Genetic Analysis of Ethylene Signal Transduction in *Arabidopsis thaliana*: Five Novel Mutant Loci Integrated into a Stress Response Pathway

Gregg Roman, Barry Lubarsky, Joseph J. Kieber,[1] Madge Rothenberg and Joseph R. Ecker

Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018

Manuscript received October 12, 1994
Accepted for publication November 17, 1994

ABSTRACT

The response of *Arabidopsis thaliana* etiolated seedlings to the plant hormone ethylene is a conspicuous phenotype known as the triple response. We have identified genes that are required for ethylene perception and response by isolating mutants that fail to display a triple response in the presence of exogenous ethylene. Five new complementation groups have been identified. Four of these loci, designated *ein4, ein5, ein6* and *ein7,* are insensitive to ethylene. The fifth complementation group, *eir1,* is defined by a novel class of mutants that have agravitropic and ethylene-insensitive roots. Double-mutant phenotypes have allowed the positioning of these loci in a genetic pathway for ethylene signal transduction. The ethylene-response pathway is defined by the following loci: *ETR1, EIN4, CTR1, EIN2, EIN3, EIN5, EIN6, EIN7, EIR1, AUX1* and *HLS1. ctr1-1* is epistatic to *etr1-3* and *ein4,* indicating that *CTR1* acts after both *ETR1* and *EIN4* in the ethylene-response pathway. Mutations at the *EIN2, EIN3, EIN5, EIN6* and *EIN7* loci are all epistatic to the *ctr1* seedling phenotype. The *EIR1* and *AUX1* loci define a root-specific ethylene response that does not require *EIN3* or *EIN5* gene activity. *HLS1* appears to be required for differential cell growth in the apical hook. The *EIR1, AUX1* and *HLS1* genes may function in the interactions between ethylene and other plant hormones that occur late in the signaling pathway of this simple gas.

ETHYLENE is a simple gaseous molecule that regulates many complex processes in plant growth and physiology. The effect of ethylene on pea seedling development, studied by DIMITRY NELJUBOV in 1901, was the first demonstration that a gas could act as a signaling molecule in a biological system (described in ABELES *et al.* 1992). Ethylene since has been shown to play a fundamental role in fruit ripening, germination, sex determination, leaf abscission, flower senescence, responses to mechanical stress and several different pathogenic responses (MATTOO and SUTTLE 1991; ABELES *et al.* 1992). These effects are regulated by factors that control either the biosynthesis or the perception of this gas. The biosynthetic pathway for ethylene has been established and several of the rate-limiting enzymes have been cloned (YANG and HOFFMAN 1984; SATO and THEOLOGIS 1989; SPANU *et al.* 1991). In contrast, the mechanisms by which plants perceive and respond to this hormone are just beginning to be revealed (KIEBER and ECKER 1993).

In the presence of ethylene, *Arabidopsis thaliana* seedlings undergo dramatic morphological changes referred to as the triple response (KNIGHT *et al.* 1910). This seedling phenotype consists of an exaggerated curvature of the apical hook, radial swelling of the hypocotyl and an inhibition of root and hypocotyl elongation (BLEECKER *et al.* 1988; GUZMAN and ECKER 1990). It long has been suggested that these changes, which result from stress-induced ethylene, are necessary for the seedling to penetrate the soil without damaging the apical meristem (DARWIN and DARWIN 1881). The formation of the triple response relies on the plant's ability to respond to ethylene. The silver ion (Ag^+), a very effective noncompetitive inhibitor of ethylene action (BEYER 1976), and *trans*-cyclooctene, a strong competitive inhibitor of ethylene responses (SISLER 1990), inhibit the triple-response phenotype in *A. thaliana* (GUZMAN and ECKER 1990).

One mechanism by which ethylene may regulate hypocotyl and root length is by its effects on the structure of the extracellular matrix (APELBAUM and BURG 1971; LANG *et al.* 1982). Ethylene treatment of pea epicotyls causes the orientation of cellulose microfibrils in the secondary cell wall to shift from a transverse to a longitudinal direction (APELBAUM and BURG 1971; LANG *et al.* 1982; YUAN *et al.* 1994). This change may be responsible for the increased lateral growth of these cells. Ethylene also affects the polar transport of the auxin indole acetic acid (IAA), thus controlling the levels of this hormone in various target tissues (BURG and BURG 1967; BEYER 1973). Modified levels of auxin may result in reduced cell elongation and, therefore, reduce the length of the seedling. Ethylene also has been shown

Corresponding author: Joseph R. Ecker, Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018. E-mail: jecker@atgenome.bio.upenn.edu

[1] *Present address:* University of Illinois, Laboratory for Molecular Biology, Chicago, IL 60607-7020.

to inhibit the rate of cell division in pea seedlings (APELBAUM and BURG 1972). The effect of ethylene on the apical hook appears complex and may involve intricate interactions with auxin to coordinate the rate of elongation between cells throughout the hook structure (SCHWARK and SCHIERLE 1993; A. LEHMAN and J. R. ECKER, unpublished results).

Some of these and other effects of ethylene on plant growth and development are likely to be mediated by changes in gene expression. Several genes whose transcription is induced by ethylene have been identified (ECKER and DAVIS 1987; LAWTON *et al.* 1994; MELLER *et al.* 1993). Complex patterns of ethylene induced gene expression have been found in tomato fruit ripening (LINCOLN and FISHER 1988), and there is some evidence that ethylene also acts posttranscriptionally (THEOLOGIS 1992).

Mutations that affect the triple response in Arabidopsis have defined a large number of genes involved in the synthesis and perception of ethylene (BLEECKER *et al.* 1988; GUZMAN and ECKER 1990; HARPHAM *et al.* 1991; VAN DER STRAETEN *et al.* 1993). Four mutant loci with an ethylene-insensitive phenotype have been identified and characterized genetically: *etr1* (BLEECKER *et al.* 1988), *ein2* (GUZMAN and ECKER 1990), *ein3* (KIEBER *et al.* 1993; M. ROTHENBERG, G. ROMAN and J. R. ECKER, unpublished data) and *ain1* (VAN DER STRAETEN *et al.* 1993). Additional screens identified mutants that overproduce ethylene (*eto1, eto2* and *eto3*) and have a constitutive triple response phenotype (KIEBER *et al.* 1993), as well as mutants that failed to form an exaggerated apical hook (hookless, *hls1*; GUZMAN and ECKER 1990). Mutations at the *CTR1* locus result in severe constitutive triple-response phenotypes that are not reverted by inhibitors of ethylene biosynthesis or action (KIEBER *et al.* 1993). Several pleiotropic mutants have been identified with auxin defects that are also insensitive to ethylene in the seedling root (LINCOLN *et al.* 1990; PICKETT *et al.* 1990).

The *CTR1* gene was cloned and found to show similarity to the Raf family of protein kinases, implicating a kinase cascade in this ethylene response (KIEBER and ECKER 1993; KIEBER *et al.* 1993). The dominant *etr1* mutant gene also has been cloned; it shows significant similarity to the *SLN1* gene of *Saccharomyces cerevisiae* and the bacterial two-component histidine kinases (CHANG *et al.* 1993; OTA and VARSHAVSKY 1993).

In this study, we describe mutations in four new loci, all of which produce an ethylene-insensitive phenotype. We also describe a novel class of ethylene-insensitive and agravitropic root mutants. A genetic framework has been established for the action of these genes on the basis of epistasis interactions. Most of the mutations that we and others have described act in linear pathway. Genes that control branch points leading to tissue-specific phenotypes occur late in this pathway; these branches may provide critical insights into the nature of hormonal interactions in plants.

MATERIALS AND METHODS

Plant material: Arabidopsis plants were grown on Metro-Mix 200 (Grace-Sierra Horticultural Products, Milpitas, CA) under the following conditions: 23°, 100–200 $\mu E/m^2/sec$ constant light. Plants were fertilized once between 2 and 4 wk with either a modified Hoaglands solution (FELDMANN and MARKS 1987) or Pete's Lite 14–15–16 (Grace-Sierra Horticultural Products, Milpitas, CA). Plants were out-crossed as previously described (GUZMAN and ECKER 1990). All mutants (with the exception of *ctr1-5, ein3-2* and *ein6*) were isolated from the *A. thaliana* strain Columbia. Wassilewskija is the parental strain of both *ctr1–5* and *ein3–2* (FELDMANN and MARKS 1987; KIEBER *et al.* 1993). The *ein6* mutant was isolated from mutagenized Landsberg seed. X-ray and diepoxybutane mutagenesis were performed as described (KIEBER *et al.* 1993). Fast neutron treated seed were a gift of LAURA CONWAY (University of Pennsylvania). Surface-sterilized seedlings were grown on medium pH 5.7 containing MS salts (GIBCO, Gaithersburg, MD) supplemented with 1 mg/ml thiamine, 0.5 mg/ml pyridoxine, 0.5 mg/ml nicotinic acid, 100 mg/liter inositol, 10 g/liter sucrose and 0.8% bacto-agar (DIFCO, Detroit, MI). Surface sterilization of seeds was performed as previously described (GUZMAN and ECKER 1990). Mutagen treated seeds were plated at a density of ~5000 seeds per 132 mm diameter petri dish. Assays for ethylene insensitivity or constitutive triple response were as previously described (GUZMAN and ECKER 1990).

Genetic mapping: The *ein2–1* mutant was mapped in a cross to the Landsberg marker line DP28. DNA was prepared from F_3 Ein$^-$ families and scored with RFLPs (CHANG *et al.* 1988; NAM *et al.* 1990) and the ATCTR1 **S**imple **S**equence **L**ength **P**olymorphism (SSLP; BELL and ECKER 1994). The *ein4, ein5–1* and *ein7* mutants were crossed onto the M10 marker line (KOORNNEEF and STAM 1992). The genotypes of the F_2 progenies from the *ein4* cross were determined by testing the F_3 progenies for segregation of mutant phenotypes; the F_3 seedlings were then used for SSLP and **C**leaved **A**mplified **P**olymorphic **S**equences (CAPS; KONIECZNY and AUSUBEL 1993) mapping. An *ap1 ein5–1* double mutant was isolated from the F_2 progenies of the cross to M10; this plant was then crossed with CS2 (*dis1, an*). F_3 families were tested for segregation of the flanking *ap1,* and *dis1* markers and were mapped with nga280 (BELL and ECKER 1994). F_3 families were also progeny tested in the *ein7* cross for the segregation of *ap1* and nga280. The *ein6* mutant was mapped in a cross to the wild-type Columbia strain. The *eir1* locus was mapped in a cross to the W100 marker strain (KOORNNEEF *et al.* 1987).

DNAs for the SSLP and CAPS mapping experiments were prepared from a single rosette leaf from each F_2 progeny or a minimum of 20 F_3 seedlings (EDWARDS *et al.* 1991). The ATCTR1 and nga280 SSLPs were amplified using a capillary thermocycler (Idaho Technologies, Idaho Falls, ID) with the following conditions: 94°, 1 sec; 56° 1 sec; 72°, 15 sec; 3 mM magnesium, 50 cycles. The LFY CAPS products were amplified on a capillary thermocycler (Idaho Technologies, Idaho Falls, ID) with the following conditions: 94°, 2 sec; 55°, 2 sec; 72°, 60 sec; 3 mM magnesium, 50 cycles. All other amplifications were performed as previously described (KONIECZNY and AUSUBEL 1993; BELL and ECKER 1994). Map distances were determined using the Map Manager v2.5 program (MANLY 1993).

Determination of epistasis and isolation of double mutants: A list of mutant strains used in this analysis is provided

in Table 1. Most strains were backcrossed at least twice to remove potential background effects on the ethylene phenotype before double mutant construction. Chi-square analysis was performed on the F_2 segregation ratios to examine possible epistasis relationships. Double mutants then were isolated to demonstrate the genetic interaction between the mutant phenotypes.

The *etr1–3 ctr1–1*, *ein3–2 ctr1–1*, *ein5–1 ctr1–1*, *ein7 ctr1–1*, *ein2–1 eir1–1*, *ein3–1 eir1–1*, *ein5–1 eir1–2*, *ctr1–1 eir1–1*, *ctr1–1 aux1–21*, *ein2–1 eto1–1*, *ein2–1 hls1–1* and *eir1–1 aux1–21* double mutants were obtained by progeny testing F_2 plants of each parental mutant phenotype to find a plant of the genotype m1/m1, +/m2; the double mutants were identified as seedlings with a new phenotype in the F_3 generation. The genotype of each double mutant was verified by failure to complement the hypostatic mutation. The *ctr1–1 hls1–1*, *eto1–1 hls1–1* and *eir1–1 hls1–1* double mutants were identified in F_2 progenies as seedlings expressing both mutant phenotypes. The *ctr1–5* allele was created by the insertion of a kanamycin-resistant marked T-DNA into the *CTR1* gene (KIEBER *et al.* 1993). This selectable marker was used to isolate recombinants between *ein2–1* and *ctr1–5*. F_2 seeds from a *ctr1–5* by *ein2–1* cross were examined for ethylene insensitive seedlings in the presence of 50 μg/ml kanamycin. Kanamycin-resistant (kan^r) F_2 Ein^- plants were progeny tested for the presence of both the kan^r marker and Ein^- phenotype. The double mutant was then verified by the failure to complement *ctr1–1*.

Quantifying the seedling phenotypes: Seedling measurements were obtained using a WILD dissecting scope with an ocular micrometer. Hook angles were quantified using a protractor reticle (Edmunds Scientific, Edison, NJ). The severity of the triple-response phenotype was measured as elongation of root or hypocotyl in the presence either of 10 μl/liter ethylene or in hydrocarbon-free air. Statistical analysis was carried out with the Statview 512+ software (BrainPower Inc., Ventura, CA). The Student's *t*-test was used for statistical comparison of the means. Sensitivity to 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma) was assayed as inhibition of root elongation. Seedlings were surface sterilized and plated on MS agar media containing 2,4-D. The seedlings were vernalized at 4° for 4 days before germination and root elongation was measured after 5 days at 23° in the dark. Gravity response was measured using a stage protractor on an Olympus dissecting scope. Surface-sterilized seeds were plated along a straight line marked on the petri dish. Seedlings were vernalized for 4 days at 4° and then placed perpendicular to the horizontal line. After 3 days in the dark at 23°, the angle of root growth was measured as the deviation from vertical; a perfect positive gravitropic response (completely vertical root) was measured as 0°.

RESULTS

Identification of five novel mutant loci affected in ethylene responses: Wild-type *A. thaliana* seedlings undergo drastic morphological changes in the presence of ethylene. The total seedling length is reduced dramatically and the degree of curvature in the apical hook becomes highly exaggerated when grown in ethylene (Figure 1). To identify components of the ethylene signal transduction pathway in Arabidopsis, we screened between 10,000 and 15,000 3-day-old etiolated seedlings from each of 23 independent X-ray-mutagenized and 20 independent diepoxybutane-mutagenized M_2 pools for ethylene insensitivity. Each of the M_2 populations represented ~1500 M_1 plants. All putative mutants were back crossed to the parent strain and placed into complementation groups (Table 2). Five novel complementation groups were identified, *ein4*, *ein5*, *ein6*, *ein7* and *eir1*.

ein4: The *ein4* mutant was identified in a diepoxybutane-mutagenized population. This mutant has a strong ethylene-insensitive phenotype in all tissues examined (Figure 1). When back crossed to wild type, *ein4* segregated as a dominant mutant (Table 2). Linkage was detected between *ein4* and chromosome *3* SSLPs and CAPS: nga172, nga162, nga126, GAPC, and GAPA (Table 3A). The chromosomal position of this mutation is 1.5 ± 1.1 cM south of GAPC on the top of chromosome *3*. This location is distinct from any previously described ethylene mutants (Figure 2).

ein5: The *ein5* locus is represented by two recessive ethylene insensitive alleles from independent X-ray-mutagenized M_2 pools (Table 2). Both *ein5–1* and *ein5–2* alleles segregate as recessive mutations. The *ein5* alleles have a less severe ethylene-insensitive phenotype than *ein2-1* and *ein4* (Figure 1). *ein5–1* was mapped to chromosome *1* with the visible markers *ap1*, *dis1* and the SSLP nga280 (KOORNNEEF and STAM 1992; BELL and ECKER 1994). A single recombination event was detected out of 102 chromosomes scored between nga280 and *ein5–1*. This chromosome was also recombinant at the flanking *ap1* marker, which positions *ein5* 1 ± 1 cM north of nga280 (Figure 2). A recessive 1-aminocyclopropane-1-carboxylic acid (ACC)-insensitive mutation (*ain1*), which maps close to this area of chromosome *1*, has been identified (VAN DER STRAETEN *et al.* 1993). The application of micromolar quantities of ACC to etiolated Arabidopsis seedlings induces the triple-response phenotype; the seedlings can readily convert this precursor to ethylene (YANG and HOFFMAN 1984). Because all of the Ein^- mutants are also insensitive to ACC (G. ROMAN and J. R. ECKER, data not shown), the *ain1* and *ein5* mutations may be allelic.

ein6: The *ein6* mutant was identified in a screen of fast neutron-mutagenized seeds of the Landsberg strain. This mutant is recessive, and has significantly reduced gametophytic transmission (Table 2). Fast neutron-mutagenesis frequently induces large chromosomal aberrations (HAWKINS 1979; SHIRLEY *et al.* 1992), and this may account for the reduced transmission of *ein6*. The phenotype of *ein6* is substantially weaker than either *ein2–1* or *ein4* (Figure 1). *ein6* was mapped to the bottom of chromosome *3* in a cross to wild-type Columbia and lies 31.6 ± 6 cM south of the GL1 CAPS marker and 17.6 ± 4.4 cM north of the nga112 SSLP (Figure 2).

ein7: The *ein7* mutant was identified in an X-ray-mutagenized population. The phenotype of seedlings homozygous for *ein7* is comparable in severity with that of

TABLE 1

Arabidopsis mutant strains

	Ecotype	Phenotype[b]	Mutagen	Reference
A. Strains[a]				
aux1-7	Columbia	Aux^-	EMS	PICKETT *et al.* (1990)
aux1-21	Columbia	Aux^-	X-ray	This paper
aux1-22	Columbia	Aux^-	DEB	This paper
ctr1-1	Columbia	Ctr^-	DEB	KIEBER *et al.* (1993)
ctr1-5	Wassilewskija	Ctr^-, kan^r	T-DNA	KIEBER *et al.* (1993)
ein2-1	Columbia	Ein^-	EMS	GUZMAN and ECKER (1990)
ein2-6	Wassilewskija	Ein^-	Agrobacterium	ROMAN and ECKER, unpublished data
ein3-1	Columbia	Ein^-	EMS	ROTHENBERG *et al.* unpublished data
ein3-2	Wassilewskija	Ein^-, kan^r	T-DNA	ROTHENBERG *et al.* unpublished data
ein4	Columbia	Ein^-	DEB	This paper
ein5-1	Columbia	Ein^-	X-ray	This paper
ein5-2	Columbia	Ein^-	X-ray	This paper
ein6	Landsberg	Ein^-	Fast neutron	This paper
ein7	Columbia	Ein^-	X-ray	This paper
eir1-1	Columbia	Eir^-	DEB	This paper
eir1-2	Columbia	Eir^-	X-ray	This paper
eto1-1	Columbia	Eto^-	EMS	GUZMAN and ECKER (1990)
etr1-3	Columbia	Ein^-	EMS	GUZMAN and ECKER (1990)
hls1-1	Columbia	Hls^-	EMS	GUZMAN and ECKER (1990)
ein2-1 tt4	Mixed	Ein^-, Tt^-		ROMAN and ECKER, unpublished data
eir1-1 ap1	Mixed	Eir^-, Ap^-		ROMAN and ECKER, unpublished data
DP28	Landsberg	Dis^-, Clv^-, Tt^-		ABRC[d]
W2	Landsberg	Dis^-, An^-		ABRC
W100	Landsberg	Tt^-, and more		ABRC
M10	Landsberg	Ap^-, Clv^-		ABRC
B. Double mutants[a]				
aux1-21 ctr1-1		Aus−, Ctr^-[c]		This paper
aux1-21 eir1-1		Aux^-		This paper
ctr1-5 ein 2-1		Ein^-		This paper
ctr1-1 ein3-2		Ein^-		This paper
ctr1-1 ein5-1		Ein^-		This paper
ctr1-1 ein7		Ein^-		This paper
ctr1-1 eir1-1		Eir^-, Ctr^-[c]		This paper
ctr1-1 etr1-3		Ctr^-		This paper
ctr1-1 hls1-1		Hls^-, Ctr^-[c]		This paper
ein2-1 eir1-1		Ein^-, Eir^-		This paper
ein2-6 eir1-1		Ein^-, Eir^-		This paper
ein2-1 eto1-1		Ein^-		This paper
ein2-6 eto 1-1		Ein^-		This paper
ein2-1 etr1-3		Ein^-		This paper
ein2-1 hls1-1		Ein^-, Hls^-		This paper
ein3-1 eir1-1		Ein^-, Eir^-		This paper
ein5-1 eir1-2		Ein^-, Eir^-		This paper
eir1-1 hls 1-1		Eir^-, Hls^-		This paper

[a] All mutant strains are homozygous unless otherwise indicated.

[b] Phenotypes are as follows: Aux^- auxin resistant root, agravitropic root, and ethylene insensitive root; Ctr^-, constitutive triple response; Ein^-, ethylene insensitive; Eir^-, ethylene insensitive root and agravitropic root; Eto^-, triple response due to ethylene overproduction; Hls^-, hookless, lack of an apical hook; kan^r, kanamycin resistant; Tt^-, transparent testa, clear seed coat; Ap^-, apetela, flowers lacking petals; Dis^-, distorted trichomes; An^-, angustifolia, long narrow leaves; Clv^-, clavata, club shaped siliques.

[c] The *ctr1-1* background in these double mutants mimics treatment with saturating amounts of ethylene.

[d] Arabidopsis Biological Resource Center, Ohio State University.

the *ein5* mutants (Figure 1), whereas seedlings heterozygous for this mutation have a less severe phenotype. F_2 progenies of the backcross to wild-type Columbia segregated 46 strong Ein^- seedlings, 104 weak Ein^- and 33 Ein^+ seedlings, a ratio that is not significantly different from 1:2:1 (Table 2; $P > 0.05$). Eight F_2 individuals from each phenotypic class were progeny tested; weak Ein^- seedlings were heterozygous for *ein7*,

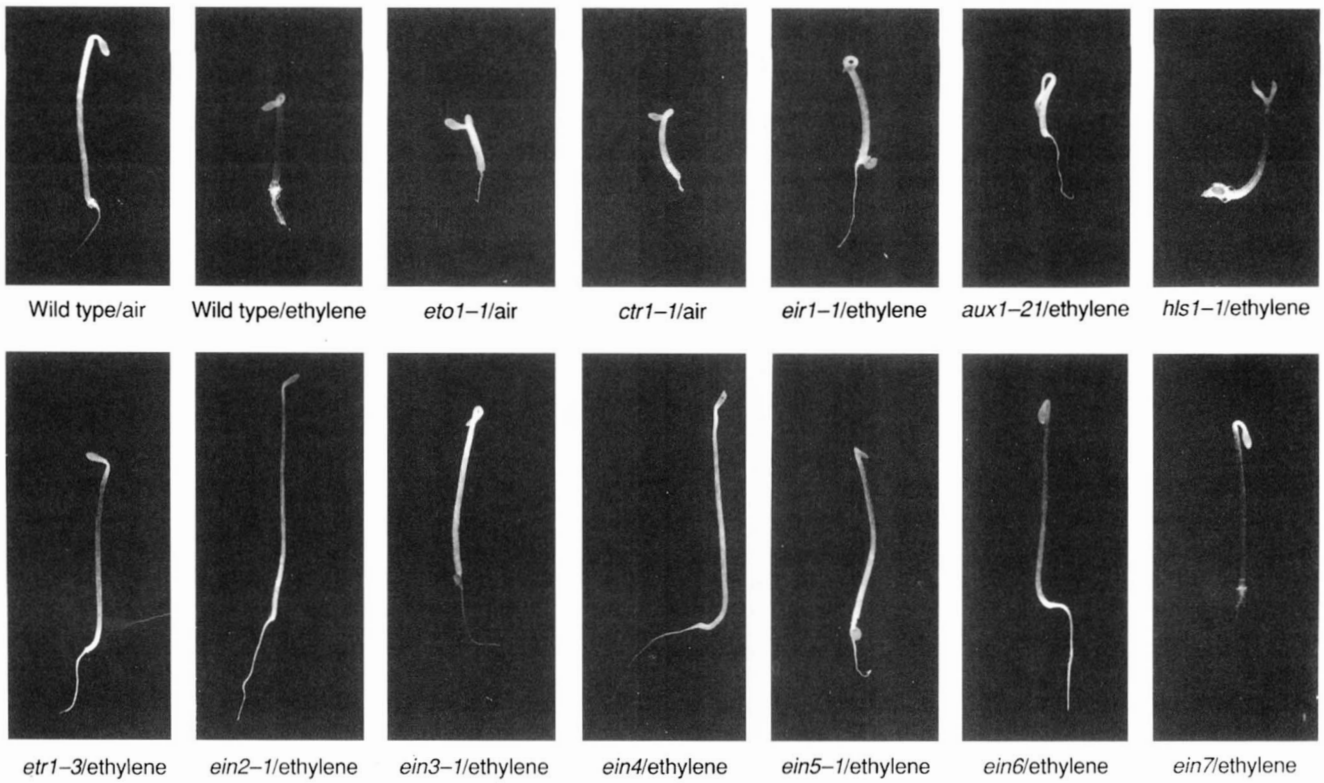

FIGURE 1.—Phenotypes of the ethylene-response mutants. The phenotypes of wild-type and mutant seedling are shown. Seedlings were germinated and allowed to grow in the dark for 3 days in the presence of either hydrocarbon-free air or 10 μl/liter ethylene. Wild-type seedlings grown in air develop a thin and elongated hypocotyl and root; in ethylene, these seedlings develop the triple response (BLEECKER *et al.* 1988; GUZMAN *et al.* 1990). The triple response includes a thickening of the hypocotyl, inhibition of root and hypocotyl elongation and an exaggeration of the curvature in the apical hook. *ctr1–1* and *eto-1* display the triple-response phenotype in the absence of exogenous ethylene (Ctr^- and Eto^-). *eir1–1* and *aux1–21* roots are insensitive to ethylene (Eir^- and Aux^-). *aux1–21* is defective in the formation of an exaggerated apical hook, whereas *hls1–1* is completely hookless (Hls^-) in ethylene. *etr1–3, ein2–1, ein3–1, ein4, ein5–1, ein6* and *ein7* display an ethylene-insensitive (Ein^-) seedling phenotype.

whereas seedlings displaying a strong Ein^- phenotype were all homozygous for this mutation. These data strongly suggest that *ein7* is a semidominant mutation. *ein7* was mapped 4.2 ± 2.4 cM north of nga280 and 17.7 ± 4.9 cM north of *ap1* on the bottom of chromosome *1*. Because *EIN5* maps to a similar position, we examined the phenotype of *ein7/ein5* plants. *ein7/ein5–1* and *ein7/ein5–2 trans*-heterozygotes have a weak Ein^- phenotype (Table 2D). These results do not confirm or repudiate allelism between these genes because *ein7* is semidominant. Consequently we have given this mutation its own locus designation.

eir1: *eir1* mutations specifically affect the ethylene insensitivity of the root. The hypocotyl and apical hook of mutant seedlings are sensitive to ethylene, but the root is moderately insensitive to this hormone. *eir1–1* was isolated from a diepoxybutane-mutagenized M_2 pool and *eir1–2* from an X-ray-mutagenized pool. Both *eir1* alleles are recessive (Table 2). *eir1–1* was mapped to the bottom of chromosome *5* between the *tt3* morphological marker and the LFY3 CAPS marker (Figure 2). *eir1–1* was mapped 10.8 ± 2.8 cM from *tt3* by progeny testing F_2 Eir^- plants in a cross of *eir1–1* by the W100 multiply marked line (KOORNNEEF *et al.* 1987). The distance between *eir1–1* and the LFY3 CAPS marker was found to be 6.1 ± 2.6 in these same F_3 families. The least frequent class of recombinants was recombinant for both LFY3 and *tt3*, indicating that *eir1–1* is flanked by these markers.

The auxin-resistant *aux1, axr1* and *axr2* mutations produce ethylene insensitive roots that have an altered gravitropic response (MAHER and MARTINDALE 1980a; LINCOLN *et al.* 1990; PICKETT *et al.* 1990; WILSON *et al.* 1990). Two alleles of *aux1* were identified in our screens (Table 2); the *aux1–21* allele was isolated from an X-ray-mutagenized pool and the *aux1–22* allele from a diepoxybutane-mutagenized pool. To test if *EIR1* is a member of this class of genes, *eir1* alleles were assayed for root growth on 0.05 μM 2,4-D. Wild-type levels of auxin sensitivity were observed in both cases (Figure 3). Similar results were obtained with another auxin, indole butyric acid (IBA; data not shown). The seedling roots of both *eir1* alleles failed to respond to gravity when grown on vertically oriented petri plates (Figure

TABLE 2

Genetic segregation of new ethylene response mutants

Strain/ generation	Female	Male	Ein$^+$	Ein$^-$	χ^2
ein4					
F_1	Wild type	*ein4*	0	8	
F_2			13	36	0.11
ein5					
F_1	Wild type	*ein5-1*	10	0	
F_2			41	13	0.02
F_1	Wild type	*ein5-2*	5	0	
F_2			53	19	0.07
F_1	*ein5-1*	*ein5-2*	0	4	
F_1	*ein5-1*	*ein2-1*	13	0	
F_1	*ein5-1*	*ein3-1*	4	0	
ein6					
F_1	Wild type	*ein6*	9	0	
F_2			374	38	54.7[b]
F_1	*ein6*	*ein2-1*	2	0	
F_1	*ein6*	*ein3-2*	4	0	
F_1	*ein6*	*ein5-2*	5	0	
ein7					
F_1	Wild type	*ein 7*	0	6[c]	
F_2			33	46/104[d]	5.3[e]
F_1	*ein5-1*	*ein7*	0	1	
F_1	*ein5-2*	*ein7*	0	3	
eir1					
F_1	Wild type	*eir1-1*	19	0	
F_2			48	18	0.19
F_1	Wild type	*eir1-2*	9	0	
F_2			101	24	2.24
F_1	*eir1-1*	*eir1-2*	0	12	

Strain/ generation	Female	Male	Aux$^+$	Aux$^-$	χ^2
aux1 alleles					
F_1	Wild type	*aux1-21*	8	0	
F_2			20	5	0.07
F_1	Wild type	*aux1-22*	15	0	
F_2			31	14	1.08
F_1	*aux1-7*	*aux1-21*	0	15	
F_1	*aux1-7*	*aux1-22*	0	7	
F_1	*aux1-21*	*aux1-22*	0	10	

[a] Wild type denotes the wild-type Columbia strain.
[b] $P < 0.05$.
[c] These *ein7* heterozygotes had phenotypes intermediate between that of the parental *ein7* homozygote and wild-type Columbia.
[d] Of these 150 Ein$^-$ plants, 104 had the intermediate phenotype seen in the F_1, whereas 46 had a phenotype similar to the *ein7* parental strain.
[e] A 1:2:1 ratio of Ein$^-$:intermediate:Ein$^+$ was tested.

4). Thus *EIR1* represents a new class of genes, in that it appears to mediate the responses of seedling roots to ethylene and gravity but, unlike the *AUX1* gene, does not affect the sensitivity of roots to exogenously applied auxin.

Mapping of the *EIN2* and *ETO1* loci: As part of our effort to define genetic loci involved in ethylene signal transduction, we reexamined the map position of the previously identified *ein2* locus using additional RFLPs (GUZMAN and ECKER 1990). The *ein2-1* mutation was found to segregate with multiple RFLPs on the top of chromosome *5* (Table 3). This gene lies between the ATCTR1 and ubq6121 markers. The *eto1-1* mutant was mapped with visible markers to the bottom of chromosome *3*. *eto1-1* mapped 5.4 ± 1.2 cM from *tt5*, 17.4 cM from *gl1*, and 12.7 cM from

TABLE 3

Molecular genetic mapping of the *EIN4* and *EIN2* loci

Gene/genetic marker	No. of F_2 progenies	Map distance[a]
EIN4		
nga172	41	6.7 ± 2.5
GPAC	81	1.2 ± 0.9
nga126	45	6.7 ± 2.6
nga162	47	7.5 ± 2.7
GAPA	64	21.9 ± 3.7
EIN2		
ubq6121	38	1.3 ± 1.3
g3715	47	0
ATHCTR1	102	0.5 ± 0.5
m217	28	2.6 ± 1.9
g3837	47	3.2 ± 2.2
m291	44	11.6 ± 3.5

[a] Map distances and errors were determined by Map Manager (MANLY 1993).

tt6. The data are most consistent with *eto1-1* being north of *tt5* (Figure 2).

Double-mutant analysis: Epistasis relationships between mutations in a biochemical or regulatory pathway can provide information about the way these genes interact without any *a priori* knowledge of their molecular identity (FERGUSON *et al.* 1987; CHORY 1990). To define the relationship between ethylene mutants, double mutants were isolated as described in METHODS AND MATERIALS and confirmed by test crosses (Table 4). The triple-response phenotypes of wild-type, single and double mutant strains were quantified in the presence and absence of ethylene (Table 5).

etr1 and ein2 are epistatic to eto1: *eto1* mutants display a triple-response phenotype in the absence of exogenously added ethylene because of a 10-fold overproduction of ethylene (GUZMAN and ECKER 1990; KIEBER *et al.* 1993). When the F_2 plants from a *eto1-1* by *etr1-3* cross were grown in the presence of ethylene, 75% of these plants were ethylene insensitive ($\chi^2 = 0.02$, $P > 0.1$), which indicates that *etr1-3* is not masked by *eto1-1*. In contrast, the Eto$^-$ phenotype segregated at a ratio that was significantly less than 1 Eto$^-$:3 Eto$^+$ ($\chi^2 = 58.38$, $P < 0.05$), and closely approximated a 1 Eto$^-$:15 Eto$^+$ ($\chi^2 = 0.56$, $P > 0.1$). This is the expected result if *etr1-1* is epistatic to *eto1-1*.

ein2-1 is also epistatic to *eto1-1*. The *eto1-1 ein2-1* double mutant has a strong ethylene-insensitive phenotype (Table 5). The double mutant also lacks the strong triple response phenotype of *eto1-1* when grown in air. Together these data indicate that both *ein2-1* and *etr1-3* mutants are epistatic to *eto1-1* and are therefore insensitive to endogenously produced ethylene.

ctr1 is epistatic to etr1 and ein4: The *ctr1-1* allele has a constitutive triple-response phenotype when grown in air. Because this phenotype is not reverted by antagonists of ethylene biosynthesis or action (KIEBER *et al.* 1993), *CTR1* is believed to play a role in ethylene signal transduction. *ctr1-1* is transmitted at a reduced frequency; *ctr1-1/ctr1-1* plants segregate at 17.3% instead of 25% in F_2 families from heterozygous plants (KIEBER and ECKER 1994). The *ctr1-1 etr1-3* double mutant has a constitutive triple-response phenotype (Table 5). The length of the *ctr1-1 etr1-3* double mutant was not significantly different from *ctr1* in the absence of exogenous ethylene ($t = 0.05$, $P > 0.1$), and the hook of these plants was also very similar to that of *ctr1-1* ($t = 1.31$, $P > 0.1$). *ctr1-1* is also epistatic to the dominant *ein4* mutation (Table 6). The segregation of Ein$^-$ seedlings in the F_2 of the *ctr1-1* by *ein4* cross was significantly <75% ($\chi^2 = 90.8$; $P < 0.01$), but not different than the 62% expected if *ctr1-1* were masking its phenotype ($\chi^2 = 1.70$; $P > 0.1$). The Ctr$^-$ phenotype segregated significantly greater than the 4.3% expected if the dominant *ein4* mutation were epistatic to this locus ($\chi^2 = 336.78$; $P < 0.01$); *ctr1* was found in the F_2 at ~17.3%, as is the case in the absence of *ein4* ($\chi^2 = 1.13$; $P > 0.1$). Therefore, the data are most consistent with *ctr1-1* masking the phenotype of *ein4*. The *ctr1-1 ein4* double mutant is needed to verify this tentative order of gene action.

etr1 and ein2 are not additive: Two experiments were performed to examine the interaction between *etr1-3* and *ein2-1*. In the first experiment, the *ein2-1 etr1-3* double mutant was grow in the presence of ethylene. Under these conditions, the length of the double mutant was slightly, but significantly less than that of *ein2-1* (Table 5; $t = 4.28$, $P < 0.01$). In the second experiment, freshly harvested *ein2-1 etr1-3*, *etr1-3* and *ein2-1* seeds were grown on 10 μM ACC. Under these conditions, the *ein2-1 etr1-3* double-mutant seedlings (16.3 ± 0.4 mm) were significantly longer than *etr1-3* (13.7 ± 0.6 mm; $t = 3.29$, $P < 0.01$), but were shorter than *ein2-1* seedlings (17.6 ± 0.9 mm) although this difference was not significant ($t = 1.23$, $P > 0.1$). Thus, in both experiments, *etr1* and *ein2* did not interact in an additive manner.

ein2, ein3, ein5, ein6 and ein7 loci act downstream of ctr1: The *ein2* and *ctr1* loci are located within a one cM region of each other on the top of chromosome 5 (Table 3B; Figure 2). To generate double mutants between these genes, we took advantage of *ctr1-5*, a T-DNA-induced allele of *ctr1* that contains a dominant kanamycin-resistant marker (kan^r; KIEBER *et al.* 1993). This marker permitted us to detect recombination events between *ein2-1* and *ctr1-5* as kan^r Ein$^-$ seedlings in the F_2. From ~10,000 F_2 seedlings, 3 were selected as being unequivocally kan^r and ethylene insensitive. All three failed to segregate Ctr$^-$ plants in the F_3, suggesting that *ein2-1* masks the *ctr1-5* phenotype. One of these plants was found to be homozygous *ctr1* (Table 4), and the prog-

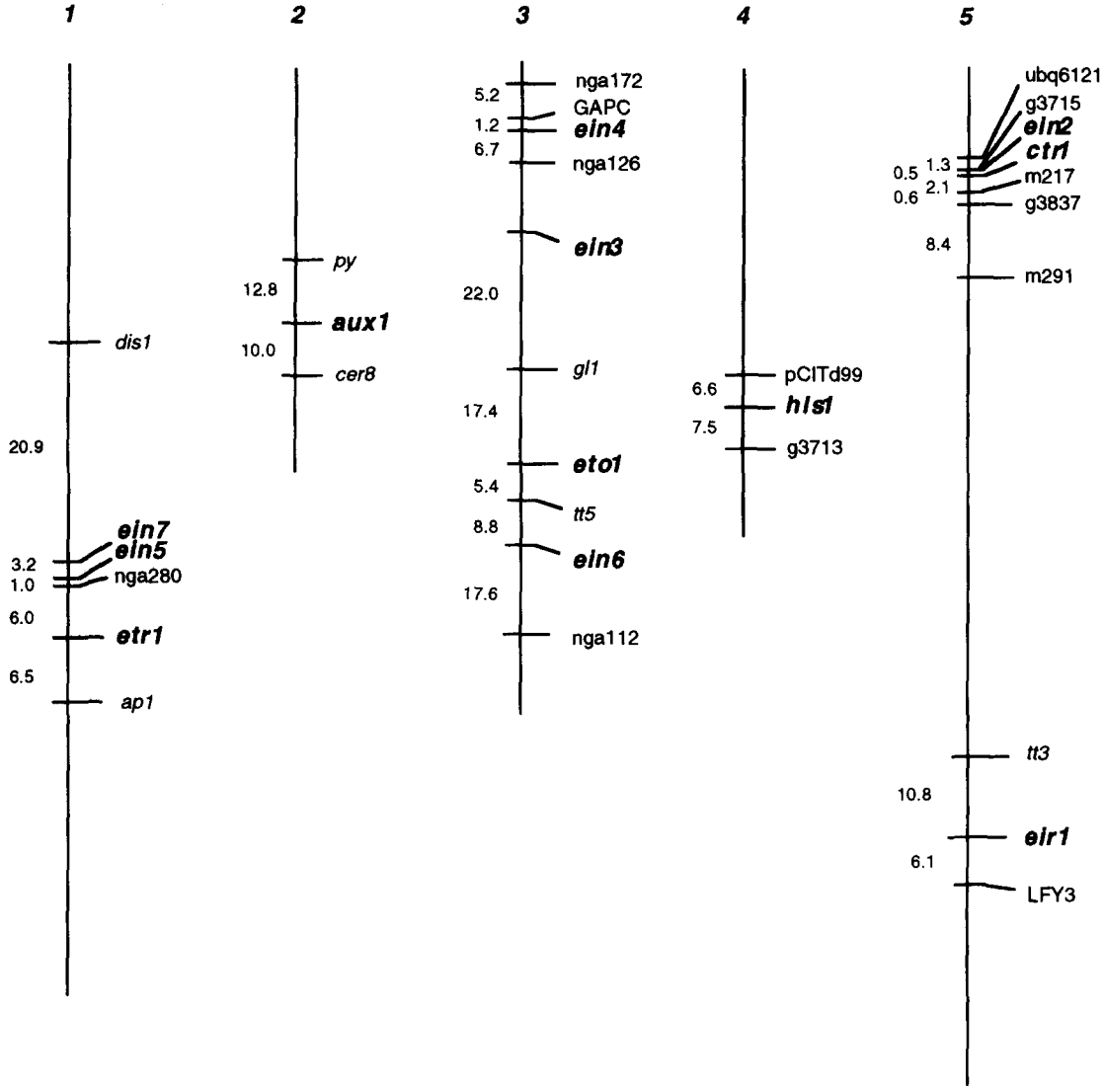

FIGURE 2.—Map positions of ethylene-response mutants. A map of the five *A. thaliana* chromosomes is shown with the locations of the ethylene-response mutants. The genetic mapping data for these mutant loci are as follows: *ein2, ein4, ein5, ein6, ein7, eto1, eir1* (this paper), *ein3* (M. ROTHENBERG, G. ROMAN and J. R. ECKER, unpublished data), *hls1* (A. LEHMAN and J. R. ECKER, unpublished data), *ctr1* (KIEBER *et al.* 1993), *etr1* (CHANG *et al.* 1993) and *aux1* (MAHER and MARTINDALE 1980b). The molecular markers are as follows: m217, m291, ubq6121 (CHANG *et al.* 1988); g3713, g3715, g3837 (NAM *et al.* 1990); GAPC and LGY3 (KONIECZNY and AUSUBEL 1993); nga280, nga172, nga126, nga112 and ATCTR1 (BELL and ECKER 1994).

eny of this plant were confirmed to be ethylene insensitive (Table 5). The *ctr1-1 ein3-2* and *ctr1-1 ein5-1* double mutants were also insensitive to 10 μl/liter ethylene (Table 5). The *ctr1-1 ein7* double mutant also displayed an Ein$^-$ phenotype (Table 5). The ability of *ein2-1, ein3-2, ein5-1* and *ein7* to mask the phenotype of the *ctr1* indicates that all of these Ein$^-$ loci act after *CTR1* in the ethylene signal transduction pathway.

The *ein6* mutation also was crossed onto *ctr1-1*. F_3 families from plants with each mutant phenotype in the F_2 were progeny tested for segregation of a novel phenotype. Two out of eight Ctr$^-$ F_2 plants produced ethylene insensitive seedlings, but none of the eight Ein$^-$ plants tested produced seedlings with a Ctr$^-$ phenotype (data not shown). The segregation of an Ein$^-$ seedling from a parent homozygous for *ctr1-1* strongly suggests that *ein6* can mask the seedling effects of the *ctr1-1* mutant.

None of the F_2 progeny of intercrosses between *ein3-1* and *ein5-1*, *ein3-1* and *ein5-2*, *ein3-2* and *ein2-1*, *ein3-2* and *ein6* and *ein2-1* and *ein5-1* displayed an additive phenotype (data not shown). Thus, there is no evidence for a second ethylene response pathway, or for branches in this pathway.

eir1 and aux1 interactions with ctr1: Both *eir1-1* and *aux1-21* displayed an elongated root in the presence of ethylene whereas root elongation in *ctr1-1* was inhibited in air (Table 5). In the absence of exogenous

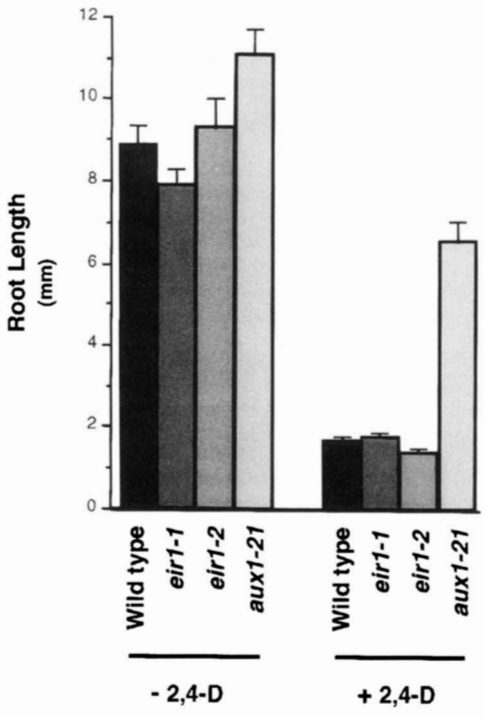

FIGURE 3.—Inhibition of root elongation by auxin. Wild-type, *eir1* and *aux1* seedlings were grown in the presence or absence of 0.05 μM 2,4-D. Root elongation was measured after 5 days growth. Each value is the mean ± SE of 15 seedlings. *aux1–21* demonstrated auxin resistance in this root-growth assay, consistent with the phenotype of other *aux1* mutant alleles (MAHER and MARTINDALE 1980a; PICKETT *et al.* 1990).

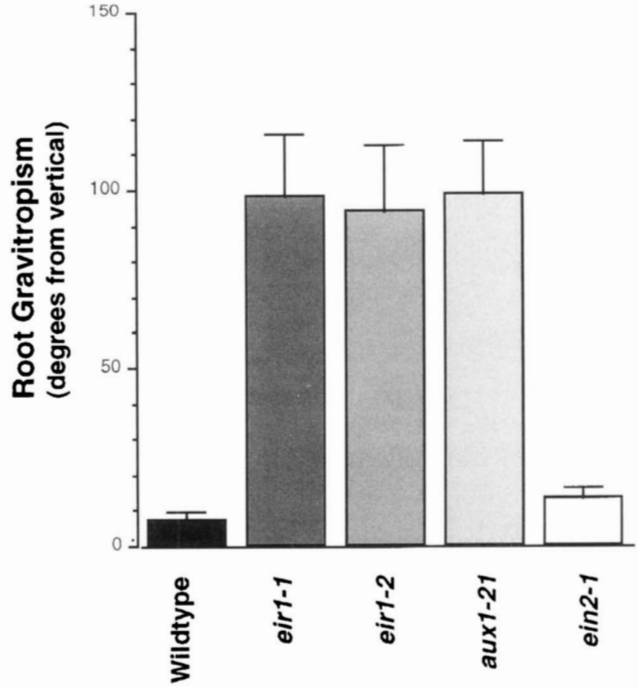

FIGURE 4.—Agravitropic response of ethylene mutants. Wild-type, *eir1, aux1* and *ein2* seedlings were grown on vertical plate as described in MATERIALS AND METHODS. The primary vector of root growth was measured relative to vertical such that positively gravitropic roots were at 0° and negatively gravitropic roots were at 180°. Each value is the mean ± SE of 20 seedlings. *aux1–21* seedlings display an agravitropic root phenotype, consistent with the phenotype of other *aux1* mutant alleles (MAHER and MARTINDALE 1980a; PICKETT *et al.* 1990.

ethylene, the seedling root of the *ctr1–1 eir1–1* double mutant was significantly longer than the *ctr1–1* root (Table 5; $t = 17.24$, $P < 0.01$). *ctr1–1 eir1–1* plants have a significantly shorter root than *eir1–1* in the presence of 10 μl/liter ethylene ($t = 6.76$, $P < 0.01$). The inability of *eir1–1* to completely block the short root phenotype of *ctr1–1* may be due to either the leakiness of the *eir1–1* mutation or to parallel functions of *ctr1* and *eir1*. The molecular nature of the *eir1–1* mutation may clarify the relationship between these two loci.

The phenotype of the *ctr1–1 aux1–21* double mutant in the absence of exogenous ethylene mimics the phenotype of *aux1–21* in the presence of 10 μl/liter ethylene (Table 5). The root phenotype of this double mutant was significantly longer than *ctr1–1* ($t = 20.55$, $P < 0.01$) but not significantly different from *aux1–21* in ethylene ($t = 1.55$, $P > 0.1$). The *aux1–21* apical hook is less severe in the presence of ethylene than wild-type Columbia or air grown *ctr1–1* (Table 5). The apical hook phenotype of *aux1–21* is also epistatic to *ctr1–1* (Table 5). Thus, the *aux1–21* mutant appears to act after *ctr1* in the inhibition of root elongation and the exaggeration of the apical hook.

eir1 is additive with ein3 and ein5: The effects of both *ein3–1* and *eir1–1* on root length are additive in the *ein3–1 eir1–1* double-mutant root, indicating that these genes function in separate pathways. When grown in ethylene, the root length of the *ein3–1 eir1–1* double mutant was significantly longer than either *ein3–1* (Table 5; $t = 8.09$, $P < 0.01$) or *eir1–1* (Table 5; $t = 9.24$, $P < 0.01$). In ethylene, the *eir1–1* root length was ~1.9 mm longer than wild-type Columbia. This was very close to the difference observed between the *ein3–1 eir1–1* and *ein3–1* roots when grown in ethylene (2.0 mm; Table 5). Additionally, the difference in length between *ein3–1* and wild-type Columbia seedling roots when grown in ethylene was ~2.1 mm, which closely approximated the difference seen between *ein3–1 eir1–1* and *eir1–1* (2.2 mm; Table 5). These data indicate an additive effect on ethylene insensitivity between the *ein3–1* and *eir1–1* mutants.

The *ein5–1 eir1–2* double mutants have significantly longer roots in ethylene than either *ein5–1* (Table 5; $t = 16.75$, $P < 0.01$) or *eir1–2* (Table 5; $t = 12.0$, $P < 0.01$). The effects of *ein5* and *eir1* on root length are additive in the *ein5–1 eir1–2* double mutant (Table 5). *ein3–1 eir1–1* and *ein5–1 eir1–2* double mutants also

TABLE 4

Testcrosses of the double mutants

Double mutant	Testcross	Generation	No. examined	Phenotype[a]
eto1-1 ein2-1	× *eto1-1*	F_1	19	Eto^-
eto1-1 ein2-6	× *eto1-1*	F_1	6	Eto^-
ctr1-1 etr1-3	× Wild type[b]	F_1	15	Ein^-
		F_2	8 families	seg. Ein^- and Ctr^-
ctr1-5 ein2-1	× *ctr1-1*	F_1	14	Ctr^-
		F_2	10 families	Ctr^- seg. Ein^-
ctr1-1 ein3-2	× *ctr1-1*	F_1	7	Ctr^-
ctr1-1 ein5-1	× *ctr1-1*	F_1	12	Ctr^-
		F_2	6 families	Ctr^- seg. Ein^-
ctr1-1 ein7	× *ctr1-1*	F_1	7	Ctr^{-1}
eir1-1 aux1-21	× *eir1-1*	F_1	28	Eir^-
		F_2	8 families	Eir^- seg. Aux^-
	× *aux1-21*	F_1	22	Aux^-
eir1-1 ein2-1	× *eir1-1/ap1*	F_1	11	Eir^- Ap^+
		F_2	8 families	Eir^- seg. Ein^- and Ap^-
eir1-1 ein2-6	× *eir1-1/ap 1*	F_1	6	Eir^- Ap^+
eir1-1 ein3-1	× *eir1-1/ap1*	F_1	30	Eir^- Ap^+
		F_2	8 families	Eir^- seg. Ein^- and Ap^-
eir1-2 ein5-1	× *eir1-1*	F_1	19	Eir^-
eir1-1 ctr1-1	× *eir1-1/ap1*	F_1	9	Eir^- Ap^+
aux1-21 ctr1-1	× *ctr1-1*	F_1	23	Ctr^-
	× *aux1-21*	F_1	8	Aux^-
etr1-3 ein2-1	× Wild-type	F_1	27	Ein^-
	× *ein2-1, tt4*	F_1	15	Ein^- Tt^-
		F_2	15 families	Ein^- seg. Tt^-
ein2-1 hls 1-1	× *ein2-1, tt4*	F_1	11	Ein^- Tt^-
	× *hls1-1*	F_1	17	Hls^-

[a] A minimum of 50 F_2 progenies were scored for each F_2 family. The segregation of recessive mutations in F_2 families was not significantly different from the expected frequency of 25% (data not shown).
[b] Wild type is the wild-type Columbia strain.

have agravitropic roots, a phenotype of *eir1* alleles (data not shown). The additive interactions between the *eir1* and *ein3* and the *eir1* and *ein5* mutations suggest that the *EIR1* gene is involved in a pathway of ethylene inhibition of root growth which is distinct from that of the *EIN3* and *EIN5* loci.

eir1 is not additive with ein2 and aux1: The *ein2–1 eir1–1* double mutant has a root length in ethylene that is significantly longer than *eir1–1*, but is not significantly different from that of the *ein2–1* root (Table 5; $t = 0.46$, $P > 0.1$). The primary root of the *ein2–1 eir1–1* double mutant is agravitropic, a phenotype typical of *eir1–1* but not *ein2–1*. These data suggests that *EIN2* and *EIR1* act within the same pathway effecting ethylene induced inhibition of root cell elongation, and that *EIR1* also functions in a seperate pathway involved in the root response to gravity.

In ethylene, *aux1–21 eir1–1* double-mutant roots were identical in length to *eir1–1* roots, but were significantly shorter than *aux1–21* roots (Table 5; $t = 31.23$, $P < 0.01$). In a separate experiment, the *aux1–21 eir1–1* double-mutant seedlings were grown on vertically oriented plates containing 100 μM ACC. In this experiment, *aux1–21 eir1–1* roots were significantly longer (3.3 ± 0.1 mm) than *eir1–1* roots (2.6 ± 0.1 mm; $t = 4.31$, $P < 0.01$), but were identical in length to *aux1–21* roots (3.2 ± 0.1 mm; $t = 1.07$, $P > 0.1$). Thus in both experiments, *aux1–21* and *eir1–1* did not have additive effects on root elongation in the presence of ethylene. These results indicate that *EIR1* functions in a root-specific ethylene-response pathway that includes the *AUX1* and *EIN2* loci. In several experiments, the *ein3 eir1–1* root length in ethylene closely approximated the level of insensitivity seen in *ein2;* this would suggest that *ein2* is epistatic to both *ein3* and *eir1* functions and may act before these loci in the ethylene signal transduction pathway.

hls1 interaction with the ethylene mutants: Wild-type Columbia seedlings have a moderate apical hook when grown in the absence of ethylene and develop an exaggerated hook in the presence of this hormone (Figure 1; Table 5). In contrast, *hls1–1* seedlings have almost no hook in ethylene (Figure 1; Table 5). This defect in hook formation in *hls1–1* is epistatic to the *eto1*, *ctr1*, *ein2* and *etr1* loci. The *eto1–1 hls1–1* hook in air is indistinguishable from *hls1–1* (6 ± 2°; $t = 1.53$, $P >$

TABLE 5

Quantifying the ethylene response phenotype

Strain	Ethylene				Air			
	Root length	Hypocotyl length	Total seedling	Hook angle	Root length	Hypocotyl length	Total seeding	Hook angle
Columbia	1.5 ± 0.1	3.0 ± 0.1	4.5 ± 0.1	250 ± 8	3.9 ± 0.2	4.8 ± 0.1	8.7 ± 0.2	114 ± 9
Landsberg	2.1 ± 0.1	2.7 ± 0.1	4.8 ± 0.1	233 ± 18	nd[b]	nd	nd	nd
Wasslewskija	1.0 ± 0.1	3.1 ± 0.1	4.1 ± 0.1	270 ± 6	4.0 ± 0.2	6.1 ± 0.1	10.1 ± 0.2	166 ± 7
aux1-21	4.7 ± 0.2	3.4 ± 0.1	8.1 ± 0.3	197 ± 8	6.3 ± 0.3	6.0 ± 0.1	12.3 ± 0.3	126 ± 6
ctr1-1	0.8 ± 0.0	2.8 ± 0.1	3.6 ± 0.1	247 ± 5	0.9 ± 0.0	3.1 ± 0.1	4.0 ± 01	246 ± 10
ctr1-5	0.4 ± 0.0	1.9 ± 0.1	2.3 ± 0.1	252 ± 5	0.6 ± 0.0	2.4 ± 0.1	3.0 ± 0.1	237 ± 8
ein2-1	6.1 ± 0.2	6.5 ± 0.2	12.6 ± 0.2	39 ± 4	5.8 ± 0.2	6.9 ± 0.2	12.7 ± 0.3	43 ± 7
ein3-1	3.6 ± 0.1	5.2 ± 0.1	8.8 ± 0.2	118 ± 7	5.4 ± 0.3	5.9 ± 0.1	11.3 ± 0.4	77 ± 7
ein3-2	3.1 ± 0.1	5.5 ± 0.1	8.5 ± 0.2	176 ± 6	5.2 ± 0.3	6.3 ± 0.2	11.4 ± 0.3	152 ± 8
ein4	7.1 ± 0.2	7.3 ± 0.3	14.4 ± 0.3	64 ± 9	6.8 ± 0.3	6.9 ± 0.3	13.7 ± 0.4	45 ± 5
ein5-1	2.5 ± 0.1	4.8 ± 0.1	7.3 ± 0.1	144 ± 10	5.6 ± 0.2	5.3 ± 0.2	11.0 ± 0.3	89 ± 8
ein5-2	2.6 ± 0.1	4.6 ± 0.2	7.2 ± 0.2	156 ± 10	4.3 ± 0.2	5.6 ± 0.2	9.9 ± 0.3	113 ± 10
ein6	3.5 ± 0.1	6.2 ± 0.2	9.7 ± 0.2	95 ± 6	7.0 ± 0.2	6.0 ± 0.2	13.0 ± 0.2	47 ± 4
ein7	2.9 ± 0.1	5.2 ± 0.1	8.1 ± 0.2	176 ± 4	5.2 ± 0.2	6.7 ± 0.2	11.9 ± 0.2	137 ± 8
eir1-1	3.4 ± 0.1	3.1 ± 0.1	6.5 ± 0.1	282 ± 7	5.2 ± 0.9	6.2 ± 0.1	11.4 ± 0.1	106 ± 7
eir1-2	3.0 ± 0.1	3.1 ± 0.1	6.1 ± 0.1	261 ± 7	4.8 ± 0.3	5.7 ± 0.2	10.5 ± 0.4	109 ± 10
eto1-1	1.4 ± 0.1	3.3 ± 0.1	4.7 ± 0.2	244 ± 10	1.9 ± 0.1	3.3 ± 0.1	5.3 ± 0.1	239 ± 8
etr1-3	4.6 ± 0.2	6.1 ± 0.1	10.7 ± 0.3	89 ± 7	4.9 ± 0.3	5.5 ± 0.2	10.4 ± 0.3	96 ± 5
hls1-1	0.9 ± 0.1	3.4 ± 0.1	4.3 ± 0.2	5 ± 1	3.3 ± 0.1	5.1 ± 0.1	8.4 ± 0.2	4 ± 1
aux1-21 ctr1-1	3.9 ± 0.2	3.6 ± 0.1	7.5 ± 0.2	178 ± 8	4.2 ± 0.2	3.3 ± 0.1	7.5 ± 0.2	194 ± 6
aux1-21 eir1-1	3.4 ± 0.1	3.8 ± 0.1	7.2 ± 0.2	138 ± 7	4.9 ± 0.2	5.4 ± 0.1	10.3 ± 0.2	124 ± 6
ctr1-1 ein3-2	3.4 ± 0.1	5.4 ± 0.1	8.8 ± 0.2	176 ± 7	4.3 ± 0.3	5.9 ± 0.2	10.1 ± 0.4	158 ± 9
ctr1-1 ein5-1	2.4 ± 0.0	5.5 ± 0.1	7.9 ± 0.1	141 ± 9	2.5 ± 0.1	4.8 ± 0.1	7.3 ± 0.1	134 ± 10
ctr1-1 ein7	3.2 ± 0.1	5.3 ± 0.1	8.5 ± 0.2	129 ± 9	3.0 ± 0.1	5.4 ± 0.1	8.4 ± 0.1	139 ± 9
ctr1-1 eir 1-1	1.9 ± 0.1	3.3 ± 0.1	5.2 ± 0.1	236 ± 9	2.6 ± 0.1	3.7 ± 0.1	6.2 ± 0.1	254 ± 8
ctr1-1 etr1-3	0.8 ± 0.0	3.2 ± 0.1	4.0 ± 0.1	228 ± 9	1.1 ± 0.1	2.9 ± 0.1	4.0 ± 0.1	228 ± 9
ctr1-1 hls1-1[a]	0.6 ± 0.1	3.0 ± 0.1	3.6 ± 0.2	4 ± 1	0.6 ± 0.0	2.5 ± 0.1	3.1 ± 0.2	6 ± 2
ctr1-5 ein2-1	6.0 ± 0.3	8.3 ± 0.2	14.3 ± 0.5	35 ± 3	5.5 ± 0.2	6.8 ± 0.1	12.3 ± 0.2	35 ± 5
ein2-1 eir1-1	6.2 ± 0.2	7.7 ± 0.2	13.9 ± 0.3	37 ± 7	6.7 ± 0.3	6.7 ± 0.1	13.4 ± 0.3	31 ± 3
ein2-1 eto1-1	6.1 ± 0.2	7.3 ± 0.2	13.4 ± 0.4	33 ± 3	6.4 ± 0.2	7.4 ± 0.2	13.8 ± 0.3	36 ± 6
ein2-1 etr1-3	4.5 ± 0.1	6.9 ± 0.1	11.4 ± 0.2	35 ± 5	4.8 ± 0.1	6.6 ± 0.2	11.5 ± 0.2	23 ± 3
ein2-1 hls1-1	6.0 ± 0.2	5.4 ± 0.1	11.4 ± 0.3	5 ± 1	5.7 ± 0.1	4.8 ± 0.2	10.5 ± 0.2	4 ± 1
ein3-1 eir1-1	5.6 ± 0.2	5.8 ± 0.1	11.4 ± 0.3	144 ± 6	7.1 ± 0.2	8.0 ± 0.2	15.1 ± 0.3	90 ± 7
ein5-1 eir1-2	4.4 ± 0.1	4.8 ± 0.1	9.1 ± 0.1	151 ± 8	nd	nd	nd	nd
eto1-1 hls1[a]	0.7 ± 0.1	3.0 ± 0.2	3.7 ± 0.2	5 ± 2	1.4 ± 0.1	3.8 ± 0.2	5.2 ± 0.2	6 ± 2

All values are the means ± SE of 20 seedlings unless otherwise noted. Root length, hypocotyl length and total seeding are measured in millimeters. Hook angles are measured in degrees.

[a] Values for these genotypes are the means ± SE of 15 seedlings.

[b] Not determined.

0.1). Likewise, the *ctr1–1 hls1–1* double-mutant hook in air displays the *hls1–1* phenotype (6 ± 2°; $t = 1.28$, $P > 0.1$). The *ein2–1* mutation retains a small amount of hook curvature in air grown seedlings (43 ± 7°; Table 5). The *ein2–1 hls1–1* double mutant is statistically different from *ein2–1* ($t = 5.54$, $P < 0.01$) but not *hls1–1* ($t = 0.53$, $P > 0.1$). Although not quantified, the *etr1–1 hls1–1* and *eir1–1 hls1–1* double mutants also show the *hls1–1* phenotype (data not shown).

An additional interaction between *hls1–1* and *ctr1–1* was observed. The reduced gametophytic transmission of *ctr1–1* was not seen in crosses with *hls1–1* and *aux1–21* (Table 6). In the *ctr1–1* by *hls1–1* cross, the Ctr^- phenotypic class was significantly greater than 17.3% ($\chi^2 = 7.74$, $P < 0.05$) but not significantly different from 25% ($\chi^2 = 0.88$, $P > 0.1$). Similarly, in the cross with *aux1–21* the Ctr^- class more closely approximated 25% ($\chi^2 = 0.11$, $P > 0.1$) than 17.3% ($\chi^2 = 6.18$, $P < 0.05$). These results suggest that the reduced female gametophytic transmission in *ctr1–1* requires functional *aux1* and *hls1* loci. This interaction may account for the high chi-square values seen in these crosses.

The *ctr1* mutants have a dramatic adult phenotype that can be phenocopied in wild-type plants by continuous growth in the presence of ethylene (KIEBER *et al.* 1993). This phenotype includes reduced leaf and petiole size

TABLE 6

F_2 segregation ratios of ethylene response mutants

Cross			Phenotype							Ratio	
Female	Male	Condition	Wild type	Ein$^-$	Eto$^-$	Ctr$^-$	Hls$^-$	Eir$^-$	Double	tested[a]	χ^2
eto1-1	*ein2-1*	Ethylene	232	69						3:1	0.64
		Air	378		94					3:1	6.51[b]
eto1-1	*ein2-6*	Ethylene	98	39						3:1	0.98
		Air	168		43					3:1	2.52
eto1-1	*etr1-3*	Ethylene	59	182						1:3	0.02
		Air	263		14					3:1	58.38[b]
ctr1-1	*ein2-1*	Ethylene	375	126						3:1	0.01
		Air	413			105				3:1	3.03
ctr1-1	*etr1-3*	Ethylene	60	99						1:3	13.36[b]
		Air	151			25				3:1	1.00
ctr1-1	*ein3-2*	Ethylene	126	32						3:1	1.66
		Air	39			9				3:1	0.13
ctr1-1	*ein4-1*	Ethylene	291	431						1:3	90.80[b]
		Air	529			123				3:1	1.13
ctr1-1	*ein5-1*	Ethylene	478	170						3:1	0.53
		Air	497			73				3:1	8.27[b]
ctr1-1	*ein6*	Ethylene	58	10						3:1	2.75
		Air	91			15				3:1	0.70
ctr1-1	*ein7*	Ethylene	60	98						1:3	13.38[b]
		Air	214			18				3:1	14.62[b]
ctr1-1	*aux1-21*	Air	110			35		36	11	9:3:3:1	6.27
hls1-1	*ein2-1*	Ethylene	187	53			50		13	9:3:3:1	4.73
hls1-1	*etr1-1*	Ethylene	48	165			25		60	3:9:1:3	4.20
hls1-1	*eir1-1*	Ethylene	207				61	43	11	9:3:3:1	12.60[b]
eto1-1	*hls1-1*	Air	82		28		28		8	9:3:3:1	0.18
ctr1-1	*hls1-1*	Air	225			64	73		24	9:3:3:1	8.23[b]
eir1-1	*aux1-21*	Ethylene	177					165		9:7	2.67
eir1-1	*ein3-1*	Ethylene	152	60				38		9:4:3	2.64
eir1-1	*ein2-1*	Ethylene	207	99				73		9:4:3	0.25
eir1-1	*ein2-6*	Ethylene	89	46				41		9:4:3	3.15
eir1-2	*ein5-1*	Ethylene	129	48				40		9:4:3	1.09
etr1-3	*ein3-1*	Ethylene	106	1108						3:13	75.38[b]
ein2-1	*etr1-3*	Ethylene	49	217						3:13	0.02
ein2-1	*ein6*	Ethylene	79	55						9:7	0.48
ein5-1	*ein2-1*	Ethylene	60	48						9:7	0.04
ein5-1	*ein3-1*	Ethylene	145	92						9:7	2.46
ein6	*ein3-2*	Ethylene	156	96						9:7	3.16

[a] Ratios involving *ctr1-1* and *ein6* have been adjusted to account for the reduced transmission of these alleles (KIEBER *et al.* 1993; Table 4).

[b] $P < 0.05$.

and reduced elongation of the inflorescence; the result is a dramatically smaller plant. The *ctr1-1 eir1-1*, *ctr1-1 aux1-21* and *ctr1-1 hls1-1* double mutants share this Ctr$^-$ adult phenotype (data not shown). The *ctr1-1 etr1-3* double-mutant adult phenotype was predominantly similar to that of *ctr1-1*, but slightly larger in size (data not shown). Consistent with their seedling phenotypes, the *ctr1-5 ein2-1*, *ctr1-1 ein3-2* and *ctr1-1 ein5-1* double mutants do not have the Ctr$^-$ adult phenotype (data not shown). Interestingly, the *ctr1-1 ein7* double-mutant adult plants were intermediate in size between the two single mutants. The additive phenotype of the *ctr1-1 ein7* double mutant cosegregated with the seedling Ein$^-$ phenotype in a backcross to *ctr1-1* (data not shown). The *ctr1-1 ein7* seedlings were not intermediate between the two parental phenotypes (Table 5), suggesting a developmental change in the expression of the *ein7* mutant phenotype in the adult plants.

DISCUSSION

In this study, we employed the triple-response phenotype to identify genes involved in ethylene signal transduction. The triple response consists of inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and an exaggeration in the curvature of the

apical hook (Figure 1). Five novel loci have been identified through mutations that are deficient in this ethylene response. The examination of double-mutant phenotypes has provided information on the relative order of these mutations, and previously identified mutations, in the ethylene response pathway. Our results demonstrate that ≥10 genes are involved in the development of the triple response. Most of these genes affect all aspects of the seedling and adult ethylene responses; these genes are most likely involved in ethylene signaling. Other mutations define genes that affect only a subset of ethylene responses. The phenotypes of these mutations, and their relative positions in the ethylene signal transduction pathway, suggests that the development of the triple response involves hormonal interactions.

Components of the ethylene signal-transduction pathway: The *EIN4* locus is represented by a single mutant with a severe ethylene-insensitive phenotype that is dominant to wild-type. The *ein4* mutation has been genetically mapped to the top of chromosome *3;* this map position is distinct from all other ethylene mutants and thus, is not a dominant allele of a previously identified locus.

The *EIN5* gene is represented by two recessive alleles that are the least insensitive of all the Ein$^-$ mutants. This locus is very close to the previously identified *ain1* mutation on chromosome *1* (Van Der Straeten *et al.* 1993). The *ain1* mutant is also recessive and shows moderate insensitivity to the ethylene precursor ACC. It will be possible to determine if these loci belong to the same complementation group when *ain1* becomes available. The *ein7* mutation is semidominant and is tightly linked to the *EIN5* locus. The dominance of *ein7* makes it difficult to establish if it is an allele of *ein5.* Clustering of hormone mutant loci within one cM has been found in Arabidopsis for the *ein2* and *ctr1* genes on chromosome *5* and for the *ga4* and *gai* genes on chromosome *1* (Figure 2; Koornneef *et al.* 1985). For this reason, we tentatively give *EIN7* its own locus designation.

The *EIN6* locus is represented by a single ethylene-insensitive, recessive mutation that maps to the bottom of chromosome *3. ein6/+* plants produce less than the expected number of Ein$^-$ seedlings; this effect may be the result of a multilocus chromosomal aberration created during the fast neutron-mutagenesis, rather than a product of the ethylene insensitivity of this mutant. Additional alleles are required for the phenotypic characterization of this locus.

The *ein2* and *ein3* mutations are recessive and have been described previously (Guzman and Ecker 1990; Kieber *et al.* 1993). *EIN2* maps within one cM of *CTR1.* The *CTR1* gene is represented by five independent recessive mutations that have a constitutive triple-response phenotype (Kieber *et al.* 1993). This gene has been cloned and shown to be similar to the Raf family of serine/threonine kinases. It is likely that all of the *ctr1* mutants are the result of a severe reduction-of-function or a loss-of-function mutation. These mutants demonstrate that the activity of this putative serine/threonine kinase is to negatively regulate the ethylene activation of gene expression and is required for normal cell growth.

Several mutant loci that are defective in tissue-specific aspects of the ethylene-response phenotype have been identified. *EIR1* is a novel locus that is defined by two independent recessive mutations. *eir1* seedlings have moderate defects in the ethylene inhibition of root elongation and more severe defects in root gravitropism. The hypocotyl and apical hook of Eir$^-$ seedlings appear to have a normal ethylene response. These seedlings also display normal sensitivity to the synthetic auxin 2,4-D. *aux1* mutants have a gravity and root-elongation phenotype that is similar to *eir1* mutants, but differ from *eir1* in that they are also resistant to exogenous auxin (Maher and Martindale 1980a; Pickett *et al.* 1990). New alleles of *aux1* (*aux1–21* and *aux1–22*) have a mild defect in the formation of an exaggerated apical hook in the presence of ethylene. The *hls1–1* mutant has an almost complete deficiency in the ability to form an apical hook, a differential cell-growth process; hypocotyl and root elongation in this mutant exhibit wild-type ethylene sensitivity (Guzman and Ecker 1990).

Order of gene action within the response pathway: We have used the phenotypes of several double mutants to build a framework for the action of these genes within an ethylene-response pathway (Figure 5). The earliest steps of this pathway are defined by the *ETR1* and *EIN4* loci, both of which appear to act before *CTR1.* The *etr1* mutants all have Ein$^-$ phenotypes and are dominant to the wild-type alleles (Bleecker *et al.* 1988; Guzman and Ecker 1990; Chang *et al.* 1993). This locus recently has been cloned by a map-based strategy and found to be similar to bacterial two-component histidine kinases (Chang *et al.* 1993). It is unclear whether the *etr1* mutants result from gain-of-function or dominant-negative mutations. This question remains crucial in determining the role of the putative histidine kinase in ethylene signaling. The primary sequence of *ETR1* is similar to the *SLN1* histidine kinase in *Saccharomyces cerevisiae* (Ota and Varshavsky 1993). Both gene products are predicted to have very similar structures (Chang *et al.* 1993; Ota and Varshavsky 1993), and mutations of both genes are suppressed by mutations in putative members of a MAP kinase cascade (Kieber *et al.* 1993; Maeda *et al.* 1994). The *SLN1* gene product directly phosphorylates and inactivates the *SSK1* gene product in response to high osmolarity, which results in the inactivation of a MAP kinase phosphorylation cascade (Maeda *et al.* 1994). The *HOG1* gene product

is the MAP kinase family member that is involved in this response to osmotic stress (MAEDA *et al.* 1994). Interestingly, *HOG1*-related MAP kinases also are involved in mammalian responses to osmotic and lipopolysaccharide stress signals (GALCHEVA-GARGOVA *et al.* 1994; HAN *et al.* 1994). The ethylene-signal transduction pathway therefore may represent a generalized stress response system that is conserved in budding yeast and mammals.

We do not yet know the position of *ETR1* and *EIN4* relative to each other, although the similarities between *ETR1* and *SLN1* suggests putative functions for the *EIN4* gene. *EIN4* appears to act before *CTR1*. *EIN4* also may act after *ETR1* and have the analogous function in ethylene-signal transduction in Arabidopsis as *SSK1* has in the osmolarity stress response in yeast. Another intriguing possibility is that these genes have redundant functions. Sequences similar to *ETR1* have been found within the Arabidopsis expressed sequence tag collection (NEWMAN *et al.* 1994). This redundancy would account for the absence of recessive alleles at these loci. Alternatively, *EIN4* also may act before *ETR1* within the ethylene-signal transduction pathway. Indirect evidence suggests that the ethylene receptor contains a transition metal that could coordinate this simple gas (BURG and BURG 1967; SISLER 1990). The *ETR1* gene product does not appear to have any structure suggestive of an ethylene binding domain (CHANG *et al.* 1993). Therefore, there may be other proteins which act before *ETR1* that are required for ethylene perception.

The *EIN2* locus acts after *CTR1* in the ethylene signal transduction pathway (Figure 5). In addition to ethylene insensitivity, *ein2* mutants are also deficient in the development of disease symptoms upon infection with virulent *Pseudomonas syrringae* pv. tomato and *Xanthomonas campestris*. Interestingly, mutations at the *ETR1* locus do not have this phenotype (BENT *et al.* 1992). There are several possible explanations for why *EIN2*, which acts later in the ethylene signal transduction pathway, has a phenotype not seen in the upstream gene, *ETR1*. Because *etr1-3* has a less severe phenotype than the *ein2* mutants that were tested for disease tolerance, it is possible that *etr1-3* develops disease symptoms because of the remaining ethylene sensitivity in these plants. Another possibility is that the pathways for sensitivity to pathogens and ethylene are distinct and that *EIN2* participates in both of these events.

EIN3, *EIN5*, *EIN6* and *EIN7* act after *CTR1* in the ethylene-signal transduction pathway (Figure 5). The *ein3*, *ein5* and *ein6* mutants have a significantly less severe Ein$^-$ phenotype than does *ein2-1*. In addition, LAWTON *et al.* (1994) found that the ethylene-induced Hevein-like gene (HEL) was induced by ethylene to higher levels in *ein3* than in either *etr1-1* or *ein2-1*. The sequence of *ein3-1* predicts that it gives rise to a truncated protein that should result in a severe reduction-of-function or a loss-of-function (M. ROTHENBERG and J. R. ECKER, unpublished results). Therefore, the weak phenotype of *ein3-1* can not be attributed to a simple model of reduced activity, but must be explained by the function of this gene within ethylene signal transduction. Thus, the *EIN3* locus affects only a subset of the functions of *EIN2*. The molecular identity of *EIN5* has yet to be determined. It is possible that the *ein5* mutants have a weak phenotype because they are leaky;

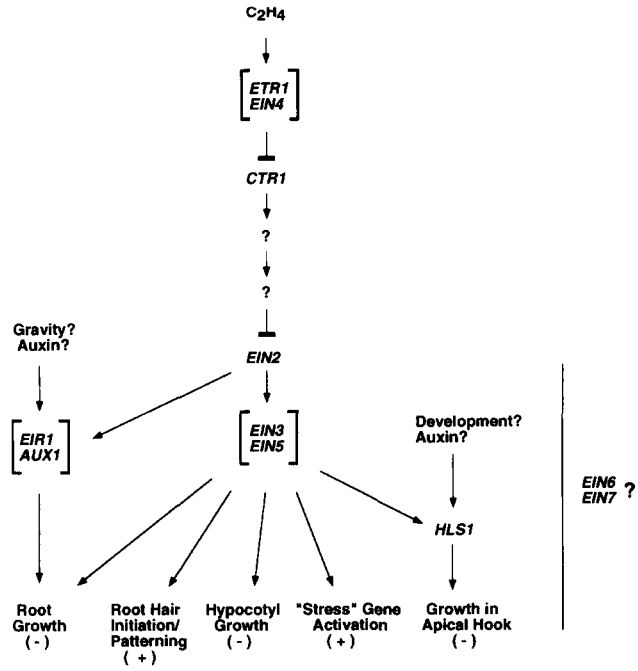

FIGURE 5.—Genetic pathway for ethylene-response mutants. A model for the genetic pathway of ethylene signal transduction is shown that is consistent with epistatic relationships. *ctr1-1* masks the phenotype of *etr1-3* and *ein4;* therefore, *CTRL1* is shown acting after *ETR1* and *EIN4*. In this model, the *etr1* and *ein4* mutations are assumed to be dominant negative; therefore, these genes would negatively regulate *CTR1*. *CTR1* negatively regulates the ethylene-response pathway (KIEBER *et al.* 1993). These negative control points are indicated by a bar. Similarity between the ethylene-response pathway and the mammalian and yeast stress responses suggests additional proteins acting after *CTR1*. These putative proteins are indicated by question marks. *ein2, ein3, ein5, ein6, ein7, hls1, eir1* and *aux1* are all epistatic to *ctr1*, and likely act after *CTR1* in the ethylene-response pathway. The *EIR1/AUX1* effect on root growth is distinct from the *EIN3/EIN5* root response and therefore is shown in a separate pathway affecting root growth. *EIN2* is required for the *EIN3/EIN5* and *EIR1/AUX1* responses and is shown acting before these genes. *EIN7* and *EIN6* are shown outside of the genetic pathway because these loci have not been characterized for *EIR1/AUX1* interactions. *hls1, eir1* and *aux1* are defective in gravity or auxin responses. Brackets indicate uncertain gene order. Responses that are regulated positively by ethylene are indicated (+) as are negatively regulated responses (−). Root hair initiation and patterning requires genes within the ethylene signal-transduction pathway (DOLAN *et al.* 1994; J. SCHIEFELBEIN, personal communication).

however it is also possible that this gene only affects a portion of the *EIN2* functions.

The *EIN6* and *EIN7* loci are only defined by single-mutant alleles. The *ctr1-1 ein7* double mutant displays an adult phenotype intermediate between *ctr1-1* and *ein7*. The interpretation of this adult phenotype remains equivocal without more information about the nature of the semidominant *ein7* mutation. The moderate Ein$^-$ phenotypes seen in the *ein6* and *ein7* mutants may be explained by reduction-of-function mutations. These moderate Ein$^-$ phenotypes also could be because *EIN6* and *EIN7* only affect a subset of the *EIN2* functions.

A subset of *EIN2* functions not included in an *EIN3/EIN5* pathway are the *EIR1/AUX1* effects on root elongation. The additivity seen between *eir1* mutants and both *ein3-1* and *ein5-1* demonstrates that these genes produce ethylene insensitivity by separate processes. *eir1-1* failed to display such additivity with *ein2-1* and *aux1-21*, suggesting that these loci function in a common response pathway. The relative positions of *EIN2*, *EIR1* and *AUX1* loci within this root response pathway are equivocal. Both *AUX1* and *EIN2* act after *CTR1* but the results are not as clear for *EIR1*. The *eir1-1* allele was not completely epistatic to *ctr1-1*. This effect could result from either a leaky mutation at the *eir1* locus or an additive effect between the *ctr1* and *eir1* mutants. In the latter case, *EIR1* would likely function before both *EIN2* and *AUX1* in this root response. The agravitropic phenotype of both *eir1* alleles and *aux1* alleles would suggest that these gene products are responding to additional stimuli, such as gravity or auxin.

Seedling roots perceive the gravity stimulus at the root cap; this physical stimulus is then transduced to the adjacent tissue (DARWIN and DARWIN 1881). The response to this signal is differential cell elongation between the top and bottom of a horizontal, gravity-stimulated root; this results in a downward bending of the root tip (JACKSON and BARLOW 1981). Several lines of evidence point to auxin as the signal transmitted from the root cap to the region of cell elongation (FELDMAN 1985; EVANS *et al.* 1994). Inhibitors of IAA transport also disrupt gravitropism in seedling roots (LEE *et al.* 1984). Gravity-stimulated roots rapidly induce auxin-regulated genes (MCCLURE and GUILFOYLE 1989). Furthermore, all Arabidopsis mutants that are resistant to exogenous auxin are also defective in root gravitropism (MAHER and MARTINDALE 1980a; LINCOLN *et al.* 1990; PICKETT *et al.* 1990; M. ESTELLE, personal communication).

A possible function for the *EIR1/AUX1* pathway may be in the regulation of auxin in response to gravity. This model predicts that *AUX1* will function in auxin signal transduction. The sensitivity of *eir1* mutants to applied auxin is consistent with *EIR1* functioning in auxin transport rather than in auxin signal transduction, because the IAA transport system is not required in the presence of high concentrations of exogenous auxin. The ethylene insensitivity of *aux1* and *eir1* roots may reflect the role of ethylene in regulating gravity responses and auxin transport. Several studies have shown that ethylene can inhibit the polar flow of auxin in different tissues (BURG and BURG 1967; BEYER 1973; KANG *et al.* 1967; SUTTLE 1988). Inhibitors of ethylene action and biosynthesis slowed gravitropic responses in several plant species (WHEELER and SALISBURY 1980). Ethylene production also is induced immediately and transiently after gravity stimulation of tomato and pea seedlings (HARRISON and PICKARD 1984; PICKARD 1985). Although the processes involved in these gravity responses are poorly understood, further molecular and physiological characterization of the *eir1* and *aux1* mutants may provide critical insights.

The *hls1-1* allele has a severe deficiency in the formation of the apical hook (GUZMAN and ECKER 1990). The shape of the apical hook is defined by differential cell elongation (KANG *et al.* 1967; SILK and ERICKSON 1978; ABELES *et al.* 1992). Ethylene controls several responses that require differential cell elongation, such as epinasty and thigmomorphogenesis (ABELES *et al.* 1992). As in the root gravitropic response, inhibitors of auxin transport disrupt the formation of the apical hook in both *Phaseolis vulgaris* and Arabidopsis (SCHWARK and SCHIERLE 1993; A. LEHMAN and J. R. ECKER, unpublished results). In addition, auxin and ACC are asymmetrically localized in the Phaseolis apical hook (SCHWARK and BOPP 1993). Together, these results suggest that the formation of the apical hook requires an auxin gradient and involves regulation of the ethylene precursor ACC. All Ein$^-$ mutants, after 3 days of growth, have some degree of apical hook curvature. Arabidopsis seedlings treated with inhibitors of ethylene action also have greatly reduced apical hooks (GUZMAN and ECKER 1990; G. ROMAN and J. R. ECKER, unpublished results). The Hls$^-$ phenotype is retained in all double-mutant combinations and is consistent with the *HLS1* gene responding to factors in addition to ethylene (Figure 5). Further genetic and molecular characterization of the *HLS1* locus and the Hls$^-$ phenotype may provide a valuable paradigm for hormonal interactions and the role of ethylene in controlling differential cell elongation.

Summary: We have utilized the Arabidopsis triple response to identify a minimum of 10 genes involved in ethylene signal transduction. In building a genetic framework for the action of these genes, we are developing models that will help facilitate our understanding of the molecular requirements for ethylene responsiveness. The ethylene signal-transduction pathway in Arabidopsis appears to be primarily linear and is defined by the *ETR1, EIN4, CTR1, EIN2, EIN3, EIN5, EIN6* and *EIN7* genes. The downstream branches identified

by the *HLS1, EIR1* and *AUX1* genes may involve interactions with other hormonal or developmental signals.

The authors acknowledge SCOTT POETHIG and MARGARET AHMAD for critical review of the manuscript. G.R. was supported by a National Institutes of Health predoctoral training grant (PHS 5T32GM67229). J.J.K. was supported by a National Science Foundation Postdoctoral Fellowship in Plant Biology (DCB-9008323). This research was supported in part by grants from the National Science Foundation (IBN-9205342) and the Department of Energy (DE-FG02-93ER20104) to J.R.E.

LITERATURE CITED

ABELES, F. B., P. W. MORGAN and M. E. SALTVEIT JR., 1992 *Ethylene in Plant Biology.* Academic Press, Inc., New York.

APELBAUM, A., and S. P. BURG, 1971 Altered cell microfibrillar orientation in ethylene-treated *Pisum sativum* stems. Plant Physiol. **48:** 648–652.

APELBAUM, A., and S. P. BURG, 1972 Effects of ethylene on cell division and deoxyribonucleic acid synthesis in *Pisum sativum.* Plant Physiol. **50:** 117–124.

BELL, C. J., and J. R. ECKER, 1994 Assignment of thirty microsatellite loci to the linkage map of Arabidopsis. Genomics **19:** 137–144.

BENT, A. J., R. W. INNES, J. R. ECKER and B. J. STASKAWICZ, 1992 Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent Pseudomonas and Xanthomonas pathogens. Mol. Plant-Microbe Interact. **5:** 372–378.

BEYER, E. M., 1973 Abscission. Plant Physiol. **52:** 1–5.

BEYER, E. M., 1976 A potent inhibitor of ethylene action in plants. Plant Physiol. **58:** 268–271.

BLEECKER, A. B., M. A. ESTELLE, C. SOMERVILLE and H. KENDE, 1988 Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana.* Science **241:** 1086–1089.

BURG, S. P., and E. A. BURG, 1967 Molecular requirements for the biological activity of ethylene. Plant Physiol. **42:** 144–152.

CHANG, C., J. L. BOWMAN, A. W. DEJOHN, E. S. LANDER and E. M. MEYEROWITZ, 1988 Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana.* Proc. Natl. Acad. Sci USA **85:** 6856–6860.

CHANG, C., S. F. KWOK, A. B. BLEECKER and E. M. MEYEROWITZ, 1993 Arabidopsis ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science **262:** 539–544.

CHORY, J., 1992 A genetic model for light-regulated seedling development in Arabidopsis. Development **115:** 337–354.

DARWIN, C., and F. DARWIN, 1881 *The Power of Movement in Plants.* Appleton-Century-Crofts, New York.

DOLAN, L., C. M. DUCKETT, C. GRIERSON, P. LINSTEAD, K. SCHNEIDER *et al.*, 1994 Clonal relationships and cell patterning in the root epidermis of Arabidopsis. Development **120:** 2465–2474.

ECKER, J. R., and R. W. DAVIS, 1987 Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA **84:** 5202–5206.

EDWARDS, K., C. JOHNSTONE and C. THOMPSON, 1991 A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349.

EVANS, M. L., H. ISHIKAWA and M. A. ESTELLE, 1994 Responses of Arabidopsis roots to auxin studied with high temporal resolution: comparison of wild-type and auxin-response mutants. Planta **194:** 215–222.

FELDMAN, L. J., 1985 Root Gravitropism. Physiol. Plant. **65:** 341–344.

FELDMANN, K. A., and M. D. MARKS, 1987 Agrobacterium-mediated transformation of germinating seeds of Arabidopsis thaliana: a non-tissue culture approach. Mol. Gen. Genet. **208:** 1–9.

FERGUSON, E. L., P. W. STERNBERG and H. R. HORVITZ, 1987 A genetic pathway for the specification of the vulva cell lineages of *C. elegans.* Nature **362:** 259–267.

GALCHEVA-GARGOVA, Z., B. DERIJARD, I-H. WU and R. J. DAVIS, 1994 An osmosensing signal transduction pathway in mammalian cells. Science **265:** 806–808.

GUZMAN, P., and J. R. ECKER, 1990 Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2:** 513–523.

HAN, J., J.-D. LEE, L. BIBBS and R. J. ULEVITCH, 1994 A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science **265:** 808–811.

HARPHAM, N. V. J., A. W. BERRY, E. M. KNEE, G. ROVEDA-HOYOS, I. RASKIN *et al.*, 1991 The effect of ethylene of the growth and development of wild-type and mutant *Arabidopsis thaliana* (L.) Heynh. Ann. Bot. **68:** 55–61.

HARRISON, M., and B. G. PICKARD, 1984 Burst of ethylene upon horizontal placement of tomato seedlings. Plant Physiol. **75:** 1167–1169.

HAWKINS, R. B., 1979 Double strand-breaks and DNA-to-protein crosslinks induced by fast neutrons in bacteriophage DNA. Int. J. Radiat. Biol. **35:** 1–13.

JACKSON, M. B., and P. W. BARLOW, 1981 Root geotropism and the role of growth regulators from the cap: a re-examination. Plant Cell Environ. **4:** 107–123.

KANG, B. G., C. S. YOCUM, S. P. BURG and P. M. RAY, 1967 Ethylene and carbon dioxide: mediation of hypocotyl hook-opening response. Science **156:** 958–959.

KIEBER, J. J., and J. R. ECKER, 1993 Ethylene gas: it's not just for ripening any more! Trends Genet. **9:** 356–362.

KIEBER, J. J., and J. R. ECKER, 1994 Molecular and genetic analysis of the constitutive ethylene response mutant ctr1, pp 193–201 in *Nato ASI Series, Vol. H 81: Plant Molecular Biology; Molecular Genetic Analysis of Plant Development and Metabolism,* edited by G. CORUZZI and P. PUIGDOMENECH. Springer-Verlag, Berlin.

KIEBER, J. J., M. ROTHENBERG, G. ROMAN, K. A. FELDMANN and J. R. ECKER, 1993 *CTR1,* a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell **72:** 1–20.

KNIGHT, L. I., ROSE, R. C. and W. CROCKER, 1910 Effects of various gases and vapors upon etiolated seedlings of the sweet pea. Science **31:** 635–636.

KONIECZNY, A., and F. AUSUBEL, 1993 A procedure for quick mapping of Arabidopsis mutants using ecotype specific markers. Plant J. **4:** 403–410.

KOORNNEEF, M., and P. STAM, 1992 Genetic Analysis, pp 83–99 in *Methods in Arabidopsis Research,* edited by C. KONCZ, N.-H. CHUA and J. SCHELL. World Scientific, Singapore.

KOORNNEEF, M., A. ELGERSMA, C. J. HANHART, E. P. and E. P. VAN LOENEN-MARTINET, 1985 New linkage data of chromosome *one* with a case of very close linkage of three genes affecting plant height. Arabidopsis Inf. Serv. **22:** 43–48.

KOORNNEEF, M., C. J. HANHART, E. P. VAN LOENEN-MARTINET and J. H. VAN DER VEEN, 1987 A marker line that allows the detection of linkage on all Arabidopsis chromosomes. Arabidopsis Inf. Serv. **23:** 46–50.

LANG, J. M., W. R. EISINGER and P. B. GREEN, 1982 Effects of ethylene on the orientation of microtubules and cellulose microfibrils of pea epicotyl cells with polyamellate cell walls. Protoplasma **110:** 5–14.

LAWTON, K. A., S. S. POTTER, S. UKNES and J. RYALS, 1994 Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6:** 581–588.

LEE, J. S., T. J. MULKEY and M. L. EVANS, 1984 Inhibition of polar calcium movement and gravitropism in roots treated with auxin-transport inhibitors. Planta **160:** 536–543.

LINCOLN, J. E., and R. L. FISHER, 1988 Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol. Gen. Genet. **212:** 71–75.

LINCOLN, C., J. H. BRITTON and M. ESTELLE, 1990 Growth and development of the *axr1* mutants of Arabidopsis. Plant Cell **2:** 1071–1080.

MAEDA, T., S. M. WURGLER-MURPHY and H. SAITO, 1994 A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242–245.

MAHER, E. P., and S. J. B. MARTINDALE, 1980a Mutants of *Arabidopsis thaliana* with altered responses to auxin and gravity. Biochem. Genet. **18:** 1041–1053.

MAHER, E. P., and S. J. B. MARTINDALE, 1980b Further studies of auxin resistance in *Arabidopsis thaliana.* Arabidopsis Inf. Serv. **17:** 40–47.

MANLY, K. F., 1993 A MacIntosh program for storage and analysis

of experimental genetic mapping data. Mamm. Genome **4:** 303–313.

MATTOO, A. K., and J. C. SUTTLE, 1991 *The Plant Hormone Ethylene.* CRC Press, Boca Raton, FL.

MCCLURE, B. A., and T. GUILFOYLE, 1989 Rapid redistribution of auxin-regulated RNAs during gravitropism. Science **243:** 91–93.

MELLER, Y., G. SESSA, Y. EYAL and R. FLUHR, 1993 DNA-protein interactions on a cis-DNA element essential for ethylene regulation. Plant Mol. Biol. **23:** 453–463.

NAM, H-G., J. GIRAUDAT, B. DEN BOER, F. MOONAN, W. D. B. LOOS *et al.*, 1989 Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana.* Plant Cell **1:** 699–705.

NELJUBOV, D., 1901 Ueber die horizontale Nutation der Stengel von Pisum sativum und einiger anderer. Pflanzen Beih. Bot. Zentralbl. **10:** 128–139.

NEWMAN, T., F. J. DE BRUIJN, P. GREEN, K. KEEGSTRA, H. KENDE *et al.*, Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous Arabidopsis cDNA clones. Plant Physiol. **106:** 1241–1255.

OTA, I., and A. VARSHAVSKY, 1993 A yeast protein similar to bacterial 2-component regulators. Science **262:** 566–569.

PICKARD, B. G., 1985 Early events in geotropism of seedling shoots. Annu. Rev. Plant Physiol. **36:** 55–75.

PICKETT, F. B., A. K. WILSON and M. ESTELLE, 1990 The *aux1* mutation of Arabidopsis confers both auxin and ethylene resistance. Plant Physiol. **94:** 1462–1466.

SATO, T., and A. THEOLOGIS, 1989 Cloning the mRNA encoding 1-amino-cyclopropane-1-carboxylate synthtase, the key enzyme for ethylene biosynthesis in plants. Proc. Natl. Acad. Sci. USA **86:** 6621–6625.

SCHWARK, A., and M. BOPP, 1993 Interaction of ethylene and auxin in the regulation of hook growth II. The role for ethylene in different growing regions of the hypocotyl hook of *Phaseolus vulgaris.* J. Plant Physiol. **142:** 585–592.

SCHWARK, A., and J. SCHIERLE, 1993 Interaction of ethylene and auxin in regulation of hook growth. I. The role of auxin in different growing regions of the hypocotyl hook of *Phaseolus vulgaris.* J. Plant Physiol. **142:** 562–570.

SHIRLEY, B. W., S. HANELY and H. M. GOODMAN, 1992 Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis *transparent testa* mutations. Plant Cell **4:** 333–347.

SILK, W. K., and R. O. ERICKSON, 1978 Kinematics of hypocotyl curvature. Am. J. Bot. **65:** 310–319.

SISLER, E. C., 1990 Ethylene-binding components in plants, pp 81–100 in *The Plant Hormone Ethylene,* edited by A. K. MATTOO and J. C. SUTTLE. CRC Press, Baco Raton, FL.

SPANU, P., D. REINHARDT and T. BOLLER, 1991 Analysis and cloning of the ethylene-forming enzyme from tomato by functional expression of its mRNA in *Xenopus laevis* oocytes. EMBO J. **10:** 2007–2013.

SUTTLE, J. C., 1988 Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of N-1-naphthylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol. **88:** 795–799.

THEOLOGIS, A., 1992 One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell **70:** 181–184.

VAN DER STRAETEN, D., A. DJUDZMAN, W. VAN CAENEGEGHEM, J. SMALLE and M. VAN MONTAGU, 1993 Genetic and physiological analysis of a new locus in Arabidopsis that confers resistance to 1-aminocyclopropane-1-carboxylic acid and ethylene and specifically affects the ethylene signal transduction pathway. Plant Physiol. **102:** 401–408.

WHEELER, R. M., and F. B. SALISBURY, 1980 Gravitropism in plant stems may require ethylene. Science **209:** 1126–1128.

WILSON, A. K., F. B. PICKETT, J. C. TURNER and M. ESTELLE, 1990 A dominant mutation in Arabidopsis confers resistance to auxin, ethylene, and abscisic acid. Mol. Gen. Genet. **222:** 377–383.

YANG, S. F., and N. E. HOFFMAN, 1984 Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. **35:** 155–189.

YUAN, M., P. J. SHAW, R. M. WARN and C. W. LLOYD, 1994 Dynamic reorientation of cortical microtubules, from transverse to longitudinal, in living plant cells. Proc. Natl. Acad. Sci. USA **91:** 6050–6053.

Communicating editor: J. CHORY