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Short communication

The mRNA for an ETR1 homologue in tomato is constitutively expressed in vegetative and reproductive tissues

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Abstract

Dominant mutations in the *Arabidopsis ETR1* gene block the ethylene signal transduction pathway. The *ETR1* gene has been cloned and sequenced. Using the ETR1 cDNA as a probe, we identified a cDNA homologue (eTAE1) from tomato. eTAE1 contains an open reading frame encoding a polypeptide of 754 amino acid residues. The nucleic acid sequence for the coding sequence in eTAE1 is 74% identical to that for ETR1, and the deduced amino acid sequence is 81% identical and 90% similar. Genomic Southern blot analysis indicates that three or more *ETR1* homologues exist in tomato. RNA blots show that eTAE1 mRNA is constitutively expressed in all the tissues examined, and its accumulation in leaf abscission zones was unaffected by ethylene, silver ions (an inhibitor of ethylene action) or auxin.

The phytohormone ethylene elicits a broad range of physiological responses including fruit ripening, abscission and senescence [15]. The physiology of ethylene action is well documented. However, understanding the molecular mechanism of ethylene action has been more elusive. Nevertheless, recent exploitation of the simpler molecular genetics of *Arabidopsis* is beginning to unravel some of the mystery of ethylene signal transduction. Several *Arabidopsis* mutants affecting ethylene signal transduction have been identified [1, 3, 6, 9]. Among these, the *etr1* mutant is one of the best characterized. Mutant alleles of the *ETR1* gene are dominant to the wild-type allele and are insensitive to ethylene. *Etr1* mutants fail to show a range of ethylene responses including seed germination, the triple response and peroxidase activity [1], suggesting that the *ETR1* gene is essential for an ethylene response. The *ETR1* gene and mRNA in *Arabidopsis* have been cloned and sequenced [4]. The deduced ETR1 sequence has high similarity with the family of 'two-component' regulators known to perceive and transduce external signals in prokaryotes.

The hierarchy of the protein products of the

The nucleotide sequence data reported will appear in EMBL and GenBank Nucleotide Sequence Databases under the accession number U41103.

mutant *Arabidopsis* genes in the ethylene signal transduction pathway has been established by genetic crosses of the various mutants [17]. Double mutation analysis places the ETR1 gene product at an early step in the ethylene signal transduction pathway [6]. It has been suggested that the ETR1 gene product is an ethylene receptor. Saturable ethylene binding in the *etr1–1* mutant was approximately one-fifth of that in the wild type [1]; however, interpretation of the results of the ethylene binding assay was complicated by the fact that wild type plants produced only one-seventh of the ethylene produced by *etr1–1* mutants after incubation in ethylene [1]. Recently, the wild-type ETR1 protein was expressed in yeast and found to bind ethylene with a high affinity [20]. Moreover, when expressed in yeast, the mutant etr1–1 protein lacked detectable ethylene binding.

Irrespective of the promise of using ethylene mutants of *Arabidopsis*, other aspects of ethylene action such as climacteric fruit ripening and flower and leaf abscission are difficult to study in *Arabidopsis*. Tomato, on the other hand, is a model plant for studies on the role of ethylene in fruit ripening and is becoming so for abscission. Yen *et al.* [23] used the *Arabidopsis* ETR1 cDNA as a probe on RFLP blots to place the *ETR1* gene on a tomato genetic map. Their results suggested the existence of several possible ETR1 homologues in tomato. One of the ETR1 loci on the tomato RFLP map was linked to the *Never-ripe* (*Nr*) mutant of tomato. The *Nr* tomato mutant shows many classic ethylene-resistant phenotypes including a reduced triple response, delayed abscission and altered fruit ripening [13]. Lanahan *et al.* [13] suggested that the *Nr* mutation in tomato may be homologous to the ETR1 gene in *Arabidopsis*.

We have used the *Arabidopsis* ETR1 cDNA as a probe to clone putative homologues from tomato. Here, we describe the cloning, nucleotide sequence and genomic Southern blot analysis of one of these clones. In addition, we present RNA blot data on the level of transcripts for this ETR1 homologue during abscission and fruit ripening, and the effects of ethylene, auxin and silver thiosulfate on its accumulation in abscission zones.

Cloning and sequencing of the tomato ETR1 homologue

A cDNA library made from tomato leaf abscission zone mRNA [12] was screened with ^{32}P-labeled *Arabidopsis* ETR1 cDNA [4]. Hybridization conditions were 42 °C in 5× SSPE, 5× Denhardt's solution, 0.4% SDS, 20% (v/v) formamide and 500 μg/ml of denatured salmon sperm DNA [19]. The final wash of the plaque lifts was performed in 0.1× SSC, 0.5% SDS at 42 °C [19]. A 1 kb cDNA fragment, TAE1, was isolated. Preliminary DNA sequencing indicated that TAE1 lacks ca. 1.7 kb of the 5′ end of the original transcript (Fig. 1A). We then screened a tomato fruit cDNA library (Stratagene, La Jolla, CA) using TAE1 as a probe. Thirty cDNA clones were obtained. Minipreps of the subcloned plasmid DNAs of these thirty clones were again screened with the TAE1 probe to identify those that were most similar and longer than the original TAE1 clone. The 5′ and 3′ ends of 5 clones were partially sequenced using an Applied Biosystem model 373 DNA Sequencer (Foster City, CA). The nucleotide sequence of the 3′ end of one of these cDNA clones, TFE21, was found to be identical with TAE1. The TFE21 clone was then fully sequenced and found to be ca. 2.3 kb long. We estimated that the TFE21 clone was missing ca. 500 bp of the 5′ end (Fig. 1A). We then extended the TFE21 clone in the 5′ direction by reverse transcription of polysomal RNA isolated from leaf abscission zones followed by PCR using gene-specific primers [7]. The 5′ extension gave a composite cDNA length of 2681 bp, which is approximately the size of the transcript estimated from RNA blots. The composite cDNA, eTAE1, contains an open reading frame starting with an ATG codon which encodes a polypeptide of 754 amino acid residues (Fig. 1B) with a deduced molecular mass of 84 kDa and a pI of 7.9. The nucleic acid sequence of the eTAE1 coding sequence is 74% identical to the *Arabidopsis*

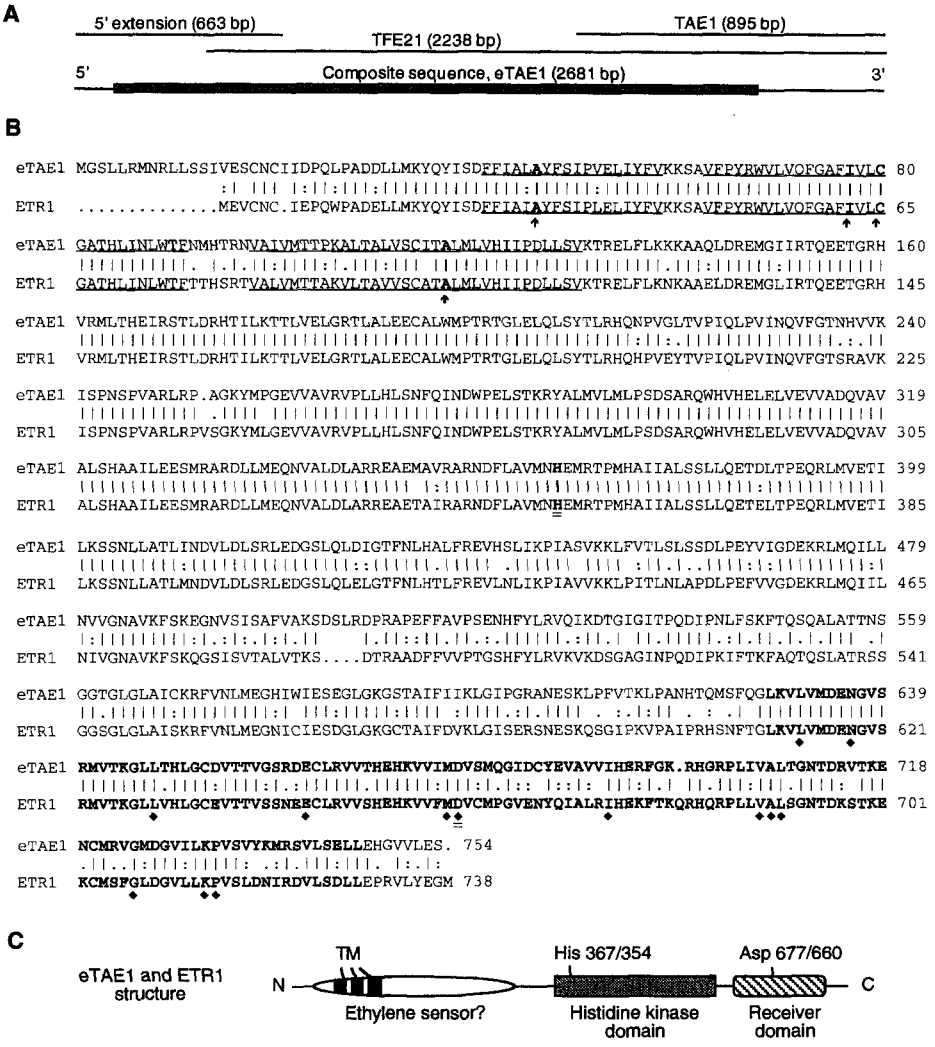

Fig. 1. A. Relative map of overlapping cDNA clones including the 5′ extension and composite eTAE1 sequence. Boxed region in the composite sequence demarcates a 2262 bp coding sequence. B. Comparison of deduced amino acid sequence for eTAE1 with that of the *Arabidopsis* ETR1 sequence. Sequence comparison was done using the GAP program of the Genetics Computer Group (GCG) software (Madison, WI). Single-underlined sequences indicate potential transmembrane domains. Double-underlined amino acids mark the conserved histidine and aspartate residues that may be phosphorylated *in vivo*. Amino acid residues in bold type marked by arrows in the N-terminus are sites of allelic mutations in the *Arabidopsis* ETR1 gene. Bold type in the C-terminus demarcates the putative receiver domains found in the two component systems. Amino acids marked by solid diamonds are conserved amino acids in the receiver domains of eTAE1 and ETR1 and the *Saccharomyces* SLN1 and SSK1 sequences. C. Structural drawing of eTAE1 and ETR1. TM, transmembrane domains; numbers indicate the position of the amino acid residues (eTAE1/ETR1).

ETR1 sequence and the deduced polypeptide for eTAE1 is 81% identical and 90% similar to that for ETR1 (Fig. 1B).

Structural domains in the eTAE1 protein

The amino-terminal half of the deduced protein sequence for eTAE1 does not share significant

similarity to any other translated sequence in the GenBank and EMBL databases except *Arabidopsis* ETR1 (Fig. 1B). Moreover, like ETR1, the first 150 amino acids of eTAE1 contain three transmembrane domains (marked by a single underline in Fig. 1B). Also of interest is that all the amino acids that were mutated in the mutant alleles of ETR1 in *Arabidopsis* [4] are conserved in tomato eTAE1 (indicated by arrows in Fig. 1B). These amino acid residues may be useful in generating dominant ethylene-insensitive mutants of tomato.

The carboxy-terminal half of the deduced protein sequence for eTAE1 shows a high degree of identity to the bacterial 'two-component' regulator family of genes [4] and the recently described yeast osmolarity sensory gene, SLN1, which also shows similarity with the bacterial two-component system [3, 16]. In bacteria, the two protein components, referred to as sensory protein kinase and the response regulator, are involved in a variety of adaptive responses [18]. *ETR1* and eTAE1 along with the yeast SLN1 [3] have sequence similarities both to the sensor histidine kinase domain and the receiver response regulator domain of the two component family (Fig. 1C). However, all three genes lack an output domain.

Comparison of the ethylene signal transduction pathway in *Arabidopsis* to the osmolarity response pathway in yeast has led to a suggestion that the two may be similarly regulated [3]. The putative receiver domain of eTAE1 (119 bp) is 71%, 30% and 22% identical to the *Arabidopsis* ETR1mRNA [4] and the *Saccharomyces* SLN1 [3, 16] and SSK1 [14] receiver domains, respectively. Although the overall sequence identity of eTAE1 and the yeast receiver domains is low, there are some highly conserved residues in this region (Fig. 1B, solid diamonds).

Recently, Hua *et al.* [10], using the ETR1 cDNA clone as a probe, identified another *Arabidopsis* gene, *ERS*, showing sequence identity with the *ETR1* gene. The deduced ERS amino acid sequence shows similarity with the amino-terminal domain and putative histidine kinase domain of *ETR1*; however, most interesting in regard to comparison with eTAE1, the *Arabidopsis ERS* gene lacks a receiver domain. Nevertheless, when a missense mutation identical to the dominant *etr1*-4 mutation [4] was introduced into the *ERS* gene, the altered *ERS* gene, when transferred into wild-type *Arabidopsis*, conferred dominant ethylene insensitivity to *Arabidopsis* [10]. The function of the ERS protein and its interaction with the ETR1 protein in *Arabidopsis*, and their potential homologues in tomato, pose interesting questions.

Tissue-specific and hormonal regulation of eTAE1 transcript accumulation

To determine if eTAE1 transcript accumulation is dependent on developmental cues that trigger ethylene-responsive events, such as abscission and fruit ripening, we examined expression of eTAE1 during leaf and flower abscission and fruit ripening. Polysomal RNA was extracted from leaf abscission zones, petioles, stems, flower abscission zones, pedicels, and pericarp tissue from green, breaker, light red and ripe tomato fruit as described by Kalaitzis *et al.* [12]. RNA samples were electrophoresed, blotted and probed with ^{32}P-labeled TAE1. A 2.7 kb mRNA was identified in all the tissues examined (Fig. 2).

The same blot probed with TAE1 was reprobed independently with tomato abscission polygalacturonase (TAPG1) [12] and tomato fruit polygalacturonase (TFPG) [5]. TAPG1 expression increases specifically in abscission zones during leaf and flower abscission but not fruit. TFPG, on the other hand, is expressed only in ripening fruit (Fig. 2). These tissue-specific and highly induced mRNAs are included for comparison to the more constant TAE1 hybridization signal (Fig. 2).

Although TAE1 hybridization was observed in all the RNA extracts, the signal strength varied slightly among the samples. The relative signal strength of hybridization to RNA from different stages of fruit development was remarkably constant and that of flower abscission zones and pedicels was also fairly constant before and after

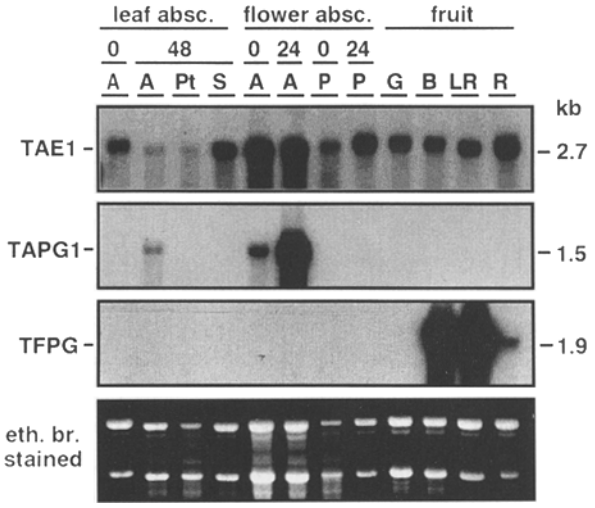

Fig. 2. RNA blot analysis of the expression of TAE1 in various tissues. Leaf abscission zones (A), petiole (Pt), stems (S) were prepared from explants treated with ethylene for 48 h. Lanes labeled 0 included RNA extracted from leaf and flower abscission zones, and pedicels prior to ethylene treatments. Flower abscission zone (A) and pedicels (P) were collected from explants treated with ethylene for 24 h. In addition, pericarp tissues from green (G), breaker (B), light red (LR) and ripe (R) tomato fruits were collected for RNA extraction. Ten μg of polysomal RNA was loaded per lane, electrophoresed and blotted onto nylon membrane. Hybridization conditions were 42 °C in 5 × SSPE, 5 × Denhardt's solution, 0.4% SDS, 50% formamide, and 500 μg/ml of denatured salmon sperm DNA. The final wash was performed at 0.1 × SSC, 0.1% SDS at 50 °C. The same blot was sequentially probed with TAE1, TAPG1 (tomato abscission polygalacturonase) and TFPG (tomato fruit polygalacturonase). Before reprobing with the next probe the blot was washed free of radioactivity with 0.1 × SSPE at 100 °C. The bottom panel shows the ethidium bromide-stained gel used to prepare the blot.

a 24 h ethylene exposure (Fig. 2). However, the hybridization signal for TAE1 in ethylene-induced leaf abscission zones is less than in uninduced abscission zones. Moreover, the signal strength in petioles is less than in stems. Prior to formation of the separation layer in leaf abscission zones (0 h in Fig. 2), we collected ca. 4 mm of tissue at the juncture of the stem and petiole in the region where we expect the separation layer to form. The difference in signal strength between uninduced and induced leaf abscission zones shown in Fig. 2 may simply reflect a difference in the amounts of stem and petiole tissue in the two samples. In a separate experiment in which total RNA was extracted and used for RNA blot analysis, hybridization to the TAE1 probe increased slightly after exposure to ethylene (Fig. 3) which is the opposite response seen when polysomal RNA was used. As suggested above for RNA blots with polysomal RNA (Fig. 2), the differences seen for total RNA from uninduced compared to induced leaf abscission zones (Fig. 3) may simply reflect differences in the amount of stem and petiole tissue in these two samples.

Ethylene is used to initiate abscission in many plants and is considered to be the natural regulator of abscission [11]. Therefore, an increase or decrease in the abundance of TAE1 mRNA in ethylene-induced leaf abscission needs to be more directly addressed. To determine if ethylene indeed affects the accumulation of TAE1 transcripts in leaf abscission, we treated tomato explants with silver thiosulfate (STS), an inhibitor of ethylene action [2] and binding [8]. The stem end of tomato explants with leaves attached were placed in 2 mM STS for 5 h in the greenhouse to allow uptake of STS by the plant. The leaves were then cut off leaving a ca. 5 cm petiole stump. The stem end of explants was then placed in 0.2 mM STS and exposed to 25 μl/l ethylene for 48 h. The

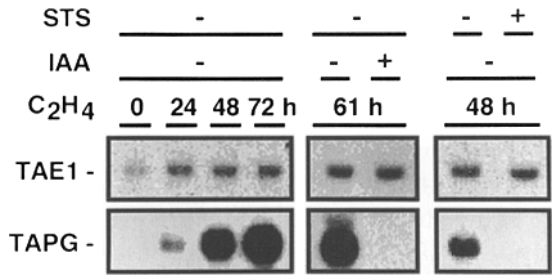

Fig. 3. Effect of IAA and silver thiosulfate (STS) on TAE1 mRNA expression in tomato leaf abscission zones. Explants were treated with sodium thiosulfate (–) or silver thiosulfate (+) and exposed to 25 μl/l ethylene for 48 h. The petiole stumps of a separate batch of explants were dipped in lanolin paste containing 0 μM (–) or 50 μM (+) IAA and the explants exposed to 25 μl/l ethylene for 61 h. A separate batch of explants not treated with STS or IAA was exposed to 25 μl/l ethylene for 0, 24, 48 and 72 h. Total RNA was isolated from each treatment and 10 μg loaded per lane. Hybridization and washing conditions were as described in the legend to Fig. 2.

petioles of explants treated in this manner did not abscise. Total RNA was extracted from the abscission zones of these explants and used for RNA blot analysis. PG mRNA, an indicator of abscission, was not expressed in abscission zones of STS-treated explants (Fig. 3); however, hybridization to TAE1 was unaffected by the STS treatment. This observation suggests that TAE1 transcript level in abscission zones is independent of ethylene.

We also examined the effect of auxin treatment on TAE1 transcript level in leaf abscission zones. Auxin is an antagonist of abscission [21]. In fact, auxin treatment blocks abscission in the presence of saturating concentrations of ethylene [22]. IAA (50 μM) in a lanolin paste was applied to the petiolar stumps of tomato explants and 5 h later exposed to 25 μl/l ethylene for 61 h. As with the STS treatment, auxin treatment inhibited petiole abscission and PG mRNA accumulation but had no effect on TAE1 hybridization.

Number of tomato genes related to eTAE1

Southern genomic blot analysis of *Arabidopsis* DNA suggests a family of *ETR1*-related genes [3]. To estimate the number of genes in tomato that share identity with eTAE1 and therefore *ETR1*, tomato genomic DNA was digested with *Eco*RI, *Hin*dIII and *Xba*I, and then electrophoresed and blotted. The blot was hybridized at low stringency with ^{32}P-labeled TFE21 cDNA and washed sequentially at 42 °C and 60 °C. After washing at 42 °C in 0.2× SSC and 0.1% SDS, five to nine hybridization bands were apparent in each lane (Fig. 4). A 60 °C wash resulted in the retention of approximately half of the bands. These results suggest that in tomato there is a small gene family of *ETR1* homologues. Yen *et al.* [23], using RFLP analysis, identified five distinct tomato loci sharing identity with *Arabidopsis* ETR1.

The TFE21 cDNA used to probe the genomic Southern blot shown in Fig. 4 is 2238 bp long, a nearly full-length clone. The cDNA clone used to probe the RNA blots discussed above, TAE1, is a partial 895 bp clone that includes 328 bp of 3′-untranslated sequence (Fig. 1A). The same Southern blot shown in Fig. 4 was reprobed with TAE1 using the same hybridization conditions described above and washed in 0.2× SSC and 0.1% SDS at 42 °C. A strong hybridization signal was observed for a single band in each lane of the blot (data not shown). The band in the TAE1-probed blot corresponded to the uppermost band in each lane of the blot shown in Fig. 4 (upper panel, 4 h exposure). This result suggests that at the stringency conditions used for the RNA blots, the TAE1 probe does not cross-hybridize to any other gene transcript. Other genes related to the eTAE1 gene may represent functional re-

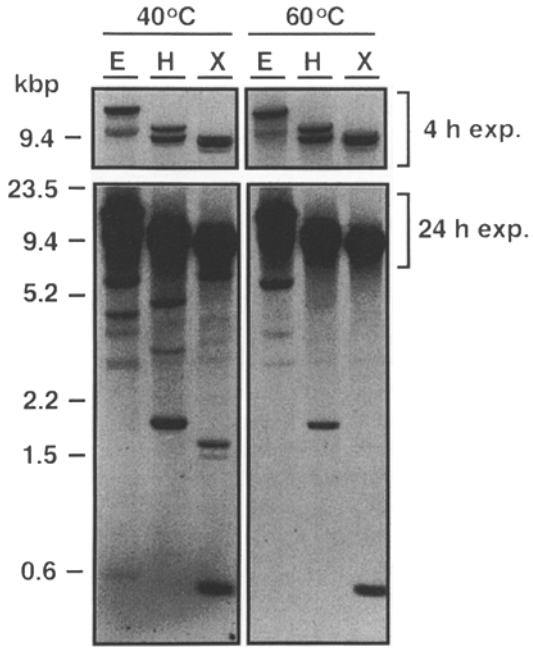

Fig. 4. Southern blot analysis of tomato genomic DNA probed with TFE21. Tomato genomic DNA (15 μg each) was digested with *Eco*RI (E), *Hin*dIII (H) and *Xba*I (X) as shown at the top of each lane. Hybridization was carried out at 42 °C in 5× SSPE, 5× Denhardt's solution, 0.4% SDS, 20% formamide, and 500 μg/ml of denatured salmon sperm DNA. The blot was washed in 0.2× SSC, 0.1% SDS at 40 °C and exposed to film (left) and then washed again in the same solution at 60 °C and reexposed to film (right). The 24 h exposures of the entire blot washed at 40 °C and 60 °C are shown in the bottom two panels and 4 h exposures of the marked regions are shown in the top two panels. The sizes of the molecular markers are shown on the left.

dundancy in tomato ETR1 homologues or each may have a specialized function of its own.

Conclusion

The eTAE1 composite sequence shows high sequence identity with the *Arabidopsis ETR1* gene (Fig. 1B), and, like *ETR1* [4], the tomato gene encoding eTAE1 is constitutively expressed. The high sequence identity of the deduced amino acid sequence for eTAE1 with ETR1 (81%) and structural similarity with other two component systems suggest that the eTAE1 gene product may be involved in the ethylene signal transduction pathway in tomato. Of particular interest is that the N-terminal half of the eTAE1 protein which comprises the putative 'input' domain is very highly conserved between eTAE1 and ETR1 (Fig. 1C) but not at all conserved in other bacterial or eukaryotic genes. The high degree of sequence identity in this region accentuates its potential importance in ethylene signal transduction and further accommodates the hypothesis that ETR1 is an ethylene receptor or interacts directly with the ethylene receptor. The TAE1 and TFE21 clones provide additional tools to be used to decipher and understand ethylene perception and signal transduction in plants.

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