

## ***ETR2* is an *ETR1*-like gene involved in ethylene signaling in *Arabidopsis***

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Contributed by Elliot M. Meyerowitz, March 2, 1998

**ABSTRACT** The plant hormone ethylene regulates a variety of processes of growth and development. To identify components in the ethylene signal transduction pathway, we screened for ethylene-insensitive mutants in *Arabidopsis thaliana* and isolated a dominant *etr2-1* mutant. The *etr2-1* mutation confers ethylene insensitivity in several processes, including etiolated seedling elongation, leaf expansion, and leaf senescence. Double mutant analysis indicates that *ETR2* acts upstream of *CTR1*, which codes for a Raf-related protein kinase. We cloned the *ETR2* gene on the basis of its map position, and we found that it exhibits sequence homology to the ethylene receptor gene *ETR1* and the *ETR1*-like *ERS* gene. *ETR2* may thus encode a third ethylene receptor in *Arabidopsis*, transducing the hormonal signal through its “two-component” structure. Expression studies show that *ETR2* is ubiquitously expressed and has a higher expression in some tissues, including inflorescence and floral meristems, petals, and ovules.

The simple gas ethylene plays an important role in regulating growth and development in higher plants. It is involved in basic cellular processes such as cell elongation, cell division, and cell death, as well as in physiological processes such as seed germination, senescence, and fruit ripening (1). Ethylene is also a signal for plants to adapt to a changing environment. It mediates stress responses such as wound response and pathogen response (2, 3). A number of mutants defective in ethylene responses have been isolated in *Arabidopsis thaliana* with the aid of the “triple response” assay. The “triple response” refers to the three features of an etiolated seedling grown in the presence of ethylene, which are a short and thick hypocotyl, a short root, and an exaggerated apical hook. Mutants that do not display the triple response in ethylene (categorized as ethylene insensitive) are *etr1*, *ein2*, *ein3*, *ein4*, *ein5*, *ein6*, *ein7*, and *ain1* (4). *ctr1* mutants, which exhibit the triple response in air, are categorized as constitutive ethylene responsive (5). Ethylene response defects also extend to adult plants in mutants such as *etr1* and *ctr1* (5, 6). A genetic pathway for ethylene signal transduction has been deduced from double mutant analyses. *ETR1* and *EIN4* act upstream of *CTR1*, whereas *EIN3*, *EIN5*, *EIN6*, and *EIN7* are downstream of *CTR1* (4).

The molecular cloning of some of these genes has begun to reveal the components of the ethylene signal transduction pathway. The *ETR1* gene encodes an ethylene receptor, as indicated by the ethylene-binding activity of the amino-terminal domain of *ETR1* (7). The carboxyl-terminal region consists of a putative histidine kinase domain and a receiver domain, both of which are homologous to the bacterial “two-

component” regulators (8). These regulators are sensors and transducers of a variety of signals in adaptation responses in bacteria (9). The first component, the sensor, consists of an input domain and a histidine protein kinase domain. The second component, the response regulator, has a receiver domain and an output domain. The histidine autokinase of the sensor is activated or repressed by the perception of the signal through the input domain. The phosphoryl group from the histidine residue can be transferred to the aspartate in the receiver domain, whereupon the activity of the output domain is modulated. In bacteria, the output domain usually has a DNA binding motif and regulates transcription directly. *ETR1* has both the putative histidine kinase domain and the receiver domain in the same protein. This “hybrid” structure also exists in a small fraction of known bacterial sensors. By analogy to the bacterial two-component regulators, *ETR1* may sense ethylene through the amino-terminal domain and transmit the binding signal to the histidine protein kinase domain and the receiver domain. *ETR1* appears to be a member of a small family of ethylene receptors in *Arabidopsis*. An *ETR1*-related gene, *ERS*, was discovered by cross-hybridization to *ETR1* (10). *ERS* is also implicated in ethylene sensing, as indicated by the dominant ethylene-insensitive phenotype conferred by a mutated *ERS* gene (10) and the ethylene-binding activity of the amino-terminal domain of *ERS* (A.B.B., unpublished results). *CTR1* acts downstream of *ETR1* and *ERS* and encodes a serine/threonine protein kinase related to the Raf kinases found in animals (5). *CTR1* is a negative regulator of ethylene response, as loss-of-function mutants display constitutive ethylene responses. The downstream *EIN3* gene encodes a novel nuclear-localized protein that is probably directly involved in transcriptional regulation (11).

To further elucidate the ethylene signal perception pathway, we undertook a genetic approach to isolate other genes involved in this process. We describe here the isolation and characterization of an ethylene-insensitive mutant *etr2* and the cloning of the *ETR2* gene. Its sequence similarity to *ETR1* and *ERS* together with the genetic studies suggests that *ETR2* may act as an additional ethylene receptor. Expression studies of the *ETR2* gene suggest its possible involvement in the development of different plant tissues.

### **MATERIALS AND METHODS**

**Plant Strains and Growth.** *Arabidopsis thaliana* ecotype Columbia was used in this study unless stated otherwise.

Abbreviation: cM, centimorgan.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF047975).

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Etiolated M<sub>2</sub> seedlings were screened after 3 days' growth on Murashige and Skoog (MS) medium solidified with 1% agar in the presence of 5  $\mu$ l/liter ethylene. Seedlings with long hypocotyls and roots were chosen as ethylene insensitive mutants for further characterization. Dose-response analysis was carried out as previously described (12).

**Mapping of *ETR2*.** *etr2-1* was crossed to ecotype Landsberg for mapping. Between 60 and 70 F<sub>3</sub> families homozygous for ethylene-insensitive phenotypes were used for restriction fragment length polymorphism (RFLP) (13) and simple sequence length polymorphism (SSLP) mapping (14).

**DNA Analysis.** Nucleotide sequences were determined with the use of Sequenase Version 2.0 (United States Biochemical) and synthetic oligonucleotides. A 7.3-kb fragment containing the *ETR2* gene (see below) was sequenced at least once in both directions. To identify mutations in *etr2-1*, we amplified the *ETR2* coding region from wild-type and mutant plant tissues by polymerase chain reaction (PCR). PCR products were excised from a low melting point gel and used directly for sequencing. A genomic DNA library constructed from *Sau3A* partially digested DNA of *etr2-1* was screened at high stringency [wash in 0.25 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA (0.1 $\times$  SSPE) at 65°C] with an *ETR2* probe.  $\lambda$  clones containing the *ETR2* gene were isolated. The alteration in *etr2-1* was confirmed by sequencing the *ETR2* gene isolated from this library.

**Plant Transformation.** A full-length *ETR2* genomic fragment was generated by fusing two *EcoRI* (5.5 kb and 1.8 kb) pieces into pBluescript (Stratagene). The 7.3-kb fragments isolated from wild-type and from *etr2-1* mutant plants were each excised by *BamHI/KpnI* digestion and ligated into transformation vector pCGN1547 (15). These constructs in pCGN1547 were introduced into *Agrobacterium* strain ASE (16), which was used to transform wild-type *Arabidopsis thaliana* by the *in planta* vacuum infiltration method (17). T<sub>1</sub> plants were selected on MS plates supplemented with 50 mg/liter kanamycin. About 20 transgenic plants were obtained for each construct.

**RNA Analysis.** Poly(A)<sup>+</sup> RNA was isolated from 1-week-old seedlings, roots, rosette leaves, inflorescences with flowers, and mature siliques of *Arabidopsis thaliana* ecotype Landsberg *erecta* (*Ler*) as previously described (18) by using the PolyATtract system (Promega). Poly(A)<sup>+</sup> RNA was then incubated with RNase-free DNase I for 1 hr and subsequently extracted several times with phenol and chloroform. One microgram of poly(A)<sup>+</sup> RNA was reverse-transcribed, and 1/100 of the first strand cDNA was used for PCR. Three different sets of primers were designed for amplification: *ETR2* ORF encompassing the intron (5'-AGGACAAGATCTAAGC-3' and 5'-AGGCAATCGAATCCAG-3'), the downstream untranslated region of *ETR2* (5'-CTGCACATTCTCGAGC-3' and 5'-GCAATAACTGTAGGCAGC-3'), and the ubiquitin-conjugating enzyme gene, *UBC4* (5'-AGTGGAGCTCCA-CAAG-3' and 5'-CCTTGCAGCCTCTGCG-3') (19). PCR products were separated on agarose gels and visualized by using a digital imaging system (Alpha Innotech, San Leandro, CA). DNA amounts were quantitated by using the NIH IMAGE 1.6.4 program.

To identify the 5' end of the mRNA, flower poly(A)<sup>+</sup> RNA was reverse transcribed and ligated with a linker DNA of the Marathon PCR System according to the manufacture (CLONTECH). 5' *ETR2* cDNA was amplified by using a primer (5'-CGTGGATAACCACCGCCG-3') for the first round reaction and a nested primer (5'-CTGTACTCCAGAACTG-3') for the second round reaction in combination with the linker primers. The amplified DNA was cloned into the pCR2.1 vector (Invitrogen) and sequenced.

**In Situ Hybridization.** The localization of RNA in plant tissues was analyzed by radioactive *in situ* hybridization as previously described (20) with modifications (21). The sense and antisense RNA probes were synthesized from the histidine

kinase and receiver domains of the *ETR2* gene by *in vitro* transcription in the presence of  $\alpha$ -[<sup>35</sup>S]thio-UTP. Hybridized sections were exposed on Kodak emulsion NBT2 for 6 weeks.

## RESULTS

**Phenotypic Analysis of the *etr2* Mutant.** The *etr2* (*ethylene response 2*) mutant was isolated from an ethyl methanesulfonate (EMS)-mutagenized population of 100,000 M<sub>2</sub> seedlings by using the seedling growth response as a screen (6). Dose-response analysis of hypocotyl-elongation response in dark-grown seedlings indicated a greatly reduced sensitivity to ethylene in *etr2-1* relative to the wild-type seedlings (Fig. 1A). A similar degree of insensitivity was observed for the root-growth response (data not shown). Similar to what was previously noted for the reduced-sensitivity *etr1-2* mutant (12), the *etr2-1* mutation did not appreciably alter the threshold or saturation concentrations of ethylene for the response, but rather primarily affected the degree of maximal responsiveness. Also similar to the *etr1* mutants, the *etr2-1* allele was genetically dominant over wild type as indicated by segregation of backcross progeny (data not shown), and by the dose-response curve for hypocotyl elongation in the heterozygous seedlings (Fig. 1B).

To further characterize the lesions in *etr2-1*, we examined other ethylene responses of this mutant. Ethylene inhibits leaf expansion of light-grown wild-type plants, so that wild-type plants have much smaller leaves when grown in ethylene than in air. This ethylene effect on leaf size is mostly due to an inhibition of cell expansion, and a correlation between leaf size and cell size has been demonstrated in *ctr1-1* and *ers-1* mutants (5, 10). Ethylene-insensitive mutants such as *etr1-1* and *ers-1* are not affected by this inhibitory effect of ethylene (ref. 10 and unpublished data). Moreover, *etr1-1* and *ers-1* mutants grown in air have bigger leaves than the wild type, presumably because they are insensitive to endogenous ethylene (10). Unlike *etr1* and *ers* mutants, *etr2-1* had the same leaf size as the wild type when grown in air (data not shown). However, the

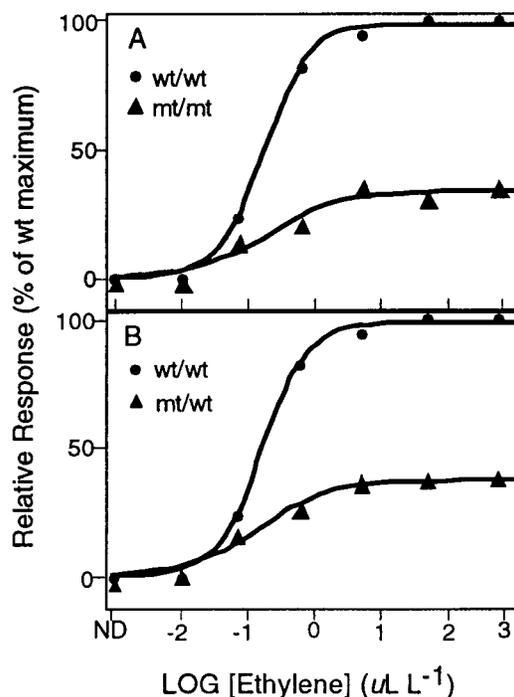


FIG. 1. Dose-response analysis of the hypocotyl elongation of the *etr2-1* mutants (homozygous in A, heterozygous in B). ●, Wild-type response; ▲, mutant response (represented as percentage of the wild-type response). ND, not detected.

*etr2-1* mutation greatly reduced the inhibitory effect of exogenous ethylene on leaf expansion, similar to, but to a lesser extent than, the *etr1-1* and *ers-1* mutations. *etr2-1* mutants grown in ethylene had only slightly reduced leaf size compared with those grown in air (data not shown), but they had much larger leaves than did the wild type grown in ethylene (Fig. 2A). Ethylene also accelerates leaf senescence of mature plants. After being treated with ethylene for 3 days, 1-month-old wild-type plants showed leaf yellowing. In contrast, *etr2-1* mutant leaves did not appear to be affected by ethylene treatment (Fig. 2B).

**Genetic Analysis of *etr2* Mutant.** Preliminary data from crosses between *etr2-1* and *etr1-1* indicated that the loci represented by these mutations are unlinked. To confirm that *etr2* represents a ethylene-response locus not previously described, the mutation was mapped by using a combination of restriction fragment length polymorphism (RFLP) and simple sequence length polymorphism (SSLP) markers. Data from more than 60 segregating F<sub>3</sub> families indicated that *etr2* mapped 7.2 centimorgans (cM) proximal to RFLP marker m560 and 27.2 cM proximal to SSLP marker nga 172 on chromosome 3. Therefore, the *ETR2* locus is distinct from *ETR1* on chromosome 1. It is also different from *EIN4*, which also resides on chromosome 3 but is more closely linked to nga 172 (22).

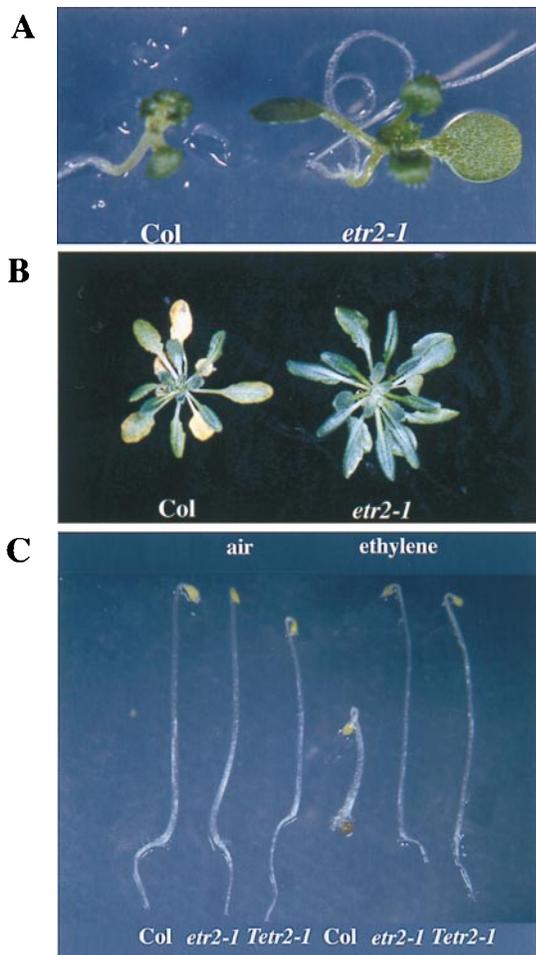


FIG. 2. Phenotypes of the *etr2-1* mutant and the *etr2-1* transgenic plants. (A) *etr2-1* grown in the presence of ethylene has larger leaves than the wild type. (B) *etr2-1* treated with ethylene has delayed leaf senescence compared with wild type. (C) Wild-type plants transformed with the mutant *etr2* gene display ethylene-insensitive phenotypes in the triple response assay. *Tetr2-1*, *etr2-1* transgenic plants.

To place the *ETR2* gene in the ethylene signal transduction pathway, we tested the epistatic relationship of *etr2* and *ctr1* by crossing *etr2-1* to *ctr1-2*. Of 514 F<sub>2</sub> progeny grown in air, 99 had the *ctr1* phenotype. This is consistent with a ratio of 1 *ctr1* to 3 wild type, taking into account that the transmission of *ctr1* is 30% reduced compared with wild type (5). Of 463 F<sub>2</sub> progeny grown in ethylene, 280 showed an ethylene-insensitive phenotype, consistent with a 9 insensitive to 7 sensitive (or constitutive responsive) ratio when *etr2* is dominant and *ctr1* is epistatic. These results indicate that *ctr1* is epistatic to *etr2*. The epistatic relationship was later confirmed by genotyping the double mutants once the *etr2-1* mutation was identified (see below).

**Isolation of the *ETR2* Gene.** The *etr2* mutation maps very close to the *SUPERMAN* (*SUP*) locus. The approximately 100-kb-long genomic DNA region containing the *SUP* gene was extensively analyzed previously, and several genes were identified in this region (21). One of these genes, *Q8*, showed sequence similarity to the *ETR1* gene. Because the phenotypes of the *etr1* and *etr2* mutants resemble each other, we investigated whether *Q8* was the *ETR2* gene. We sequenced the *Q8* gene from the *etr2-1* mutant, and we identified a C → T transversion in *etr2-1*. This change results in a leucine substitution for a proline (residue 66) that is highly conserved among the other putative ethylene receptor genes (Fig. 3B). This substitution is identical to that in the mutant *Never-ripe* (*Nr*) gene, which is a putative ethylene receptor gene in tomato (23) (Fig. 3B). To further confirm the identity of *Q8* as the *ETR2* gene, we isolated the *Q8* gene from a  $\lambda$  library constructed from *etr2-1* genomic DNA. Two 7.4-kb-long genomic DNA fragments containing the *Q8* coding region together with 3.5 kb upstream and 1.5 kb downstream were isolated from *etr2-1* and wild type, respectively, and were used to transform wild-type *Arabidopsis* plants. Six of the nine transformants containing the fragment from *etr2-1* exhibited the ethylene-insensitive phenotype (Fig. 2C), whereas none of the ten transformants with the wild-type fragment showed the insensitive phenotype. As no other genes were found in this 7.4-kb fragment, we conclude that *Q8* is the *ETR2* gene (GenBank accession no. AF047975).

**Structure of the *ETR2* Gene.** Using an *ETR2* genomic DNA fragment as a hybridization probe, we isolated several cDNA clones from a cDNA library constructed from young flower RNA (24). Comparison of the cDNA sequence and genomic DNA sequence from *Ler* showed that the *ETR2* gene consists of two exons that are interrupted by a short intron of 83 bp. 5' Rapid amplification of cDNA ends (RACE) revealed an additional intron of 97 bp in the 371-bp-long untranslated leader region and four additional out-of-frame ATGs upstream of the initiating ATG of the *ETR2* ORF.

The *ETR2* gene encodes a putative protein of 773 amino acids. A search of the GenBank database showed that *ETR2* has a high sequence similarity to the ethylene receptor gene *ETR1* (8) and putative ethylene receptor genes *ERS* (10) and *Nr* (23). The deduced *ETR2* protein has an overall structure similar to that of *ETR1*: it consists of an amino-terminal domain, a putative histidine kinase domain, and a receiver domain (Fig. 3A). *ERS* and *Nr* have only the amino-terminal domain and the putative histidine kinase domain. When sequence similarity of the full-length proteins is considered among the *Arabidopsis* gene products, *ETR1* and *ERS* are more closely related to each other (79% similarity), and *ETR2* is almost equally distantly related to *ETR1* and *ERS* (65% and 63% similarity, respectively).

The amino-terminal domain is the most conserved portion among *ETR2*, *ETR1*, and *ERS*, with *ETR2* sharing about 71% similarity to *ETR1* and *ERS* (Fig. 3B). As this domain of *ETR1* has ethylene-binding activity, *ETR2* is likely to be capable of ethylene binding as well. One unique feature of *ETR2* in this domain is the presence of an amino-terminal extension that

**A**

ETR2	MVKEIASWLLILSMVVFVSPVLAINGGGYPRCNCDEGNSFWSTENILET	50
ETR1	M--EV-----CNCIEPQWP--ADELLMKY	20
ETR2	QRVSDFLIAYVYFSIPELLLYFVSCSNV-PFKWVLFEFIAFIVLCGMTHL	99
ETR1	QYISDFPIAIAFYFSIPELLLYFVKSVAFFPYRWVLMQPGAFIVLCGATHL	70
ETR2	LHGWTYSAHPFRLLMMAFTVFKMLTALVSCATAITLITLIPDLLKVKVREF	149
ETR1	INLWTFTHSRVALVMTAKVLTAVVSCATALLVHIIIPDLLSVKTRREL	120
ETR2	MLKKKAHELGREVGLTIKKETGSPHVRMLTQEIIRKSLDRHTIITYTTLVEL	199
ETR1	FLKNKAAELEDREMLIRTQEEGRHVRMLTHEIRSTLDRHTILKTTLVEL	170
ETR2	SKTLGLQNCVMMVNDGGTMDLTHELRGRGGYGGCSVSMEDLDVVRIRE	249
ETR1	GRTLALAECCALWMPTRTGLELQLSYTLRHQHP-VEYTVPIQLPVINQVFG	219
ETR2	SDEVNVLSDVSSIARASGGGGDVEIGAVAAIRMPMLRVSDFNGE----L	295
ETR1	TSRAVKISPNPVARLRVPSGKY-MLGEVAVRVPPLHLNPFQINDWPEL	268
ETR2	S---YAILVLCVLPGGTRRDWTYQEIIEIVKVVADQVTVALDHAALVEESQL	342
ETR1	STKRVALMVLMLFSDSARQWHVHELELVEVVADQVAVALSHAAILEESMR	318
ETR2	MREKLAEQNRALQMAKRDALRASQARNAFOKTMSEGMRRPMHSILGLLSM	392
ETR1	ARDLMEQNVALDLARREAETAIRARNDPLAVINHEMRTPMHAIATLSSL	368
<b>H</b>		
ETR2	IQDEKLSDEQKMIVDVMYKTNVMSNLVGDMSDVP---DGRFGTEM----	435
ETR1	LQETELSPERQLRMVETIILKSSNPFLATLMNDVLDLSRLEDGSLQELGTFN	418
ETR2	-----KPFSLHRTIHEAACMARCLCLNGIRFLVDAEKSLPDN	473
ETR1	LHTLFRVNLNLIKPIAVVKKLP-----ITLNLAPD--LPEF	452
ETR2	VVGDERRVFQVILHMVGLVKPKRQEGSSLMFKVLKERGSLDRSDHRWA	523
ETR1	VVGDEKRLMQIILINIVGNVAVKFSKQ---GSI SVTAL----VTKSDTRAA	494
<b>N</b>		
ETR2	AWRSPASSADGDVYIRFEMNVENDSSQSFASVSSRDQEVGDV--RFSG	571
ETR1	DFP--VVPTGSHFYLRVVKVSDGAGINFDIPIKIFTKFAQTQSLATRSSG	542
<b>G1</b> <b>F</b>		
ETR2	GYGLQDLSFGVCKKVVQLIHGNIISVVGSDGSPETMSLLFRFRSIS	621
ETR1	GSGLGLAIS---KRFVNLMEGNIWI--ESDGLGKGTAFIDVKLGISER	586
<b>G2</b>		
ETR2	VHGSSESPADHHAHPSNLSRGLQVLLVDINDSNRAVTRKLEKLGCD	671
ETR1	SNESKQSGIPKVPALPR-HSNPTGLKVLVMDENGVSVMVTKGLLVHLGCE	635
ETR2	VTAVSSGFDCLTALAPGSSSPETSQVAVLDLQMAEMDGYEVAMRISR-	720
ETR1	VITVSSNEEQLRVVSHHEH-----KVVFMDCMFGVENVYQIALRIHEKF	678
ETR2	-----SWPLIVATTVSLDEEMWDKCAQIGINGVVRKPVVLRAMESELRRV	765
ETR1	TKQRHORPLLVALSGNTDKSTKCKMSFGLDGVLLKPVSLDNIIRDVLSDL	728
ETR2	LLQAD-QLL-	773
ETR1	LEPRVLYEGM	738

**B**

ETR2	MVKEIASWLLILSMVVFVSPVLAINGGGYPRCNCDEGNSFWSTENILET	50
ETR1	M--EV-----CNCIEPQWP--ADELLMKY	20
ERS	M--ES-----CDCFETHVN--QDILLVKY	20
NR	M--ES-----CDCIEALLP--TGDLLVKY	20
<b>IV</b>		
ETR2	QRVSDFLIAYVYFSIPELLLYFVSCSNV-PFKWVLFEFIAFIVLCGMTHL	99
ETR1	QYISDFPIAIAFYFSIPELLLYFVKSVAFFPYRWVLMQPGAFIVLCGATHL	70
ERS	QYISDALIALAYFSIPELLLYFVQKSAPFPYKWLMLQPGAFIVLCGATHF	70
NR	QYISDFPIAIAFYFSIPELLLYFVKSVAFFPYRWVLMQPGAFIVLCGATHF	70
<b>I</b> <b>II</b>		
ETR2	LHGWTYSAHPFRLLMMAFTVFKMLTALVSCATAITLITLIPDLLKVKVREF	149
ETR1	INLWTFTHSRVALVMTAKVLTAVVSCATALLVHIIIPDLLSVKTRREL	120
ERS	INLWTFMHSKVAIVMTIAKVSACAVVSCATALLVHIIIPDLLSVKNREL	120
NR	ISLWTFMHSKTVAVVMTISKMLTAAVSCITALLVHIIIPDLLSVKTRREL	120
<b>III</b>		

FIG. 3. Protein sequence comparison between the putative ethylene receptors. (A) Amino acid sequence alignment of ETR2 and ETR1. Similar amino acids are shaded. The five motifs found in ETR1 corresponding to those of the bacterial histidine kinases (H, N, G1, F, G2) are underlined. Three residues corresponding to the residues conserved in the bacterial receiver modules are indicated by \*. (B) Amino acid sequence alignment of the amino-terminal domains of *Arabidopsis* ETR2, ETR1, and ERS and tomato NR. Putative transmembrane segments are underlined. Amino acids that are mutated in ethylene-insensitive mutants are boxed.

may contain a fourth hydrophobic segment (Fig. 3B). A weaker sequence similarity in the putative histidine protein kinase domains is observed between ETR2 and ETR1/ERS (58%/56% similarity) than between ETR1 and ERS (79% similarity). While ETR1 and ERS have all of the five conserved motifs in the bacterial histidine protein kinases (H, N, G1, F, and G2) (9), ETR2 is more diverged from the consensus sequences (Fig. 3A). Most strikingly, the conserved histidine residue in the H motif, which is postulated to serve as a phosphorylation site in bacterial two-component proteins, is replaced by a glutamate residue in ETR2 (Fig. 3A). Sequences similar to the N motifs of ETR1 and ERS are present in ETR2, but the two N residues are absent. While the G2 motif is present in ETR2, neither the G1 motif nor the F motif is found in ETR2. The receiver domain of ETR2 has 66% similarity and 44% identity to that of ETR1. The aspartate and lysine residues conserved among the bacterial receiver domains are present in ETR2.

**Expression of the ETR2 Gene.** The low abundance of *ETR2* clones in the cDNA library (1/300,000 cDNA clones) indicated that *ETR2* is a weakly expressed gene. We applied the reverse transcription PCR (RT-PCR) method to analyze the expression of *ETR2* in various tissues. Poly(A)<sup>+</sup> RNA was isolated from seedlings, roots, leaves, flowers, and mature siliques. The total cDNA obtained from reverse transcription of poly(A) RNA was subjected to PCR using a set of *ETR2*-specific primers, which were designed to amplify DNA between two exons. As shown in Fig. 4A, the corresponding cDNA was amplified from all these tissues. Higher levels of amplification of *ETR2* were obtained from flowers and leaves. Because the cDNA for the constitutively expressed *UBC4* gene was amplified equally in experiments performed under the same conditions (Fig. 4C), *ETR2* appears to be expressed more strongly in these tissues.

In the reverse transcription PCR analysis, cDNA containing the second intron was also detected (Fig. 4A). In flowers and leaves, 14% of the total amplified cDNA was of the unspliced class, which may lead to a shorter protein product. It is very unlikely that the amplified DNA with the intron was derived from genomic DNA, as the control PCR reaction with a set of primers for the untranscribed region downstream of *ETR2* did not yield any corresponding DNA (Fig. 4B). This is also indicated by the finding that some of the cDNA clones contained this unspliced intron (data not shown).

To better localize cells expressing *ETR2*, we examined the accumulation of *ETR2* RNA by using *in situ* hybridization. *ETR2* appears to be expressed weakly but evenly in all tissues, including shoot apical meristems and leaf primordia (Fig. 5A). However, *ETR2* mRNA is more strongly detected in central

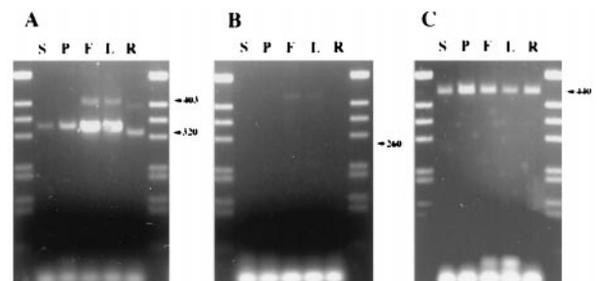


FIG. 4. Reverse transcription PCR analysis of *ETR2* expression in various plant tissues: S, seedling; P, silique; F, flower; L, leaf; and R, root. (A) Amplification of *ETR2* cDNA. The 320-bp-long DNA corresponds to the spliced cDNA, and the 403-bp-long DNA, to the unspliced cDNA. (B) Control amplification of an untranscribed region downstream of the *ETR2* gene. PCR amplification did not yield any detectable DNA of the corresponding size (260 bp), which was, however, produced by PCR amplification on the *Ler* genomic DNA (data not shown). (C) Amplification of 440-bp-long cDNA for *UBC4*, a constitutively expressed gene in these tissues (19).

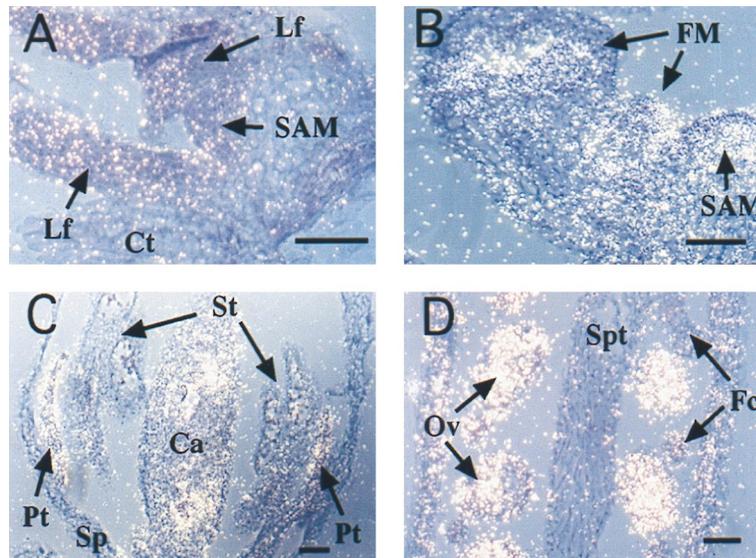


FIG. 5. Localization of *ETR2* RNA in seedlings (*A*), inflorescence shoots and young floral meristems (*B*), young flowers at stage 9 (floral stages according to ref. 25) (*C*), and carpels at stage 13–14 (*D*). Plant tissues (*Ler* ecotype) were sectioned longitudinally. Pictures were taken by a double exposure of bright- and dark-field micrographs, which show radioactive signals as bright grains. SAM, shoot apical meristem; Lf, leaf primordium; Ct, cotyledon; FM, floral meristem; Pt, petal; Sp, sepal; St, stamen; Ca, carpel; Ov, ovule; Spt, septum; Fc, funiculus. (Scale bars, 50  $\mu$ m). Hybridization of the sense RNA probe did not give any signal above the background level (data not shown).

inflorescence meristems and young floral meristems (Fig. 5*B*). In flowers, *ETR2* expression is concentrated in developing petals and ovules (Fig. 5 *C* and *D*). The expression is detected in all parts of the ovule and persists after fertilization.

## DISCUSSION

From a genetic screen for ethylene response mutants, we identified the gene *ETR2*. The *etr2* mutant exhibits a dominant ethylene-insensitive phenotype in the triple response assay, in leaf expansion, and in leaf senescence. These phenotypes are similar to those of *etr1* mutants and *ers* transgenic mutants. Like *ETR1* and *ERS*, *ETR2* acts upstream of *CTR1* in the ethylene signaling pathway as shown by the epistasis. *ETR2* maps to the well characterized region of the *SUP* locus on chromosome 3. We determined that an *ETR1*-related gene in that region is the *ETR2* gene: we identified a mutation in this gene in the *etr2-1* mutant, and reestablished the dominant mutant phenotype in wild-type plants transformed with the mutant gene.

The extensive sequence similarity and the similarity in mutant phenotype of *ETR2* to *ETR1* and *ERS* suggest that *ETR2* has a function analogous to that of *ETR1* and *ERS*. However, it is not certain that the *ETR2* gene actually encodes an ethylene receptor, as the ethylene-binding activity of *ETR2* has not been tested and only one dominant allele of *etr2* is available. It is therefore possible that the *etr2-1* mutant protein interferes with the function of the authentic ethylene receptors, while the wild-type function of *ETR2* is not related with ethylene sensing.

If *ETR1*, *ETR2*, and *ERS* all act as ethylene receptors, this redundancy could account for the absence of recessive alleles of these genes from the genetic screens. However, it remains to be explained why several ethylene receptors are required. It is possible that *ETR1*, *ETR2*, and *ERS* contribute differently to ethylene signal transduction. Sequence comparison revealed that carboxyl-terminal regions of these proteins are less conserved than the amino-terminal putative ethylene-binding domain. *ETR1* and *ERS* possess all of the signature motifs of histidine protein kinases, whereas in *ETR2*, this domain is very diverged from the consensus sequences. Another striking difference among the three proteins is the presence (*ETR1* and

*ETR2*) and absence (*ERS*) of the receiver domain. It is conceivable that the phosphoryl group from the histidine kinase domain may be transferred to the receiver domain of the same protein or other ethylene receptors or directly to unidentified downstream components. The molar ratio between ethylene receptor proteins with and without the receiver domain, thus, may determine the partitioning of phosphoryl groups between the receptors and downstream components. The presence of unspliced *ETR2* RNA, which would lead to the complete deletion of the receiver domain, invites speculation about the importance of the two ethylene receptor classes, one with and another without the receiver domain.

This potential output activity difference among these three proteins, in combination with potential differences in ethylene-binding affinity, may provide a mechanism for fine tuning ethylene responses and may thus explain the maintenance of a family of receptors with apparent redundant function. Alternatively, each gene may have specific activities in certain tissues, giving individual genes separate functions. Enhanced *ETR2* expression in petals and ovules suggests a possible tissue-specific function for *ETR2*. Ubiquitous expression of *ETR1* has been shown by RNA blots (7); however, expression of *ETR1* or *ERS* at the cellular level has not been investigated. Further characterization of the *ETR1* and *ERS* expression pattern will determine whether there are tissue-specific expression patterns for these three genes. Moreover, isolation of loss-of-function mutants for these genes will help us to understand how these genes are involved in ethylene perception in *Arabidopsis*.

The ethyl methanesulfonate-mutagenized M<sub>2</sub> seeds were provided by I. Raskin (Rutgers University). We thank X. Chen, J. Fletcher, T. Ito, S. Jacobsen, P. Kumar, C. Ohno, K. Roberg, R. Sablowski, D. Wagner, E. Ziegelhoffer, and members of Bleeker laboratory for comments on this manuscript. This work was supported by U.S. Department of Energy Grants 88ER13873 to E.M.M. and 91ER20029 to A.B.B.

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