An Ethylene-Inducible Component of Signal Transduction Encoded by *Never-ripe*

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The ripening-impaired tomato mutant *Never-ripe* (*Nr*) is insensitive to the plant hormone ethylene. The gene that cosegregates with the *Nr* locus encodes a protein with homology to the *Arabidopsis* ethylene receptor ETR1 but is lacking the response regulator domain found in ETR1 and related prokaryotic two-component signal transducers. A single amino acid change in the sensor domain confers ethylene insensitivity when expressed in transgenic tomato plants. Modulation of *NR* gene expression during fruit ripening controls response to the hormone ethylene.

The gaseous hormone ethylene (C_2H_4) regulates plant growth and development in response to internal and external cues (*1*). Ethylene affects such diverse processes as seed germination, flower initiation, fruit ripening, tissue senescence, and organ abscission. To modulate its influence, ethylene biosynthesis is controlled by positive and negative feedback mechanisms (*1*). Plants also regulate ethylene action at the level of perception. For example, many fruits must become developmentally competent before they will respond to ethylene by ripening (*2*). The mechanism of this differential responsiveness has not been determined.

Recent work with *Arabidopsis thaliana* has contributed to a more complete understanding of the ethylene signaling process, and several genes in the transduction pathway have been cloned (*3*). The *CTR1* gene encodes a putative serine-threonine protein kinase related to the Raf kinase family (*4*). The *ETR1* gene encodes a protein with homology to both components of bacterial two-component signaling systems (*5*). ETR1 forms membrane-associated dimers and, when expressed in yeast, binds ethylene (*6*, *7*).

We describe here a tomato gene that is homologous to, but distinct from, *ETR1* and the discovery that a lesion in this gene is the molecular basis of the ethylene-insensitive *Never-ripe* (*Nr*) mutant. The 5′ half of the *ETR1* coding sequence was used as a hybridization probe to screen a tomato fruit complementary DNA (cDNA) library (*8*). The largest clone isolated, designated *TXTR-14*, contains a 304-nucleotide 5′ leader sequence and an open reading frame of 635 amino acids encoding a protein with a calculated molecular size of 71 kD (*9*). This 2.4-kb cDNA is 65% identical in its nucleotide sequence, 68% identical in its amino acid sequence, and 81% similar in its amino acid sequence to the ETR1 sequence (*10*), but lacks the COOH-terminal 103 amino acids present in ETR1 (Fig. 1). This region of ETR1 is homologous to various prokaryotic response regulator conserved domains and contains a critical aspartate involved in phosphate transfer (*5*, *11*). The recently cloned *Arabidopsis ERS* gene (*12*), which also lacks this domain, shares 70% amino acid identity with the gene encoding TXTR-14.

TXTR-14 possesses all five sequence motifs characteristic of a sensor histidine protein kinase domain found in two-component systems (Fig. 1) (*5*, *11*). Like ETR1, this domain retains small but significant homology (20 to 40%) to various bacterial signal transducers such as BarA, RcsC, and ArcB, as well as to the *Saccharomyces cerevisiae* protein Sln1 (*5*, *10*). The sensory functions of ETR1, and presumably of TXTR-14, reside in their large NH_2-terminal domains, which are not homologous to any proteins in the databases (*7*, *10*). There is extensive homology between TXTR-14 and ETR1 in the hydrophobic regions near their NH_2-termini (*10*), including conservation of all four amino acids that, when mutated in ETR1, lead to dominant ethylene insensitivity (Fig. 1) (*5*). TXTR-14 is distinct from ETR1, however, by having a deletion of one amino acid in its sensor domain (position 261) and an insertion of

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Fig. 1. Amino acid sequence alignment of the predicted tomato TXTR-14 (top) and *Arabidopsis* ETR1 (bottom) proteins. Vertical bars indicate identity, colons represent close similarity, and periods signify weak similarity (*10*). Gaps (–) were introduced to maximize the alignment. Three NH_2-terminal hydrophobic regions are underlined. Amino acids shown in boldface have the following replacements in mutant alleles: Ala^{31} to Val (*etr1-3*), Ile^{62} to Phe (*etr1-4*), Cys^{65} to Tyr (*etr1-1*), Ala^{102} to Thr (*etr1-2*), and Pro^{36} to Leu (*Never-ripe*). The five boxes surround conserved motifs characteristic of the bacterial histidine kinase domain (*11*). Asterisks denote sites of histidine autophosphorylation and phosphate transfer to a conserved aspartate. Abbreviations are as follows: 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.

four amino acids in its kinase domain (positions 489 to 492).

Mapping studies in tomato have revealed at least five distinct loci with homology to *ETR1* (*13*). With the use of allele-specific polymerase chain reaction (PCR) primers and 61 F_2 individuals, *TXTR-14* was shown to cosegregate with the *Nr* locus, indicating tight genetic linkage [<0.8 centimorgan (cM)] between *TXTR-14* and *Nr* (*14*, *15*). Ethylene insensitivity in *Nr* is manifested as significant delays in fruit ripening, flower senescence, and floral abscission (*16*).

To prove that *TXTR-14* is the *NR* gene, we amplified by PCR the 5′-proximal 375 nucleotides of the *TXTR-14* coding region from *Nr* and its isogenic parental line, Pearson (*17*). Analysis of two independent PCR products from both sources revealed a single change (C to T) in *Nr* at nucleotide 411 of the cDNA sequence, resulting in a Pro to Leu substitution in the first hydrophobic region of the protein (Fig. 1). Pro^{36} is conserved in ETR1 (5) and in two additional tomato ETR1 homologs we have identified (*15*).

As final confirmation that *TXTR-14* is the *NR* gene, we stably introduced the Pro^{36} to Leu mutant *TXTR-14* cDNA (under control of the cauliflower mosaic virus enhanced 35S promoter) into wild-type tomato plants by *Agrobacterium*-mediated transformation (*18*). Ethylene-insensitive R_1 progeny were identified from kanamycin-resistant R_0 lines by the seedling triple response assay (Fig. 2) (*5*, *13*). This insensitivity phenotype was correlated with the transgene as determined by PCR analysis (*19*). Thus, a Pro^{36} to Leu mutation in the TXTR-14 protein is sufficient to confer ethylene insensitivity and is the molecular basis of the *Nr* mutant.

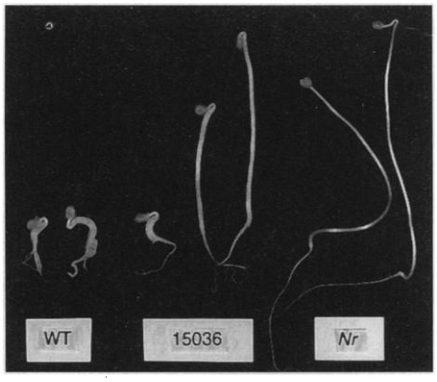

Fig. 2. Ethylene insensitivity displayed in seedlings transformed with *Nr* mutant cDNA. The ethylene response phenotype of plants grown in the dark is shown for WT, wild-type (sensitive); 15036, R_1 segregants of a tomato line transformed with the *Nr* Pro^{36} to Leu mutant *TXTR-14* cDNA (sensitive and insensitive); and *Nr*, *Nr* mutant (insensitive).

Climacteric fruit ripening is characterized by a massive, developmentally regulated, autocatalytic increase in ethylene production and the induction of specific genes (*20*). Our results indicate that *NR* mRNA in fruits is positively regulated by ethylene in a developmentally specific manner (Fig. 3) (*21*). Ethylene inducibility was demonstrated by treating mature green (MG), wild-type fruit with ethylene (*13*). Amounts of *NR* mRNA were small in the absence of ethylene but rapidly accumulated upon ethylene exposure to amounts comparable to those observed at later stages of ripening (Fig. 3A). In fruits harvested at different developmental stages, *NR* mRNA increases rapidly from a barely detectable basal level at the onset of ripening (breaker stage), showing maximal accumulation midway through ripening (pink) (Fig. 3B). This pattern is very similar to that of the developmentally and ethylene-regulated *E8* gene (*22*) (Fig. 3B) and mirrors the climacteric burst of ethylene production (*1*). In contrast, *NR* and *E8* mRNA accumulation in the *Nr* mutant is markedly reduced because of a lack of ethylene perception (Fig. 3B). Finally, ethylene treatment of staged fruits reveals that developmental competence must be achieved to allow induction of *NR* mRNA. *NR* mRNA was not inducible by ethylene in immature green fruits (31 to 43 days after pollination), but induction was possible in MG fruits (48 days after pollination) (Fig. 3C). Developmental or ethylene control has not been observed with *ETR1* or any of its known *Arabidopsis* homologs (*5*, *23*). Because ETR1 is an ethylene receptor (*7*), we speculate that regulated expression of *NR* modulates the differential ethylene sensitivity of maturing tomato fruits. This mechanism of regulating hormone sensitivity may contribute to both the acquisition of developmental competence and the altered responsiveness to ethylene observed in a variety of tissues during various stages of plant growth and development (*1*, *2*).

The work presented here describes the molecular basis of the ethylene-insensitive tomato mutant *Never-ripe*. Insensitivity is caused by a single amino acid change in a protein homologous to the *Arabidopsis* ethylene receptor ETR1. NR, a homologous protein in tomato, is substantially different because it does not contain a response regulator domain and exhibits both developmental control and ethylene-inducibility. Tomato simultaneously up-regulates both ethylene biosynthesis and expression of the signal transduction pathway, presumably to achieve rapid and coordinated maturation.

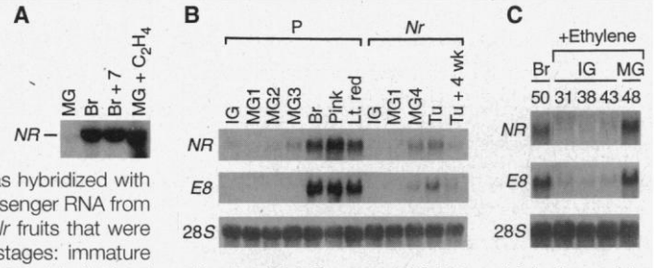

Fig. 3. Regulated expression of the *NR* gene in fruit. (**A**) *NR* mRNA in wild-type mature green (MG), breaker (Br), Br plus 7 days, and MG fruit treated 8 hours with ethylene (20 ppm). The blot was hybridized with an *NR* cDNA probe. (**B**) Messenger RNA from wild-type Pearson (P) and *Nr* fruits that were harvested at the following stages: immature green (IG), MG stages 1 to 4, Br, pink, light red, and turning (Tu). Because *Nr* fruit do not fully ripen, older fruit were staged as turning plus 4 weeks (*21*). Blots were hybridized with *NR*, *E8*, or *28S* ribosomal DNA probes (*21*). (**C**) Wild-type fruit harvested at the indicated number of days after pollination were exposed to air or air plus ethylene (100 ppm) for 24 hours. Hybridizations were performed as above.

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18. *TXTR-14* cDNA containing the *Nr* Pro^{36} to Leu mutation was subcloned behind the cauliflower mosaic virus enhanced 35*S* promoter and upstream of a pea *rbcs-E9* gene 3′ end [H. J. Klee *et al.*, *Plant Cell* **3**, 1187 (1991)]. The vector was mobilized into *Agrobacterium* and used to generate kanamycin-resistant plants essentially as described [S. McCormick *et al.*, *Plant Cell Rep.* **5**, 81 (1986)].
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Ethylene-Binding Sites Generated in Yeast Expressing the *Arabidopsis ETR1* Gene

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Mutations in the *ETR1* gene of *Arabidopsis thaliana* confer insensitivity to ethylene, which indicates a role for the gene product in ethylene signal transduction. Saturable binding sites for [^{14}C]ethylene were detected in transgenic yeast expressing the ETR1 protein, whereas control yeast lacking ETR1 showed no detectable ethylene binding. Yeast expressing a mutant form of ETR1 (*etr1-1*) also showed no detectable ethylene binding, which provides an explanation for the ethylene-insensitive phenotype observed in plants carrying this mutation. Expression of truncated forms of ETR1 in yeast provided evidence that the amino-terminal hydrophobic domain of the protein is the site of ethylene binding. It was concluded from these results that ETR1 acts as an ethylene receptor in *Arabidopsis*.

The gaseous hormone ethylene (C_2H_4) is involved in the regulation of developmental processes and stress responses in higher plants, including seed germination, seedling growth, leaf abscission, fruit ripening, organ senescence, and pathogen responses (*1*). Although the ethylene biosynthetic pathway is well-characterized (*2*), the means by which plants recognize and transduce the ethylene signal has not been established. An important contribution to our understanding of ethylene signal transduction has come from isolation of mutants that affect ethylene responses in *Arabidopsis thaliana* (*3*). The *ETR1* gene was identified in this manner (*4*). Four dominant mutant alleles of *ETR1* that confer insensitivity to a range of ethylene responses have been identified (*4*, *5*), and *Arabidopsis* plants containing the mutant *etr1-1* allele display one-fifth the saturable ethylene binding of that found in wild-type plants (*4*). Genetic analysis indicates that *ETR1* acts upstream of other loci that affect ethylene signal transduction (*3*, *6*). The *ETR1* gene was cloned (*5*) and found to encode a polypeptide with a hydrophobic NH_2-terminus responsible for membrane localization (*5*, *7*) and a COOH-terminal region with homology to the histidine kinases and response regulators of bacteria (Fig. 1). In bacteria, these signal transduction systems mediate responses to a wide variety of environmental stimuli (*8*). On the basis of these characteristics, the ETR1 protein has been hypothesized to function in the perception of the ethylene signal.

To aid in the biochemical characterization of the ETR1 protein, we expressed the full-length coding sequence in yeast (*9*). Immunological analysis indicated that the yeast-expressed protein, like the native protein in *Arabidopsis*, exists as a membrane-associated, disulfide-linked dimer (*7*). The capacity of yeast expressing ETR1 to bind ethylene was tested in vivo with the isotope displacement assay described by Sisler (*10*). Expression of ETR1 in yeast resulted in the creation of high-affinity binding sites for [^{14}C]ethylene (Fig. 1), and most of the labeled ethylene could be displaced by competition with unlabeled ethylene, which indicated that binding was saturable. The identity of the released compound as ethylene was confirmed by gas chromatography. Control yeast transformed with vector alone showed no saturable ethylene binding. One gram of transgenic yeast contained up to 40 pmol of ethylene-binding sites, as deduced from the calculated maximum binding capacity over several experiments (Fig. 2). *Arabidopsis* reportedly contains 1 pmol of ethylene-binding sites per gram of leaf tissue (*11*). The higher concentration of binding sites observed in yeast is consistent with immunological data that indicate that ETR1 protein expressed in yeast was present at about 100-fold greater abundance than in its native *Arabidopsis* (*7*).

The binding of ethylene in transgenic yeast was tight but reversible, with a calculated dissociation constant (K_d) of 2.4×10^{-9} M, assuming a membrane environment for the binding site. Analysis of release kinetics indicated a half-life for ethylene dissociation of 12.5 hours. This slow dissociation rate is similar to the rate observed with one class of binding activity reported from several plant sources (*11*, *12*). Ethylene binding by ETR1 was also found to be inhibited by *trans*-cyclooctene and 2,5-norbornadiene (Table 1), both competitive inhibitors of ethylene binding and responses in plants (*12*, *13*). *Cis*-cyclooctene is a less effective inhibitor of ethylene binding and responses in plants (*13*) and did not effectively inhibit ethylene binding to ETR1 in yeast.

Dose-dependent binding of [^{14}C]ethylene in transgenic yeast conformed to the hyperbolic relation predicted by Michaelis-Menton kinetics, spanning about two orders of magnitude and having a Hill coefficient (n) close to 1 (Fig. 2). The dose-dependent binding in yeast paralleled the curve for growth-inhibition responses to ethylene in *Arabidopsis* seedlings (Fig. 2), which showed a half-maximal response at a concentration of ethylene of 0.1 μl/liter (*14*). These effects of ethylene on seedling growth, which are among the most sensitive documented responses to ethylene in plants, can thus be accounted for by the binding relation observed with ETR1 in transgenic yeast. It remains to be determined whether the observed binding can also account for responses, such as chitinase induction, that operate over a higher range of ethylene concentrations in *Arabidopsis* but are also disrupted by mutations in *ETR1* (*3*, *14*).

To clarify which region of ETR1 was involved in binding ethylene, we expressed truncated forms of ETR1 (*9*) in yeast (Fig. 1). For the bacterial sensor proteins, ligand binding typically involves the NH_2-terminal portion of the protein, and the ligand-binding domain is distinct and can be dis-

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