 function results in constitutive ethylene response (*6*). Activation of CTR1 by ETR1 would be consistent with dominant ethylene insensitivity in the *etr1* mutants, as constitutive ETR1 activity in the mutants (as proposed above) would result in constitutive activation of CTR1, thereby repressing ethylene response.

The *Arabidopsis CTR1* gene has been cloned and codes for a protein kinase with amino acid sequence similarity to members of the Raf protein kinase family in mammals, *Drosophila*, and *C. elegans* (*6*). In these other

A

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                                        *
ETR1  QNVALDLARREAETAIRARNDFLAV MNHEMRTPM HAIIALSSLLQETELTPEQRL  380
BARA  QNVELDLAKKRAQEAARIKSEFLAN MSHELRTPL NGVIGFTRLTLKTELTPTQRD  329
LEMA  QNIELDLARKEALEASRIKSEFLAN MSHEIRTPL NGILGFTHLLQKSELTPRQFD  311
RPFC        RAVREARHANQAKSRFLAN MSHEFRTPL NGLSGMTEVLATTRLDAEQKE  176

ETR1  MVETILKSSNLLATLMNDVLDLSRLEDGSLQLELGTFNLHTLFREVLNLIKPIAVV  436
BARA  HLNTIERSANNLLAIINDVLDFSKLEAGKLILESIPFPLRSTLDEVVTLLAHSSHD  385
LEMA  YLGTIEKSADNLLSIINEILDFSKIEAGKLVLDNIPFNLRDLLQDTLTILAPAAHA  367
RPFC  CLNTIQASARSLLSLVEEVLDISAIEAGKIRIDRRDFSLREMIGSVNLILQPQARG  232

ETR1  KKLPITLNLAPDLPEFVVGDEKR LMQIILNIVGNA VKFSKQGSI (26) LRVK  510
BARA  KGLELTLNIKSDVPDNVIGDPLR LQQIITNLVGNA IKFTENGNI (15) IEVQ  448
LEMA  KQLELVSLVYRDTPLALSGDPLR LRQILTNLVSNA IKFTREGTI (15) LRIS  430
RPFC  RRLEYGTQVADDVPDLLKGDTAH LRQVLLNLVGNA VKFTEHGHV (16) LRFD  296

ETR1  VKDSGAGIN PQDIPK IFTKF AQTQSLATRSSG GSGLGL AISKRFVNLMEGNI  562
BARA  IRDTGIGIP ERDQSR LFQAF RQADASISRRHG GTGLGL VITQKLVNEMGGDI  500
LEMA  VQDTGIGLS SQDVRA LFQAF SQADNSLSRQPG GTGLGL VISKRLIEQMGGEI  482
RPFC  VEDTGIGVP MDMRPR LFEAF EQADVGLSRRYE GTGLGT TIAKGLVEAMGGSI  348
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B

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ETR1  LKVLVM DE NGVSRMVTKGLLVHLGCEVTTVSSNEECLRV  648
BVGS  LRVLVV DD HKPNLMLLRQQLDYLGQRVVAADSGEAALAL 1011
RCSC  MMILVV DD HPINRRLLADQLGSLGYQCKTANDGVDALNV  847
LEMA  PRVLCV DD NPANLLLVQTLLEDMGAEVVAVEGGYAAVNA  695

                    *
ETR1  VSHEH-KVVFM D VCMPGVENYQIALRIH (10) PLLVA  690
BVGS  WHEHAFDVVIT D CNMPGINGYELARRIR (12) CILFG 1056
RCSC  LSKNHIDIVLS D VNMPNMDGYRLTQRIR  (5) LPVIG  885
LEMA  VQQEAFDLVLM D VQMPGMDGRQATEAIR (10) LPIVA  738

ETR1  LSGNTDKSTKEKCMSFGLDGVLL K PVSLDNIRDVLSDLL  729
BVGS  FTASAQMDEAHACRAAGMDDCLF K PIGVDALRQRLNEAA 1095
RCSC  VTANALAEEKQRCLESGMDSCLS K PVTLDVIKQSLTLYA  924
LEMA  LTAHAMANEKRSLLQSGMDDYLT K PISERQLAQVVLKWT  777
```

Fig. 3. Amino acid sequence alignments of the predicted ETR1 protein with the conserved domains of several bacterial histidine kinases and response regulators. Amino acids are shown in boldface type at positions where there are at least two identities among the bacterial proteins compared with ETR1. (**A**) The deduced ETR1 amino acid sequence (residues 326 to 562) aligned with the histidine kinase domains of *E. coli* BarA (*24*), *P. syringae* LemA (*25*) and *X. campestris* RpfC (*26*). Boxes surround the five conserved motifs characteristic of the bacterial histidine kinase domain as compiled by Parkinson and Kofoid (*10*). The asterisk (*) indicates the conserved histidine residue that is the site of autophosphorylation. Numbers and positions of amino acids not shown are given in parentheses. (**B**) The deduced ETR1 amino acid sequence (residues 610 to 729) aligned with the response regulator domains of *B. parapertussis* BvgS (*32*), *P. syringae* LemA (*25*), and *E. coli* RcsC (*27*). Amino acids are shown in boldface type where there are at least two identities with ETR1. Boxes surround the four highly conserved residues in bacterial response regulators (*11*). The asterisk (*) indicates the conserved aspartate residue that is the site of phosphorylation. Numbers and positions of amino acids not shown are given in parentheses. For alignment purposes, a gap (–) was introduced in the ETR1 sequence.

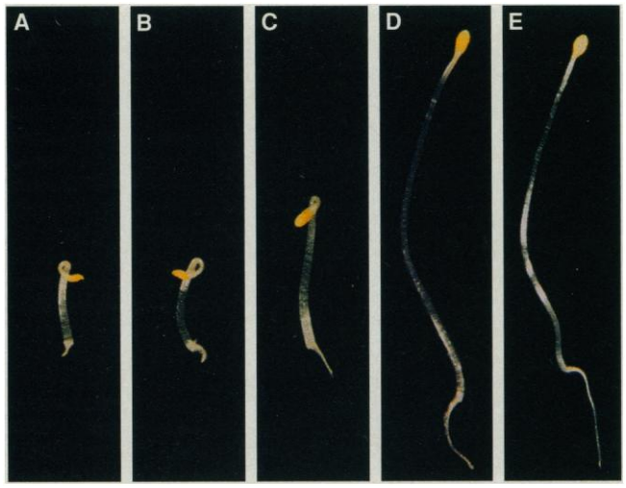

Fig. 4. Ethylene insensitivity displayed in seedlings transformed with the dominant *etr1-1* mutant gene. The ethylene response phenotype of dark-grown seedlings (*3*) is shown for (**A**) wild type (ethylene-sensitive), (**B**) a control line after transformation with the wild-type *ETR1* gene, (**C** and **D**) two lines after transformation with the mutant *etr1-1* gene, and (**E**) the *etr1-1* mutant (ethylene-insensitive). The phenotype in (C) typifies partial ethylene insensitivity (transformant line 21), whereas that in (D) typifies complete insensitivity (transformant line 12).

eukaryotes, similar signal transduction cascades for cell differentiation and growth have been identified, usually starting with cell surface tyrosine kinase receptors and proceeding to Raf activation (*45*). Raf interacts with the upstream Ras protein in these functionally conserved pathways (*46*). It is plausible that ETR1 interacts with the CTR1 Raf homolog, as the three-dimensional structure of the bacterial response regulator CheY, which is the inferred structure of all bacterial response regulators (*11*), is strikingly similar to that of Ras (*47*). The supposed pairing of ETR1 and CTR1—a two-component system homolog and a Raf homolog—in the same signal transduction pathway would be a novel combination of signaling components. Alternatively, it is possible that ETR1 is not an immediate component of the ethylene signal transduction pathway, but rather a regulator of the pathway. The yeast SLN1 protein, which is another eukaryotic example of the two-component systems, is potentially involved in proteolytic regulation of cellular processes (*48*). This suggests that ETR1 could conceivably regulate the stability of components of the ethylene signaling pathway.

At least two eukaryotic proteins in addition to ETR1 and SLN1 show sequence similarity to the histidine kinase domain of two-component systems. In both of these proteins—phytochrome in plants (*49*) and branched-chain α-ketoacid dehydrogenase (BCKDH) in rat (*50*)—the similarity to bacterial histidine kinases is less than that of ETR1, and there is no response regulator domain. Phytochrome is a photoreceptor whose molecular mechanism is unclear. BCKDH catalyzes the phosphorylation and inactivation of a key regulatory enzyme of the valine, leucine, and isoleucine catabolic pathways. Restriction of dietary protein results in an adaptive increase in BCKDH activity (*50*). The target protein of BCKDH is phosphorylated at serine residues (*50*). Further investigation is necessary to determine whether any of these eukaryotic proteins are functionally similar or identical to the bacterial proteins. Although their biochemical activities may have diverged, sensors and response regulators could prove to be widespread in plants and other eukaryotes, and to participate in a diversity of signaling tasks.

REFERENCES AND NOTES

1. F. B. Abeles, P. W. Morgan, M. E. Saltveit, Jr., *Ethylene in Plant Biology* (Academic Press, New York, ed. 2, 1992).
2. H. Kende, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 283.
3. A. B. Bleecker, M. A. Estelle, C. Somerville, H. Kende, *Science* **241**, 1086 (1988).
4. P. Guzman and J. R. Ecker, *Plant Cell* **2**, 513 (1990).
5. D. Van der Straeten, A. Djudzman, W. V. Caeneghem, J. Smalle, M. V. Montagu, *Plant Physiol.* **102**, 401 (1993).
6. J. J. Kieber, M. Rothenberg, G. Roman, K. Feldmann, J. R. Ecker, *Cell* **72**, 427 (1993).
7. N. V. J. Harpham *et al.*, *Ann. Bot.* **68**, 55 (1991).
8. J. D. Goeschl, D. L. Rappaport, H. K. Pratt, *Plant Physiol.* **41**, 877 (1966).
9. The *ETR1* mutant alleles (all ecotype Columbia) are: *etr1-1*, formerly *etr* [*3*], *etr1-2* (G. Q. Chen and A. B. Bleecker, unpublished), *etr1-3*, formerly *ein1* [*4*], and *etr1-4* (G. Roman and J. R. Ecker, unpublished). The *etr1-1*, *etr1-2*, and *etr1-3* mutations were induced with ethyl methanesulfonate. The *etr1-4* mutation was induced with diepoxybutane.
10. J. S. Parkinson and E. C. Kofoid, *Annu. Rev. Genet.* **26**, 71 (1992).
11. J. B. Stock, A. J. Ninfa, A. M. Stock, *Microbiol. Rev.* **53**, 450 (1989).
12. The *etr1-1* mutant was crossed with two lines carrying the recessive visible markers *ap1* and *clv2* [M. Koornneef *et al.*, *J. Hered.* **74**, 265 (1983)], respectively. The F_1 progeny were allowed to self-pollinate. Out of 280 *ETR1* wild-type F_2 plants, there were 36 *AP1* wild types. Out of 107 *ETR1* wild-type F_2 plants, there were 6 *CLV2* wild types. The Kosambi mapping function [D. D. Kosambi, *Ann. Eugen.* **12**, 172 (1944)] was applied to the recombination percentages, which are given in centimorgans.
13. The *etr1-1* mutant was crossed with tester line W100 (ecotype Landsberg) [M. Koornneef, C. J. Hanhart, E. P. Van Loenen-Martinet, J. H. Van der Veen, Arabidopsis *Inf. Serv.* **23**, 46 (1987)] and the F_1 plants were allowed to self-pollinate. Linkage of RFLP markers to the *ETR1* locus was analyzed in 56 F_2 plants as in C. Chang, J. L. Bowman, A. W. DeJohn, E. S. Lander, and E. M. Meyerowitz [*Proc. Natl. Acad. Sci. U.S.A.* **85**, 6856 (1988)]. Marker λbAt315 is described in *ibid.*
14. EG4E4 and EG2G11 were isolated from a YAC library [E. Grill and C. Somerville, *Mol. Gen. Genet.* **226**, 484 (1991)] by hybridization with λbAt315. To subclone the YACs, total DNA from yeast cells harboring EG4E4 or EG2G11 was partially digested with Sau 3AI, and cloned into the Bgl II site of cosmid vector pCIT30 [H. Ma, M. F. Yanofsky, H. J. Klee, J. L. Bowman, E. M. Meyerowitz, *Gene* **117**, 161 (1992)]. Standard cloning and screening methods were used [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. A library from the *etr1-1* mutant was similarly constructed in pCIT30. The wild-type library was constructed previously [M. F. Yanofsky *et al.*, *Nature* **346**, 35 (1990)]. For each step in the chromosome walk, a gel-purified DNA fragment probe was used to isolate a set of overlapping cosmids. The cosmids were analyzed by Eco RI restriction digests and Southern (DNA) blots, and a new DNA fragment from the cosmids was then chosen for use as a subsequent probe.
15. Meiotic recombinants were obtained from the F_2 progeny of the two crosses in [*12*]. Recombinants were identified as plants that had either the wild-type phenotype, or the mutant phenotype, at both loci. Ethylene sensitivity was scored in dark-grown seedlings as described [*3*]. We obtained 74 recombinants between *ETR1* and *AP1*, and 25 recombinants between *ETR1* and *CLV2*, such that the calculated average distance between break points was roughly 20 kb for each cross. This calculation is based on an average of 200 kb per centimorgan in the *Arabidopsis* genome, that is, the *Arabidopsis* genome consists of approximately 100,000 kb [E. M. Meyerowitz, in *Methods in* Arabidopsis *Research*, C. Koncz, N.-H. Chua, J. Schell, Eds. (World Scientific Publishing, Singapore, 1992), pp. 100–118] and 520 cM [B. M. Hauge *et al.*, *Plant J.* **3**, 745 (1993)]. The breakpoints were detected by RFLP analysis, using cosmids or gel-purified restriction fragments in the walk as RFLP probes. Over the 230-kb contig, the actual density of breakpoints was consistent with the expected density on the *CLV2* side (with 5 breakpoints in approximately 100 kb), and was twice the expected density on the *AP1* side (with 12 breakpoints in approximately 120 kb).
16. We isolated poly(A)$^+$ RNA and constructed a cDNA library in lambda ZapII (Stratagene) as described [D. Weigel, J. Alvarez, D. R. Smyth, M. F. Yanofsky, E. M. Meyerowitz, *Cell* **69**, 843 (1992)] except that RNA was extracted from 4-day-old seedlings (ecotype Columbia). The primary library contained 4×10^6 plaque-forming units (pfu). After the library was amplified, 5×10^5 pfu were screened with standard methods as in [*14*]. Four Eco RI restriction fragments were used as probes: the 4.25-kb and 3.1-kb fragments shown in Fig. 1, an 8.5-kb fragment adjacent to the 4.25-kb fragment, and a 4-kb fragment adjacent to the 3.1-kb fragment.
17. Nucleotide sequences were determined with the use of Sequenase Version 2.0 (United States Biochemical Co.) and synthetic oligonucleotide primers. Both strands of the genomic DNA were sequenced, including 223 bp upstream of the presumed transcription start site, and 48 bp downstream of the polyadenylation site. λC4 was sequenced on a single strand and was 1812 base pairs long, including a poly(A)$^+$ tail of 18 bases. To obtain longer cDNAs, we used sequence-specific primers internal to λC4 to synthesize first-strand cDNA (RiboClone cDNA Synthesis System, Promega) from seedling poly(A)$^+$ RNA, which was isolated as in [*16*]. The cDNA was then amplified by PCR [R. K. Saiki *et al.*, *Science* **230**, 1350 (1985)] with the use of various pairs of

primers; 3′ PCR primers were chosen to anneal to different exons as deduced from the cDNA and genomic DNA sequences, and 5′ PCR primers were chosen to anneal to various 5′ portions of genomic DNA sequence. We used six different primers at the 5′ end; the farthest upstream primer that amplified the cDNA was primer Q (5′-AGTAAGAACGAAGAAGAAGTG). An overlapping primer, which was shifted 12 bases downstream, also amplified the cDNA. We could not amplify the cDNA with a 5′ end primer that was 98 bp farther upstream. Genomic DNA templates were used for PCR controls. The longest cDNA was considered to extend to the 5′ end of primer Q. The amplified cDNAs were sequenced directly with Sequenase Version 2.0 as follows: the PCR reactions were concentrated by ethanol precipitation, and the amplified products were separated by electrophoresis in 0.8% low melting point agarose gels. The DNA fragments were excised, and a mixture of 10 μl of excised gel (melted at 70°C), 1 μl of 10 μM primer, and 1.2 μl of 5% Nonidet P-40 was heated at 90°C for 2 min, cooled to 37°C, and then sequenced.

18. For each mutant allele, we constructed an Eco RI size-selected library in the vector lambda ZAPII. Clones of the 4.25-kb Eco RI fragment were isolated by hybridization with the wild-type fragment. These clones were converted into plasmids (pBluescript vector) by in vivo excision according to the supplier (Stratagene) and sequenced as in [*17*]. Two independent clones were sequenced on a single strand for each mutant allele. The 5′ ends (535 bp not contained on the 4.25-kb Eco RI fragment) were amplified by PCR and directly sequenced as in [*17*]. Codon differences were as follows: TGT to TAT in *etr1-1*, GCG to ACG in *etr1-2*, GCG to GTG in *etr1-3*, ATC to TTC in *etr1-4*. The positions of these missense mutations are shown in Fig. 2.
19. J. Hua, C. Chang, E. M. Meyerowitz, unpublished results.
20. For normal stringency, hybridization was done in 5x SSPE (0.9 M NaCl, 50 mM NaH_2PO_4, 40 mM NaOH, 4.5 mM EDTA, pH 7.4) at 65°C and held overnight; the most stringent wash was in 0.1x SSPE at 65°C for 20 min.
21. C. Chang, Q. Sun, E. M. Meyerowitz, unpublished results.
22. Database searches were performed at the National Center for Biotechnology Information with the use of the BLAST Network Service [S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)].
23. Sequence alignments were performed with Multalign [K. J. Fryxell and E. M. Meyerowitz, *J. Mol. Evol.* **33**, 367 (1991)]. Amino acid similarities are according to the Dayhoff table [M. O. Dayhoff, R. M. Schwartz, B. C. Orcutt, in *Atlas of Protein Sequence and Structure*, M. O. Dayhoff, Ed. (National Biomedical Research Foundation, Washington, DC, 1978), vol. 5, suppl. 3, pp. 345–352]. The sequence of the putative ETR1 histidine kinase domain is longer than the bacterial sequences by 10 to 16 residues, which were not included in the calculations.
24. S. Nagasawa, S. Tokishita, H. Aiba, T. Mizuno, *Mol. Microbiol.* **6**, 799 (1992).
25. E. M. Hrabak and D. K. Willis, *J. Bacteriol.* **174**, 3011 (1992).
26. J.-L. Tang *et al.*, *Mol. Gen. Genet.* **226**, 409 (1991).
27. V. Stout and S. Gottesman, *J. Bacteriol.* **172**, 659 (1990).
28. S. Luchi, Z. Matsuda, T. Fujiwara, E. C. C. Lin, *Mol. Microbiol.* **4**, 715 (1990).
29. A. M. Stock, J. M. Mottonen, J. B. Stock, C. E. Schutt, *Nature* **337**, 745 (1989); K. Volz and P. Matsumura, *J. Biol. Chem.* **266**, 15511 (1991).
30. C. Chang and E. M. Meyerowitz, unpublished results.
31. Sequence alignments were performed as in (*23*). Sequences varied in length by one to six residues. The additional residues were not included in the calculations.
32. B. Aricò *et al.*, *Mol. Microbiol.* **5**, 2481 (1991).
33. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982).
34. J. N. Weinstein, R. Blumenthal, J van Renswoude, C. Kempf, R. D Klausner, *J. Membr. Biol.* **66**, 203 (1982).
35. For the *etr1-1* construct, the 4.25-kb Eco RI plasmid clone containing the *etr1-1* mutation [*18*] was linearized by partial Eco RI digestion, and ligated with the 3.1-kb Eco RI fragment that was agarose gel–purified from cosmid theta 8, a subclone of YAC EG4E4 generated in the walk. The resulting plasmid, containing the two Eco RI fragments in the correct relative orientation, was linearized at polylinker site Asp^{718}, the ends were filled in by Klenow fragment enzyme, and Bam HI linkers were ligated to the blunt ends. Finally, the 7.3-kb insert was removed from the plasmid at the polylinker site by digestion with Bam HI, and ligated into the Bam HI site of binary transformation vector pCGN1547 (Calgene) [K. E. McBride and K. R. Summerfelt, *Plant Mol. Biol.* **14**, 269 (1990)]. For the control construct, the wild-type 7.3-kb fragment was purified in an agarose gel from cosmid theta 8, partially digested with Eco RI, and subcloned into the Eco RI site of pBluescript. The fragment was then removed by digestion with Bam HI and Kpn I in the polylinker, and ligated into pCGN1547 prepared by digestion with Bam HI and Kpn I. The mutant and wild-type constructs were transformed into *Agrobacterium* [M. Holsters *et al.*, *Mol. Gen. Genet.* **163**, 181 (1978)] strain ASE (Monsanto) [S. G. Rogers, H. J. Klee, R. B. Horsch, R. T. Fraley, *Methods Enzymol.* **153**, 253 (1988)]. *Arabidopsis* ecotype Nossen was transformed as described [D. Valvekens, M. Van Montagu, M. Van Lijsebettens, *Proc. Natl. Acad Sci. U.S.A.* **85**, 5536 (1988)] with the use of roots cultured in liquid rather than on solid medium. Ethylene sensitivity was assayed by measurement of hypocotyl and root length in dark-grown seedlings treated with either ethylene, as in [*3*], or the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), as in [*5*]. Identically treated wild-type and *etr1-1* mutants were used as controls. For ACC treatment, plants were germinated and grown on Murashige and Skoog basal salt mixture (MS, Sigma), pH 5.7, 0 5 mM ACC (Sigma), 1% Bacto-agar (Difco). Kanamycin resistance was measured by the extent of root elongation in 1-week-old seedlings grown on MS, pH 5.7, 50 μg/ml Kanamycin, 1% Bacto-agar
36. F. Nagy, G. Morelli, R. T. Fraley, S. G. Rogers, N.-H. Chua, *EMBO J.* **4**, 3063 (1985).
37. R. M. Amasino, A. L. T. Powell, M P Gordon, *Mol. Gen. Genet.* **197**, 437 (1984).
38. L. Herman, A. Jacobs, M. VanMontagu, A Depicker, *ibid.* **224**, 248 (1990).
39. E. K. Monson, M Weinstein, G. S. Ditta, D R. Helinski, *Proc. Natl. Acad. Sci U.S.A.* **89**, 4280 (1992).
40. K. A. Borkovich and M. I. Simon, *Cell* **63**, 1339 (1990).
41. S. P. Burg and E A. Burg, *Plant Physiol.* **42**, 144 (1967); E. C. Sisler, *Tob. Sci.* **21**, 43 (1977).
42. I. Herskowitz, *Nature* **329**, 219 (1987)
43. Y Yang and M. Inouye, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11057 (1991); A. J. Wolfe and R. C. Stewart, *ibid.* **90**, 1518 (1993); R. V. Swanson, R. B. Bourret, M. I. Simon, *Mol. Microbiol.* **8**, 435 (1993).
44. M. Driscoll and M. Chalfie, *Nature* **349**, 588 (1991).
45. J. Marx, *Science* **260**, 1588 (1993).
46. S A. Moodie, B M. Willumsen, M. J. Weber, A. Wolfman, *ibid.* **260**, 1658 (1993); L. V. Aelst, M. Barr, S. Marcus, A. Polverino, M. Wigler, *Proc. Natl. Acad Sci. U.S.A.* **90**, 6213 (1993); A. B. Vojtek, S. M. Hollenberg, J. A Cooper, *Cell* **74**, 205 (1993), X Zhang *et al.*, *Nature* **364**, 308 (1993), P. H. Warne, P. R. Viciana, J. Downward, *ibid.*, p. 352.
47. P. J. Artymiuk, D W. Rice, E. M Mitchell, P. Willett, *Protein Eng.* 4, 39 (1990); J. M. Chen, G. Lee, R. B. Murphy, P. W. Brandt-Rauf, M. R. Pincus, *Int. J. Pept. Protein Res.* **36**, 1 (1990), G S Lukat, B H. Lee, J. M. Mottonen, A. M. Stock, J. B. Stock, *J. Biol. Chem* **266**, 8348 (1991).
48. I. Ota and A Varshavsky, *Science* **262**, 124 (1993).
49. H. A. W. Schneider-Poetsch, *Photochem. Photobiol.* **56**, 839 (1992).
50. K. M. Popov, Y. Zhao, Y. Shimomura, M. J. Kuntz, R. A. Harris, *J. Biol Chem.* **267**, 13127 (1992).

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