Giovannoni, H. J. Klee, unpublished results.
16. M. B. Lanahan, H.-C. Yen, J. J. Giovannoni, H. J. Klee, *Plant Cell* **6**, 521 (1994).
17. Primers NrPCR5 (CCGGATCCATGGAATCCTGTGATTGCATTG) and TETR4A (GATAATAGGAAGATTAATTGGC) were used for PCR as described (*8*). PCR products were gel-purified, digested with Bam HI and Xho I, cloned into the Bam HI–Sal I sites of pBS (Stratagene), and sequenced.
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)].
19. Ethylene-sensitive and ethylene-insensitive seedlings were assayed for the presence of the transgene by PCR as described (*17*).
20. T. I. Zarembinski and A. Theologis, *Plant Mol. Biol.* **26**, 1579 (1994).
21. RNA isolated from frozen fruit tissue and separated by denaturing agarose gel electrophoresis [J. Wilkinson, M. Lanahan, T. Conner, H. Klee, *Plant Mol. Biol.* **27**, 1097 (1995)] was analyzed by hybridization to radiolabeled *TXTR-14*, *E8* (*22*), and 28*S* ribosomal DNA [*pZm26R* from D. A. Russell and M. M. Sachs, *Mol. Gen. Genet.* **229**, 219 (1991)] probes. Fruit developmental stages were determined according to A. A. Kader and L. L. Morris, in *Proceedings of the 2nd Tomato Quality Workshop* (University of California, Davis, CA, 1976), pp. 57–62.
22. J. E. Lincoln, S. Cordes, E. Read, R. L. Fischer, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2793 (1987).
23. C. Chang, personal communication.
24. We thank C. Chang and E. Meyerowitz for *ETR1* clones and helpful discussions, S. Tanksley for use of the tomato mapping population and restriction fragment length polymorphism probes, J. Layton, J. Rottnek, P. Delaquil, G. DeBrecht, and T. Coombe for generating transgenic plants, and S. Lee for technical assistance. Supported in part by U.S. Department of Agriculture grants 92-37300-7653 and 95-37300-1575 (J.J.G.) and 95-37304-2326 (H.J.K.).

7 August 1995; accepted 7 November 1995

Ethylene-Binding Sites Generated in Yeast Expressing the *Arabidopsis ETR1* Gene

G. Eric Schaller and Anthony B. Bleecker*

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-

Department of Botany, University of Wisconsin, Madison, WI 53706, USA.

*To whom correspondence should be addressed. E-mail: ableek@macc.wisc.edu

sociated from the histidine kinase domain (8). Consistent with such a modular structure, no ethylene binding was observed when a portion of ETR1 covering the soluble COOH-terminal region (amino acids 183 to 738), including histidine kinase and response regulator domains, was expressed in yeast. However, expression of the NH_2-terminal half of ETR1 (amino acids 1 to 400) conferred upon yeast the ability to bind ethylene. The binding site was further localized by expression of the first 165 amino acids of ETR1 (1 to 165), which still showed significant and reproducible ethylene binding. On the basis of these results, we propose that the ethylene-binding site is contained within the NH_2-terminal hydrophobic domain of ETR1.

Ethylene is hypothesized to bind to its receptor through a metal, with a transition metal such as Cu(I) considered most likely given the known interactions of olefins with transition metals (*15*). Metal coordination usually involves cysteine, histidine, or methionine residues of the protein (*16*), and in this respect the *etr1-1* mutation may be of special significance as it represents the mutation of a Cys residue in the second transmembrane domain ($Cys^{65} \rightarrow$ Tyr), resulting in a plant insensitive to ethylene (*4*, *5*). To examine the effect of alterations in Cys^{65} on ethylene binding, we expressed two site-directed mutant forms of the ETR1 protein in yeast. When Cys^{65} was converted to either Tyr or Ser, the mutant proteins showed no detectable ethylene binding in yeast (Figs. 1 and 2). Thus, a single amino acid change within the hydrophobic domain of ETR1 can perturb the protein so as to prevent ethylene binding, possibly by eliminating a ligand for the presumptive transition metal.

Several other site-directed mutations were introduced into the *ETR1* gene and the expressed protein examined for ethylene binding in yeast. Cys^{99}, like Cys^{65}, is a Cys residue in the hydrophobic region of ETR1, but in the third putative transmembrane domain rather than in the second. Cys^4 and Cys^6 are involved in a disulfide linkage between monomers of ETR1, leading to formation of a covalent dimer (*7*). Mutant proteins in which these residues were converted to Ser still retained the ability to bind ethylene (Fig. 1), which indicates that neither covalent linkage of monomers nor Cys^{99} is necessary for ethylene binding. This finding does not rule out the possibility that noncovalent dimerization is required for ethylene binding.

Although the *etr1-1* mutation completely disrupts ethylene binding in yeast, some saturable binding is still present in *etr1-1* mutant plants (*4*). Residual binding activity may be attributable to additional receptor isoforms, a possibility supported by the finding that a mutated form of the *Arabidopsis ERS* gene (67% amino acid identity with *ETR1*) conferred ethylene insensitivity on *Arabidopsis* (*17*). Functionally redundant ethylene receptors may also explain why only dominant mutants of *ETR1* have been identified genetically.

Because all four mutant alleles of ETR1 result from point mutations in the presumptive ethylene-binding domain, the dominant insensitivity observed in plants may arise from disruption of ethylene binding, essentially locking the protein in a form unable to sense ethylene. If this form is inactive, dominance could involve the formation of a poisoned complex. In that case, the level of effective (wild-type) dimers in heterozygous plants would have to be below the threshold needed to induce the seedling-growth inhibition response, even at saturating ethylene concentrations. It would also be necessary for mutant ETR1 monomers to interact with

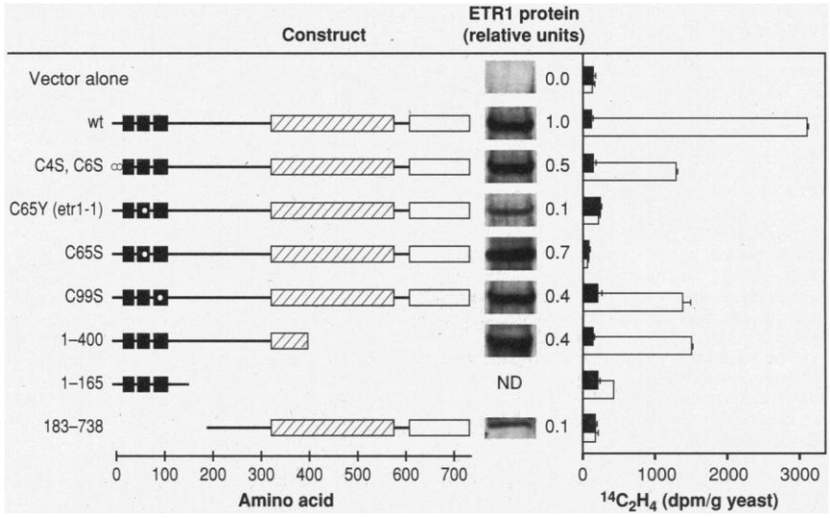

Fig. 1. Ethylene binding by yeast expressing wild-type and mutant forms of the protein ETR1. Expressed forms of ETR1 (*9*) are depicted diagramatically, with transmembrane (black), histidine kinase (hatched), and response regulator (white) domains indicated by boxes. For site-directed mutations, single-letter abbreviations for amino acids Cys (C), Ser (S), and Tyr (Y) are used. For truncations, the first and last amino acids of the expressed protein are indicated, the full-length ETR1 protein being 738 amino acids long. Transgenic yeast was incubated with 0.09 μl/liter of [^{14}C]ethylene (white bars) or with 0.09 μl/liter of [^{14}C]ethylene and 1000 μl/liter of [^{12}C]ethylene (black bars) (*10*). The difference between these two values represents the saturable binding. Samples were run in triplicate, and standard deviations are shown. ETR1 protein was identified immunologically and quantified densitometrically (*19*).

Table 1. Inhibition of ethylene binding. Saturable ethylene binding of yeast expressing ETR1 was determined at 0.08 μl/liter of [^{14}C]ethylene, in the presence of the indicated compounds.

Compound	[^{14}C]ethylene bound (%)
None	100
2,4-norbornadiene	
5 μl/liter	84
50 μl/liter	22
trans-cyclooctene	
5 μl/liter	78
50 μl/liter	24
cis-cyclooctene	
5 μl/liter	94
50 μl/liter	92
500 μl/liter	81

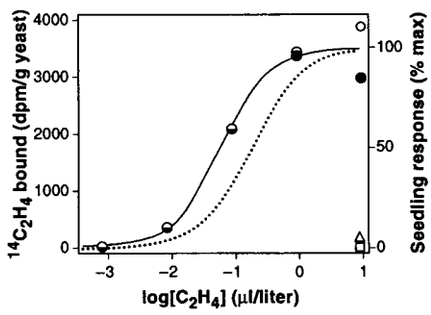

Fig. 2. Dose dependency for ethylene binding by yeast expressing ETR1. Duplicate samples of yeast (open and closed circles) expressing ETR1 were tested for binding at the concentrations of [^{14}C]ethylene indicated on the abscissa (*10*), with the saturable binding determined by subtracting the mean of two samples incubated with [^{14}C]ethylene and 1000 μl/liter of [^{12}C]ethylene. Samples were incubated with ethylene for 6 hours. The best-fit line using the Hill equation is plotted [r^2 = 0.98, n = 1.17, K_d = 0.036 μl/liter (gas phase)]. For comparison, a dose-response curve for hypocotyl growth inhibition of *Arabidopsis* seedlings by ethylene is plotted (dotted line) (*14*). Yeast expressing vector alone (square) or *etr1-1* mutant protein (triangle) was also examined for binding at 8 μl/liter of ethylene, but no significant binding was observed.

and poison subunits of any functional homologs. Alternatively, the ETR1 protein may normally be active in the absence of ethylene and negatively regulate the response pathways. Binding of ethylene would inactivate the receptor, resulting in derepression of the response pathways. Mutant receptors that fail to bind ethylene would be locked in the active state, suppressing the response pathways even when wild-type receptors were saturated with ethylene. If true, the latter model must be reconciled with the finding that *Never-ripe* (*Nr*), a mutation in tomato that seems to be attributable to a lesion in a tomato homolog of *ETR1*, shows an increase in Nr mRNA abundance in tomato fruits as they become more sensitive to ethylene-induced ripening (*18*).

REFERENCES AND NOTES

1. F. B. Abeles, P. W. Morgan, M. E. Saltveit Jr., *Ethylene in Plant Biology* (Academic Press, San Diego, CA, 1992).
2. H. Kende, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 283 (1993).
3. J. R. Ecker, *Science* **268**, 667 (1995).
4. A. B. Bleecker, M. A. Estelle, C. Somerville, H. Kende, *ibid.* **241**, 1086 (1988).
5. C. Chang, S. F. Kwok, A. B. Bleecker, E. M. Meyerowitz, *ibid.* **262**, 539 (1993).
6. J. J. Kieber, M. Rothenberg, G. Roman, K. A. Feldmann, J. R. Ecker, *Cell* **72**, 427 (1993).
7. G. E. Schaller, A. N. Ladd, M. B. Lanahan, J. M. Spanbauer, A. B. Bleecker, *J. Biol. Chem.* **270**, 12526 (1995).
8. J. S. Parkinson and E. C. Kofoid, *Annu. Rev. Genet.* **26**, 71 (1992); J. S. Parkinson, *Cell* **73**, 857 (1993).
9. ETR1 was expressed in the yeast *Saccharomyces cerevisiae* (strain LRB520) with the use of the vector pYcDE-2 with a constitutive ADC1 promoter. Constructs for full-length, single site–mutated, and truncated forms of ETR1 were made as described (*7*). For expression of the NH_2-terminal region of ETR1 representing amino acids 1 to 165, a 0.65-kb Eco RI–Dra I fragment of one of our complementary DNA clones (cETR1-9) was cloned into the Eco RI site of pYcDE-2, after addition of a 10-mer Eco RI linker to the Dra I site.
10. E. C. Sisler, *Plant Physiol.* **64**, 538 (1979). [^{14}C]ethylene (specific activity = 56.9 mCi/mmol) was obtained from American Radiolabeled Chemicals and trapped as the mercuric perchlorate complex. Yeast cells were grown to mid-log phase at 30°C, harvested by centrifugation at 1500*g* for 5 min, washed with water, and collected by vacuum filtration on glass fiber disks (1 g of yeast per disk). Samples were incubated in sealed glass chambers (Ball jelly jars) for 4 hours in the presence of [^{14}C]ethylene with or without [^{12}C]ethylene. Samples were removed, aired for 5 min, then transferred to individual chambers with 0.3 ml of mercuric perchlorate in a scintillation vial. Chambers were heated to 65°C for 90 min, then allowed to stand 24 hours to trap ethylene released from the samples. We determined the gas-phase concentration of [^{14}C]ethylene during the primary incubation by withdrawing gas with a syringe and then trapping it with mercuric perchlorate. Trapped [^{14}C]ethylene was quantified by liquid scintillation counting.
11. I. O. Sanders, N. V. J. Harpham, I. Raskin, A. R. Smith, M. A. Hall, *Ann. Bot.* **68**, 97 (1991).
12. E. C. Sisler, in *The Plant Hormone Ethylene*, A. K. Matoo and J. C. Suttle, Eds. (CRC Press, Boca Raton, FL, 1991), pp. 81–99.
13. E. C. Sisler, S. M. Blankenship, S. M. Guest, *Plant Growth Regul.* **9**, 157 (1990).
14. Q. G. Chen and A. B. Bleecker, *Plant Physiol.* **108**, 597 (1995).
15. S. P. Burg and E. A. Burg, *ibid.* **42**, 144 (1967); J. P. Collman, L. S. Hegedus, J. R. Norton, R. G. Finke, *Principles and Applications of Organotransition Metal Chemistry* (University Science Books, Mill Valley, CA, 1987).
16. J. J. R. da Silva and R. J. P. Williams, *The Biological Chemistry of the Elements* (Clarendon Press, Oxford, UK, 1991).
17. J. Hua, C. Chang, Q. Sun, E. M. Meyerowitz, *Science* **269**, 1712 (1995).
18. J. Q. Wilkinson, M. B. Lanahan, H.-C. Yen, J. J. Giovannoni, H. J. Klee, *ibid.*, **270**, 1807 (1995).
19. Total yeast protein was extracted, separated by SDS–polyacrylamide gel electrophoresis, and ETR1 visualized by protein immunoblotting as described (*7*). The antibody Ab-UNK was used, which recognizes the portion of ETR1 corresponding to amino acids 165 to 400.

20. We thank G. Polzin and J. Burstyn for synthesis of *trans*-cyclooctene and H. Kende and C. Chang for critical reading of the manuscript. This research was supported by Department of Energy (DOE) grant DE-FG02-91ER20029.000, Department of Agriculture (USDA) grant 9403009, and the DOE-NSF-USDA Collaborative Research in Plant Biology Program (grant BIR92-20331).

31 July 1995; accepted 30 October 1995

Identification of RANTES, MIP-1α, and MIP-1β as the Major HIV-Suppressive Factors Produced by CD8$^+$ T Cells

Fiorenza Cocchi,* Anthony L. DeVico, Alfredo Garzino-Demo, Suresh K. Arya, Robert C. Gallo,*† Paolo Lusso‡

Evidence suggests that CD8$^+$ T lymphocytes are involved in the control of human immunodeficiency virus (HIV) infection in vivo, either by cytolytic mechanisms or by the release of HIV–suppressive factors (HIV-SF). The chemokines RANTES, MIP-1α, and MIP-1β were identified as the major HIV-SF produced by CD8$^+$ T cells. Two active proteins purified from the culture supernatant of an immortalized CD8$^+$ T cell clone revealed sequence identity with human RANTES and MIP-1α. RANTES, MIP-1α, and MIP-1β were released by both immortalized and primary CD8$^+$ T cells. HIV-SF activity produced by these cells was completely blocked by a combination of neutralizing antibodies against RANTES, MIP-1α, and MIP-1β. Recombinant human RANTES, MIP-1α, and MIP-1β induced a dose-dependent inhibition of different strains of HIV-1, HIV-2, and simian immunodeficiency virus (SIV). These data may have relevance for the prevention and therapy of AIDS.

As documented in several viral diseases (*1*), CD8$^+$ T lymphocytes are believed to play a critical role in the containment of HIV infection, particularly during the phase of clinical latency and in long-term nonprogressors (*2*). Activated CD8$^+$ T cells derived from the peripheral blood of HIV-infected individuals (*3*), as well as from HIV- or SIV-infected nonhuman primates (*4*), secrete one or more soluble HIV-suppressive factors (HIV-SF) that may contribute to the control of HIV infection in vivo (*5*). The production of HIV-SF by CD8$^+$ T cells isolated in vitro correlates with the disease stage, showing a progressive decline in parallel with the increasing deterioration of the immune system (*6*).

We established a sensitive test system for HIV-SF (*7*), based on a CD4$^+$ T cell clone (PM1) that has a broad susceptibility to macrophage-tropic and primary HIV-1 isolates (*8*). The HIV-SF was tested on PM1 cells acutely infected with HIV-1_{BaL} (*9*), a macrophage-tropic isolate with biological properties resembling those of non–syncytia-inducing primary isolates (*8*, *9*). To identify a reproducible source of HIV-SF, we tested (i) three CD8$^+$ T cell lines immortalized in vitro with human T cell leukemia/lymphotropic virus (HTLV) type I (CD8-UI, CD8-PI, and 67-I), (ii) one HTLV-I–negative CD8$^+$ T cell line (PF382) and (iii) two CD4$^+$ T cell lines, one immortalized with HTLV-I (MT-2) and the other with HTLV-II (Vev-II) (Table 1) (*10*). No effect was seen with the culture supernatant of PF382, which suppresses the differentiation of bone-marrow precursor cells (*11*). By contrast, all three HTLV-I$^+$CD8$^+$ T cell lines produced extracellular HIV-SF, albeit at different levels. Some HIV-SF activity was also detected in the culture supernatant of MT-2, whereas Vev-II had no significant effect. To ob-

F. Cocchi, A. Garzino-Demo, S. K. Arya, R. C. Gallo, P. Lusso, Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD 20892, USA.
A. L. DeVico, Advanced BioScience Laboratories, Kensington, MD 20852, USA.

*Present address: Institute of Human Virology, Medical Biotechnology Center, University of Maryland Biotechnology Institute (UMBI), University of Maryland, Baltimore, MD 21201, USA.
†To whom correspondence and reprint requests should be addressed at Institute of Human Virology, Suite 200, Medical Biotechnology Center, UMBI, University of Maryland, Baltimore, MD 21201, USA.
‡Present address: Unit of Human Virology, DIBIT, San Raffaele Scientific Institute, 20132 Milano, Italy, and Institute of Human Virology, Baltimore, MD 21201, USA.