Five components of the ethylene-response pathway identified in a screen for *weak ethylene-insensitive* mutants in *Arabidopsis*

Jose M. Alonso*†‡, Anna N. Stepanova*†‡, Roberto Solano*§, Ellen Wisman¶, Simone Ferrari‖, Frederick M. Ausubel‖, and Joseph R. Ecker*,

*Plant Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, 92037; ¶Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824; and ‖Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

Communicated by Joanne Chory, The Salk Institute for Biological Studies, La Jolla, CA, December 31, 2002 (received for review November 22, 2002)

Five ethylene-insensitive loci (*wei1*–*wei5*) were identified by using a low-dose screen for "weak" ethylene-insensitive mutants. *wei1*, *wei2*, and *wei3* seedlings showed hormone insensitivity only in roots, whereas *wei4* and *wei5* displayed insensitivity in both roots and hypocotyls. The genes corresponding to *wei1*, *wei4*, and *wei5* were isolated using a positional cloning approach. The *wei1* mutant harbored a recessive mutation in *TIR1*, which encodes a component of the SCF protein ubiquitin ligase involved in the auxin response. *wei4*, a dominant mutant, resulted from a mutation in the ethylene receptor *ERS*, whereas *wei5*, a semidominant mutant, was caused by a mutation in the *EIN3*-related transcription factor gene *EIL1*. The simultaneous loss of functional *WEI5*/*EIL1* and *EIN3* nearly completely abolished the ethylene response in etiolated seedlings, and adult plants were highly susceptible to infection by the necrotrophic fungal pathogen *Botrytis cinerea*. Moreover, *wei5*/*eil1 ein3* double mutants were able to fully suppress constitutive signaling caused by *ctr1*, suggesting a synergistic interaction among these gene products. Unlike previously known root ethylene-insensitive mutants, *wei2* and *wei3* were not affected in their response to auxin and showed a normal response to gravity. Genetic mapping studies indicate that *wei2* and *wei3* correspond to previously unidentified ethylene pathway genes that may control cell-elongation processes functioning at the intersection of the ethylene and auxin response pathways.

The gaseous plant hormone ethylene plays an important role in diverse physiological and developmental processes ranging from seed germination to organ senescence and abscission (1, 2). After treatment with ethylene, etiolated seedlings undergo a dramatic morphological transformation called the triple response, which consists of inhibition of hypocotyl and root-cell elongation, radial expansion of cells in the hypocotyl, and exaggeration in the differential elongation rate of cells in the apical hook. This robust phenotype has been used extensively for genetic screens to identify *Arabidopsis* mutants affected in hormone biosynthesis, perception, and signal transduction pathways (3–11). The majority of signaling pathway mutants (*ein2*–*ein4*, *etr1*, *etr2*, *ers2*, *ein5*, and *ein6*) show ethylene insensitivity in all tissues of the plant and interfere with general ethylene signaling (3, 4, 8–10). However, other mutants (*aux1*, *axr1*, *eir1*, and *hls1*) are tissue-specific, and the mutations probably only disable a downstream branch of the pathway (7, 8, 11).

Ethylene is perceived by a family of receptors that are similar to bacterial two-component histidine kinases (9, 10, 12, 13). Genetic studies suggest that in the absence of ethylene, the receptors are active and positively regulate the activity of CTR1, a Raf-like serine/threonine kinase (5). In turn, the negative regulator CTR1 directly or indirectly inhibits the positive regulator EIN2, an integral membrane protein of unknown function (8, 14). EIN2 transmits the ethylene signal to the EIN3 family of DNA-binding transcription factors (15, 16). Although there are a total of six *EIN3*-like genes in *Arabidopsis*, only *EIN3* has been shown to be required for the normal ethylene response, as inferred from the hormone insensitivity of the *ein3* loss-of-function mutant (15). Transgenic studies suggest that the EIN3-like proteins EIL1 and EIL2 may also be involved in ethylene signal transduction (15). However, mutations in these genes have not been identified. EIN3 and possibly other members of the EIN3 family bind to a DNA element in the promoter of the *ERF1* gene, an ethylene-responsive element binding protein-type transcription factor (16), and presumably activate its transcription. ERF1, in turn, binds to the GCC box in the promoters of a variety of ethylene target genes (16). Because no physical interactions between CTR1 and EIN2 or EIN2 and the EIN3-like proteins have been observed, yet unknown proteins are likely to bridge these steps.

Here we describe a screen to identify additional ethylene-signaling pathway components. By using low doses of the hormone, five weak ethylene-insensitive (*wei*) mutant loci were identified. Characterization of these mutants along with positional cloning of three *wei* genes has allowed the identification of several previously unknown ethylene pathway components. The fact that only one allele belonging to each complementation group was found indicates that this screen will provide a valuable approach to uncover additional genes participating in the response to ethylene.

Materials and Methods

Strains and Growth Conditions. *Arabidopsis thaliana* accession Columbia-0 (Col-0) was the parental strain of the ethylmethanesulfonate- and En-I transposon-mutagenized populations used for the forward and reverse genetic screens, respectively. Landsberg *erecta* (L*er*) was the parental strain of the En-I populations used for forward genetic screens. For each of the hormone treatments, surface-sterilized seeds were grown on *Arabidopsis thaliana* plates [1× Murashige and Skoog salts (GIBCO), pH 6.0/1% sucrose/0.8% agar] supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC, 0, 0.1, 0.2, 0.3, 0.5, 1.0, or 10.0 μM) or 2,4-dichlorophenoxyacetic acid (0, 0.1, 1.0, or 10.0 μM) or treated with 10 ppm ethylene or with hydrocarbon-free air. Seeds were cold-treated for 3–4 days at 4°C and then placed at 24°C in darkness. Phenotypes were scored after ≈72 h. For propagation, seedlings from plates were trans-

Abbreviations: *wei*, weak ethylene-insensitive; Col, Columbia; L*er*, Landsberg *erecta*; ACC, 1-aminocyclopropane-1-carboxylic acid; SSLP, simple sequence-length polymorphic.

†J.M.A. and A.N.S. contributed equally to this work.

‡Present address: Department of Genetics, North Carolina State University, Raleigh, NC 27606.

§Present address: Departamento de Genetica Molecular de Plantas, Centro Nacional de Biotecnologia-CSIC, 28049 Madrid, Spain.

**To whom correspondence should be addressed at: Plant Biology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037. E-mail: ecker@salk.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0438070100

ferred to MetroMix-200 (Scotts, Marysville, OH) and grown to maturity at 22°C under a 16-h light/8-h dark cycle.

Mutagenesis. Ethylmethanesulfonate-mutagenized M2 families were obtained as described (4). Approximately 8,000 3-day-old M2 seedlings were screened for each of 10 independent M1 families. Sixty En-I transposon-tagged families were generated from single plants (gift of Andy Pereira, Center for Plant Breeding and Reproductive Research, Wageningen, The Netherlands) by two generations of self-fertilization of pools of ≈1,500 descendant plants. Approximately 8,000 seedlings from each of eight transposon families were initially screened for ethylene-response phenotypes.

Mutant Screen Using Low Concentrations of Hormone. Mutagenized seeds were plated on *Arabidopsis thaliana* plates supplemented with 0.5 μM ACC at a density of 1,000–1,500 seeds per 150 × 15-mm plate, cold-treated, and incubated in the dark for 3 days at 24°C. Putative ethylene-insensitive seedlings were picked and grown in MetroMix-200 to maturity. In the next generation, plants were retested in 10.0 and 0.5 μM ACC, and those displaying weak ethylene insensitivity were kept and characterized. All *wei* mutants were backcrossed to the parental strain at least once before phenotypic analysis.

Genetic Mapping of the *wei* Mutations. Mutants isolated in the Col-0 background (*wei1*, *wei2*, and *wei4*) were crossed to L*er*, whereas mutants in the L*er* background (*wei3* and *wei5-2*) were crossed to Col-0. *wei5-2* was mapped to the middle of chromosome 2 based on its cosegregation with the *erecta* mutation. *wei1–wei4* were mapped by using simple sequence-length polymorphic (SSLP) markers (17) (see *Results*). Genomic DNAs were prepared from F_3 seedlings as described (18).

PCR-Based Screen for *eil1-1* (*wei5-1*). To identify a second mutant allele of EIL1, a PCR-based screen was carried out by using pooled DNAs prepared from a population of 3,000 *Arabidopsis* insertion lines (ecotype Col-0) that contained ≈15,000 genome-insertion events of the maize transposable element En-I (19). Cosegregation of the transposon insertion and the ethylene-insensitive mutant phenotype in seedlings was confirmed by PCR analysis of 20 independent segregating descendents. The position of the transposon insertion within the *EIL1* gene was determined by sequencing of the PCR product from the mutant.

Fungal Growth and Plant Inoculation. A *Botrytis cinerea* isolate was obtained from cabbage and grown on potato dextrose agar (Difco) for 2 weeks at 24°C with a 12-h photoperiod before spore collection. Inoculation of *Arabidopsis* was performed on 4-week-old soil-grown plants by placing one 5-μl droplet of a suspension of 5×10^5 conidial spores per ml in 24 g·liter^{-1} potato dextrose broth (Difco) on each side of the midvein of each leaf. Four fully expanded leaves per plant were inoculated. Infected plants were incubated at 22–24°C with a 12-h photoperiod. High humidity was maintained by covering the plants with a clear plastic lid. The number of plants that were dead because the infection had spread to the central bud was scored 9 days after inoculation. The data were subjected to ANOVA, and *P* values were adjusted by using the Bonferroni method for multiple comparisons.

Results

Isolation of *wei* Mutants. To select the appropriate concentration to screen for *wei* mutants, we analyzed the hormone response of 3-day-old etiolated seedlings grown in the presence of various concentrations of the ethylene precursor ACC (Fig. 1). Approximately 3,000 wild-type *Arabidopsis* seedlings were examined at each of seven different hormone concentrations (see *Materials and Methods*). At 0.5 μM ACC, the seedlings showed a uniform

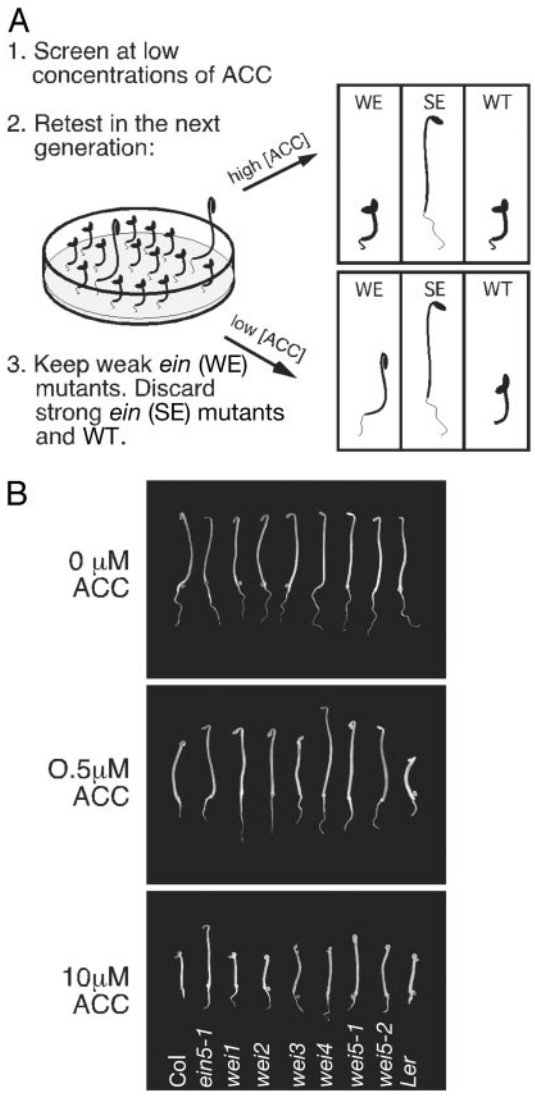

Fig. 1. Mutant screen using nonsaturating levels of ethylene. (*A*) Schematic representation of the screening strategy. Mutagenized *Arabidopsis* plants were screened at a low concentration of the ethylene precursor ACC. Plants that showed hormone insensitivity were selected and retested in the next generation. Only putative mutants that showed weak hormone insensitivity were characterized further. (*B*) Comparison of the phenotypes of *wei* mutants in different concentrations of ACC. Seedlings were grown in the dark for 3 days in the presence of the indicated concentrations of ACC and photographed.

intermediate phenotype, and this concentration was chosen for further mutant screens. Progenies of the plants selected from primary mutant screens were retested using 0.5 and 10 μM ACC (Fig. 1*A*). Two phenotypic categories of mutants were selected by comparison with the weakest known *ein* mutant (*ein5*): those that showed strong ethylene insensitivity (more resistant to ethylene than *ein5* seedlings) and those that showed weak insensitivity to the hormone (less resistant to ethylene than *ein5* seedlings). This second group was referred to collectively as *wei* mutants. Five putative mutants that showed significant ethylene insensitivity at low concentrations of ACC and a near wild-type phenotype at higher concentrations of the hormone were selected for further characterization (Fig. 1*B*). Similar phenotypes were observed when ethylene gas was used instead of ACC (data not shown).

Genetic and Phenotypic Characterization. Genetic analysis of the progeny produced from backcrosses of these five mutants to wild

type revealed that *wei1* and *wei2* are recessive mutations and *wei3* and *wei4* are dominant mutations, whereas *wei5* is a semidominant mutation. The phenotypic responses of wild type, *wei* mutants, and *ein5* to different concentrations of the ethylene precursor ACC were compared (Fig. 1*B*). In the absence of the hormone, the mutant plants were indistinguishable from wild type. Conversely, at both high and low concentrations of the hormone, the mutant seedlings showed a significant degree of ethylene insensitivity (Fig. 1*B*). To establish whether the ethylene-response defect of the mutants was more profound at lower rather than higher concentrations of the hormone, the responses were quantified. Importantly, in some of the *wei* mutants, hypocotyl and root tissues showed different degrees of ethylene sensitivity. Therefore, the effect of the ethylene treatment was assayed separately for roots and hypocotyls. At 10 μM ACC, *wei4* and *wei5* hypocotyls showed clear resistance to the hormone, although the degree of insensitivity of these mutants was significantly less than that observed in *ein5*. Conversely, the hypocotyl responses to ethylene found in *wei1*, *wei2*, and *wei3* seedlings were indistinguishable from wild type. Interestingly, hormone insensitivity in the root response to ethylene was apparent in each of the *wei* mutants. The degree of root response varied among the mutants. *wei4* roots showed a greater degree of insensitivity to ethylene than *ein5* roots, whereas the root length of *wei5* was similar to that of the *ein5* mutant. The roots of *wei1*, *wei2*, and *wei3* showed more response to the hormone but, nevertheless, when compared with wild type were clearly ethylene-insensitive (Fig. 1*B*, 2).

Overall, the differences in the sensitivity of the *wei* mutants to ethylene with respect to wild type were similar at both high and low concentrations of the hormone. The only exception was *wei4* where the difference was greater at low ACC concentrations (Fig. 2). It should also be noted that the hormone-response phenotype of the *wei3* mutant was highly variable both at high and low concentrations of ACC ranging from clear ethylene insensitivity to a near wild-type response. This variability was observed even when *wei3* plants with clear ethylene-insensitive roots were selected and propagated for several generations. Although these results can be explained by low penetrance of the mutant phenotype, it is also possible that the phenotype observed may not be due to a single mutation but may reflect the quantitative interaction of several distinct loci.

Dose-response experiments also revealed that the hypocotyls of L*er* plants were more sensitive to ethylene than those of Col (Fig. 2*A*). Interestingly, this effect was not found in the roots, where the hormone response was similar in both accessions (Fig. 2*B*).

Order of Action of Genes. To position the *wei* mutants in the ethylene pathway, we examined the epistatic relationships between the *wei* mutants and *ctr1*. The morphology of *ctr1* is similar to that of wild-type seedlings treated with saturating concentrations of ethylene (5). Double mutants were identified among F_2 or F_3 seedlings produced from crosses between each *wei* mutant and *ctr1-1*. In the case of *wei1*, *wei2*, *wei3*, and *wei5*, we identified several F_2 seedlings that showed a constitutive *wei* ethylene phenotype in the absence of the hormone (Fig. 3). As anticipated for *wei3*, the number of seedlings showing a clear *wei* phenotype was less than that expected for a fully penetrant mutant. Putative double-mutant seedlings were retested in the next generation to confirm the initial phenotype. The results of the double-mutant studies indicated that *wei1–wei3* and *wei5* are epistatic to *ctr1* in the ethylene-signaling pathway, and therefore these genes may act downstream of or at the same level as this RAF-like kinase.

When the phenotypes of the F_2 seedlings from the cross between *wei4* and *ctr1-1* were analyzed, we were unable to identify any plants showing a constitutive *wei* ethylene phenotype in the absence of the hormone. These results suggested that

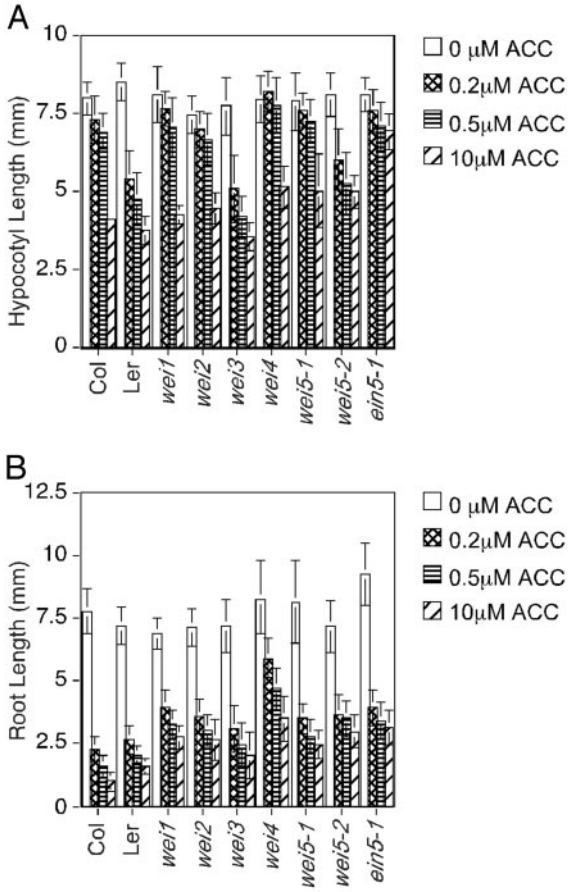

Fig. 2. Quantification of the effects of ACC on hypocotyl (*A*) and root length (*B*) of the *wei* mutants. Wild-type and mutant seedlings (30–40 per treatment per genotype) were grown in the dark in the presence of the indicated concentrations of ACC for 3 days, photographed, and measured by using the program NIH IMAGE.

ctr1 may be epistatic to *wei4*. To confirm this possibility, 10 *ctr*-like plants were crossed to wild type, and the phenotypes of F_1 progeny from each cross were examined in the presence of a saturating level of hormone (10 μM ACC). All the F_1 progeny produced from two of the crosses showed ethylene insensitivity (data not shown), implying that the genotype of the parental *ctr*-like plants was *wei4 ctr1*. These double mutants showed a phenotype indistinguishable from that of *ctr1* both in seedlings

Fig. 3. Epistasis analysis of the *wei* mutants. Double mutants were constructed between *ctr1* and five *wei* mutants. Seedlings of the corresponding double mutants were grown in the dark for 3 days in the absence of exogenously applied ethylene or ACC and photographed.

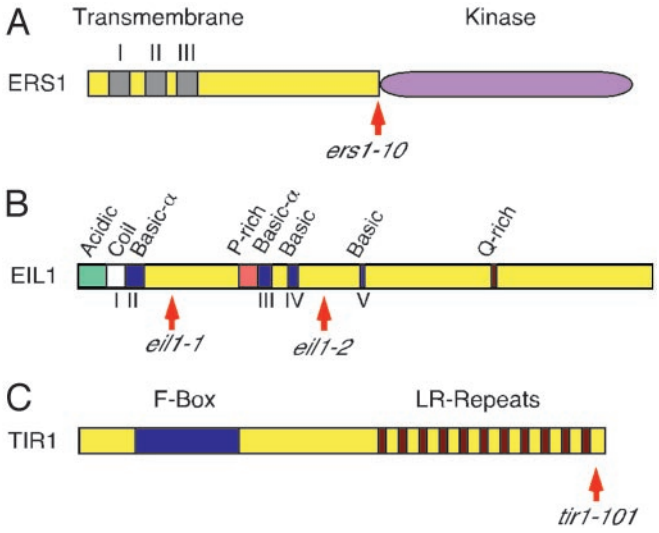

Fig. 4. Schematic representation of the ERS1 (*A*), EIL1 (*B*), and TIR1 (*C*) proteins. The approximate positions of the mutations found in *wei4*/*ers1-10* (R to C), *wei5-1*/*eil1-1* (transposon insertion), *wei5-2*/*eil1-2* (frame shift), and *wei1*/*tir1-101* (stop codon) are indicated.

and in adults. These results indicate that *wei4* may act upstream of or at the same step as *ctr1* in the ethylene-signaling pathway.

***wei4* Is Allelic to *ERS1*.** The *wei4* mutation was mapped to the bottom of chromosome 2 between the SSLP markers BIO201 and nga168 in a region known to contain the ethylene receptor gene *ERS1* (13, 20). Because all the dominant ethylene-insensitive mutants described to date belong to the ethylene receptor family and *wei4* mapped to the same region of the chromosome 2 as *ERS1*, we tested whether *wei4* was allelic to *ERS1*. Sequencing of *ERS1* in the *wei4* mutant plants identified a missense mutation that is predicted to produce a change of R320 to C. Interestingly, this amino acid is located between the hydrophobic amino end and the predicted kinase domain (Fig. 4*A*). Alignment of amino acid sequences for all the ethylene receptors showed that this arginine is conserved not only in the *Arabidopsis* ethylene receptor family but is also highly conserved in the members from other plant species (data not shown). Although several dominant mutations in other *Arabidopsis* ethylene receptors have been described, *wei4* maps outside of the hydrophobic amino-end domain of ERS1, which forms the ethylene-binding pocket. We refer to this allele of *ERS1* as *ers1-10*.

***wei5* Is Allelic to *EIL1*.** *wei5* was mapped to the middle of chromosome 2 near the *ERECTA* gene. Because the *EIN3*-like gene, *EIL1*, is also closely linked to *ERECTA* (20), we inferred that *wei5* might be allelic to *EIL1*. Sequencing of the *EIL1* gene from *wei5-2* plants revealed a 4-bp insertion at position 1,390 (GenBank accession no. AF004213) (Fig. 4*B*). Although the *wei5-2* allele of *EIL1* is in the L*er* background, we also identified a Col-0 allele of *EIL1* using a PCR-based reverse-genetics approach (see *Materials and Methods*). *wei5-1* harbors an En-I transposon insertion at position 697 (Fig. 4*B*), and the phenotype of this mutant is similar to that of the *wei5-2* allele in all of the aspects studied: resistance to ethylene, epistatic analysis, and genetic interaction with *ein3-1* (see below). The transposon insertion in *wei5-1* removes a significant part of both the putative DNA-binding domain and the dimerization domain (as predicted by similarity with the tobacco *TEIL*) (21). We renamed *wei5-1* and *wei5-2* as *eil1-1* and *eil1-2*, respectively.

***eil1* Genetically Interacts with *ein3*.** Previous studies suggest that the *EIL* genes may act in parallel with *EIN3* (15, 16). By genetically combining *ein3* with mutations in the other transcription factor family members, a further reduction of ethylene sensitivity may be evident. To test this hypothesis, we crossed the *eil1* mutants to *ein3-1*. The F_1 progeny of the crosses between *eil1-1* or *eil1-2* and *ein3-1* showed a phenotype that was intermediate between that of the *eil1* and *ein3* homozygous mutants. *eil1-1 ein3-1* and *eil1-2 ein3-1* plants were identified in the F_2 generation by PCR and DNA sequencing of candidate double-mutant plants. Quantification of the hypocotyl and root responses to ethylene in the *eil1 ein3* double-mutant seedlings revealed that these plants were almost completely hormone-insensitive and indistinguishable from the ethylene-response null mutant *ein2-5* (Table 1).

To address the requirement of *eil1* and *ein3* at later stages of plant development, we constructed a triple mutant: *ctr1-1 eil1-2 ein3-1*. Remarkably, when grown in soil this mutant was phenotypically indistinguishable from wild-type plants (data not shown), suggesting that the effect of *ctr1-1* mutation was masked completely by the *eil1-2 ein3-1* double knockout. To further investigate the role of *EIL1* and *EIN3* in mature plants, we examined the response of several ethylene mutants to a necrotrophic fungal pathogen *B. cinerea*. *B. cinerea* is known to be able to kill the ethylene-insensitive mutant *ein2-5*, whereas wild-type plants are largely resistant to this pathogen (22). Interestingly, *eil1-1* mutant plants were also resistant to *B. cinerea* infection, unlike *ein3-1*, which showed an intermediate level of susceptibility between that of wild type and *ein2-5* (Fig. 5). However, the *eil1-1 ein3-1* double-mutant plants proved to be as susceptible to the pathogen as the strong ethylene-insensitive mutant *ein2-5* (Fig. 5). Statistical analysis confirmed that the difference in average number of decayed plants between *ein3* and *ein3 eil1* or *ein2-5* plants was significant ($P < 0.015$).

Table 1. Quantification of the effects of ACC treatment on root and hypocotyl lengths in various genotypes

Background	Root length, mm*		Relative root length, %†	Hypocotyl length, mm*		Relative hypocotyl length, %†
	0 mM ACC	10 mM ACC		0 mM ACC	10 mM ACC	
Col	6.51 ± 0.85	1.88 ± 0.27	28.8	9.36 ± 1.13	4.72 ± 0.38	50.4
L*er*	5.14 ± 1.23	2.14 ± 0.50	41.6	7.86 ± 1.25	3.56 ± 0.80	45.3
eil1-1	5.42 ± 0.85	2.55 ± 0.38	47.0	9.20 ± 0.91	5.52 ± 0.49	60.0
eil1-2	5.78 ± 1.38	3.09 ± 0.63	53.5	8.07 ± 1.44	4.99 ± 1.07	61.8
ein3-1	6.14 ± 1.02	4.54 ± 0.98	73.9	8.72 ± 1.28	7.23 ± 0.83	82.9
ein2-5	6.20 ± 0.88	5.83 ± 0.98	94.0	9.44 ± 1.33	8.99 ± 1.67	95.2
eil1-1 ein3-1	5.79 ± 1.19	5.15 ± 0.88	88.9	8.36 ± 1.40	8.23 ± 1.20	98.4
eil1-2 ein3-1	6.58 ± 1.90	6.61 ± 1.59	100.4	9.24 ± 1.60	8.89 ± 1.53	96.2

*Fifty or more seedlings per treatment per genotype were measured.
†Expressed as a ratio of organ length in the presence of 10 μM ACC over the organ length in the presence of 0 μM ACC (multiplied by 100).

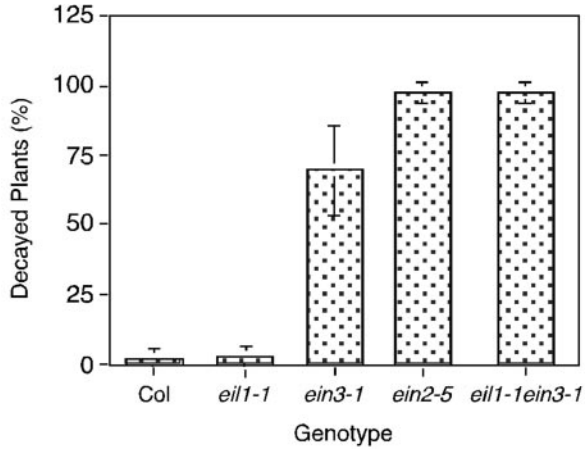

Fig. 5. Pathogen susceptibility of ethylene mutants. Rosettes of 4-week-old adult plants of Col, *eil1-1*, *ein3-1*, *ein2-5*, and *eil1-1 ein3-1* were infected with the spore suspensions of *B. cinerea*. The percentage of dead plants was scored 9 days after inoculation. The data represent averages with standard deviations of three independent experiments performed with 12 or more plants per genotype.

***wei1* Shows Altered Auxin Response.** Several mutations that interfere with auxin transport or sensitivity have also been found to affect the response to ethylene (8, 11). In these mutants, the abnormal ethylene response was restricted mainly to the root tissues, suggesting that the ethylene-induced inhibition of root-cell elongation may have depended on proper auxin biosynthesis, transport, or signaling processes. Because *wei1–wei3* showed root-specific ethylene insensitivity, we also tested their response to exogenously applied auxin. Although *wei1* seedlings displayed clear auxin insensitivity (Fig. 6), *wei2* and *wei3* phenotypes were indistinguishable from that of the wild type (data not shown). In addition, *wei2* and *wei3* also showed a normal gravitropic response (data not shown), further suggesting that *wei2* and *wei3* are root-specific ethylene-signaling components that do not affect the general auxin response.

***wei1* Is a New Allele of *tir1*.** Because of the weak phenotype of *wei1*, F_3 mapping populations from a cross of the mutant to L*er* were used for genetic mapping of this mutation. *wei1* was positioned on the bottom of chromosome 3, 8 centimorgans south of the nga6 marker. Interestingly, an auxin-insensitive mutant *tir1* has been mapped previously to this region (23). Because *wei1* is insensitive not only to ethylene but also to auxin, *wei1* and *tir1* might be alleles of the same gene. We therefore compared the phenotype of *wei1* and *tir1-1* in response to ethylene (ACC). Although both mutants showed ethylene insensitivity in the root, *wei1* was clearly more hormone-insensitive than *tir1-1* (data not shown). Similarly, the auxin insensitivity of *wei1* was stronger than that of *tir1-1* (Fig. 6). Genetic complementation analysis indicated that *wei1* and *tir1-1* were allelic (data not shown). Sequencing of the *TIR1* gene in the *wei1* mutant identified a G-to-A transition at nucleotide 2,898 (GenBank accession no. AF005047), changing W574 to a stop codon (Fig. 4*C*). The resulting TIR1 protein is predicted to lack 21 carboxyl-terminal amino acids. We named the *wei1* allele of this gene *tir1-101*.

***wei2* and *wei3* Define New Ethylene-Insensitive Loci.** The *wei2* and *wei3* mutants were mapped by using SSLP markers to the top of chromosome 5, close to the *EIN2* locus. Genetic complementation testing indicated that *wei2* and *wei3* were not alleles of the *EIN2* gene. The *wei2* mutant was further mapped to a region between the SSLP markers nga225 and nga249. Similarly, SSLP mapping placed *wei3* to a region between the markers ca72 and nga106. Because *wei2* and *wei3* are distantly located from each

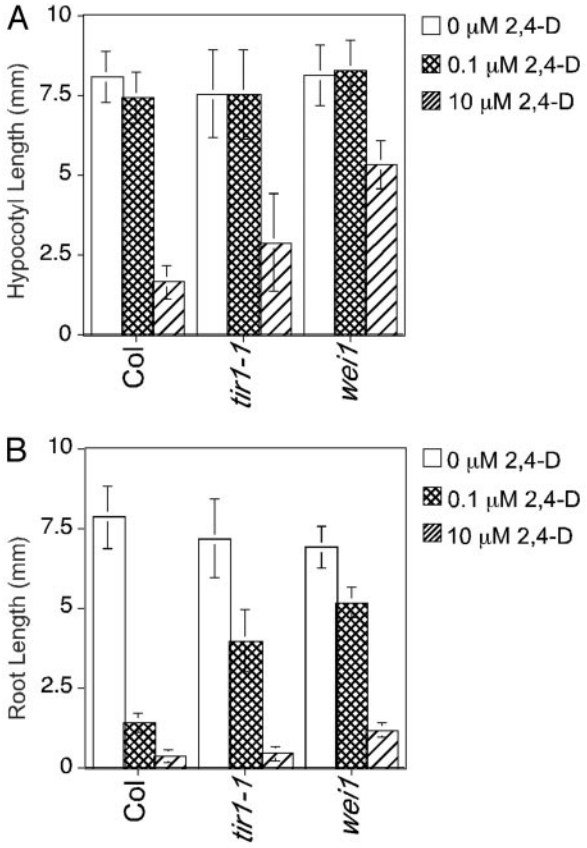

Fig. 6. Quantification of the effects of 2,4-dichlorophenoxyacetic acid on the hypocotyls (*A*) and roots (*B*) of the *wei* mutants. Wild-type and mutant seedlings (30–40 per treatment per genotype) were grown in the dark for 3 days in the presence of the indicated concentrations of 2,4-dichlorophenoxyacetic acid, photographed, and measured by using the program NIH IMAGE.

other on the chromosome 5 and no other previously known ethylene-related mutants/genes have been mapped to these regions, we conclude that *wei2* and *wei3* represent two previously uncharacterized ethylene-insensitive loci.

Discussion

In this article, we describe the results of a genetic screen that was based on nonsaturating concentrations of the ethylene precursor ACC. Five loci (*wei1–wei5*) were identified that are required for a full response to ethylene in *Arabidopsis*. *wei1*, *wei4*, and *wei5* were shown to be allelic to *TIR1*, *ERS1*, and *EIL1*, respectively, whereas *wei2* and *wei3* represent previously undiscovered ethylene-response loci. Positional cloning of *wei4* revealed that the dominant phenotype was due to a mutation in one of the ethylene receptor genes (*ERS1*). Ethylene perception is carried out by a family of five membrane-bound proteins with sequence similarity to the two-component histidine kinases (reviewed in ref. 24). The *wei4/ers1-10* mutation represents the first recovered for *ERS1* in a mutant screen. Interestingly, only strong alleles of *ers1* are obtained by creating substitution mutations in the wild-type *ERS1* gene and reintroducing these transgenes into wild-type plants (13). The identification of "native" weak alleles of *ers1* may provide important insights into gene function. The ethylmethanesulfonate-induced *ers1-10* mutation creates a *wei* plant with a predominantly root-specific phenotype, possibly providing a clue as to its normal developmental context in the plant response to ethylene.

A number of receptor mutants that confer dominant ethylene insensitivity have been identified (3, 9, 10). Each of the muta-

tions was found to affect the amino-terminal transmembrane part of the receptor protein. This region is predicted to form a hydrophobic pocket suitable for ethylene binding via copper-dependent coordination of the ethylene molecule (25–27). Although ethylene is thought to trigger a conformational change in the amino terminus that then is transmitted to the carboxyl part of the receptor, direct experimental evidence to support this model is still lacking. Remarkably, the missense mutation in *wei4/ers1-10* maps in the region between the hydrophobic amino end and the putative histidine kinase domain. Thus, this mutation may interfere with the transmission of the signal within the receptor molecule by uncoupling the input from the output domains of the protein.

EIL1 is one of six *EIN3*-like genes in *Arabidopsis* (15, 20). Although several *ein3* alleles have been identified using the triple-response assay, none of these mutants conferred complete ethylene insensitivity. These results suggest the involvement of one or more of the *EIL* genes in the ethylene response (15). This possibility is supported by previous studies demonstrating that overexpression of *EIL1* and *EIL2* was able to complement the *ein3* mutant phenotype (15). Despite this evidence, direct involvement of the *EIL*s in ethylene signaling had not been demonstrated conclusively. The isolation of *wei5/eil1* not only confirms that *EIL1* is a component of the ethylene-signaling cascade but also allows us to examine the contributions of the remaining *EIN3* family members to the ethylene response. Interestingly, *eil1 ein3* double-mutant seedlings show no morphological response to ethylene, indicating that *EIL2–EIL5* genes may not contribute to the ethylene response at this stage of development. Moreover, in adult plants the effect of these two mutations on the plant resistance to the necrotrophic fungus *B. cinerea* is synergistic. Finally, phenotypic analysis of the soil-grown adult *ctr1-1 eil1-2 ein3-1* triple-mutant plants suggests that the EIL1/EIN3 deficiency is able to mask the constitutive ethylene response of *ctr1-1*. Taken together, these results support the notion of the importance of *EIL1* and *EIN3* for plant sensitivity to the hormone ethylene throughout the plant-life cycle.

Among the ethylene-mediated responses, the growth inhibition of hypocotyl and root cells is the most apparent. Initially identified for their abnormal response to the plant hormone auxin (11), the *aux1*, *axr1–axr3*, and *eir1* mutants are also affected in the ethylene response specifically in root tissues (8, 11). These findings suggest that some ethylene responses may be mediated by the interaction between these two hormones (8). The absence of response to ethylene in these multihormone-resistant mutants suggests that ethylene may regulate cell elongation, in part, by modulating the concentration and/or sensitivity to auxin. In agreement with this suggestion, we found that plants with mutations in *TIR1*, a gene that participates in a ubiquitin-mediated degradation of general components of the auxin response (23, 28), are also altered in their response to ethylene. The near wild-type levels of ethylene sensitivity and the localized effects of these mutations suggest that other *TIR1*-like genes present in the *Arabidopsis* genome (20) might also participate in the response to ethylene/auxin.

If a functional auxin response is required for ethylene-mediated inhibition of root growth, then downstream components in the ethylene pathway may be found that function at intersection with the auxin biosynthesis, transport, or signaling pathways. In support of this idea, we previously identified two genetically downstream, tissue-specific ethylene-response mutants with alterations in auxin-mediated processes (4, 7, 8). The ethylene-insensitive root mutant *eir1* (8), an auxin efflux carrier (29–32), and the apical hookless mutant *hls1* (4, 7), a putative acetyltransferase (7), have been found to affect auxin transport in roots and auxin response gene expression in apical hooks, respectively. The two mutants described here, *wei2* and *wei3*, also show *wei* phenotypes that are restricted almost exclusively to the root tissues. As mentioned earlier, selective ethylene insensitivity of roots is often a characteristic of the mutants also affected in the response to auxin. *wei2* and *wei3*, however, show normal sensitivity to exogenously applied auxin, as well as a normal gravitropic response. The fact that these two downstream mutants are specifically affected in their response to ethylene may indicate that they function at steps connecting the general ethylene-response pathway, represented by the cascade from ETR1 to EIN3 (27), to the process of auxin-mediated growth. We anticipate that cloning of the genes affected in *wei2* and *wei3* may provide further understanding of how ethylene and auxin coordinate their biosynthesis, transport, and signaling pathways to precisely regulate uniform and differential cell-elongation processes.

We thank A. Hamilton and members of the Ecker Laboratory for critical reading of the manuscript and A. Pereira for the En-I transposon lines. J.M.A. was supported by a Spanish Ministerio de Educacion y Ciencia postdoctoral fellowship. A.N.S. was a recipient of Department of Energy Predoctoral Fellowship ER20162-A0003. R.S. was a recipient of Human Frontier Science Program Organization and Spanish Ministerio de Educacion y Ciencia postdoctoral fellowships. S.F. was a recipient of an Institute Pasteur–Fondazione Cenci Bolognetti fellowship. This work was supported by The Giovanni Armenise–Harvard Foundation, National Institutes of Health Grant GM48707 (to F.M.A.), and National Science Foundation Grant MCB-0213154, and Department of Energy Grant DE-FG03-00 ER15113 (to J.R.E.).

1. Abeles, F., Morgan, P. & Saltveit, M. (1992) *Ethylene in Plant Biology* (Academic, San Diego).
2. Johnson, P. R. & Ecker, J. R. (1998) *Annu. Rev. Genet.* **32,** 227–254.
3. Bleecker, A., Estelle, M., Somerville, C. & Kende, H. (1988) *Science* **241,** 1086–1089.
4. Guzman, P. & Ecker, J. (1990) *Plant Cell* **2,** 513–523.
5. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. (1993) *Cell* **72,** 427–441.
6. Ecker, J. R. (1995) *Science* **268,** 667–675.
7. Lehman, A., Black, R. & Ecker, J. (1996) *Cell* **85,** 183–194.
8. Roman, G., Lubarsky, B., Kieber, J., Rothenberg, M. & Ecker, J. (1995) *Genetics* **139,** 1393–1409.
9. Hua, J., Sakai, H., Nourizadeh, S., Chen, Q. H. G., Bleecker, A. B., Ecker, J. R. & Meyerowitz, E. M. (1998) *Plant Cell* **10,** 1321–1332.
10. Sakai, H., Hua, J., Chen, Q. G., Chang, C., Medrano, L. J., Bleecker, A. B. & Meyerowitz, E. M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5812–5817.
11. Estelle, M. & Klee, H. J. (1994) in *Arabidopsis*, eds. Meyerovitz, E. M. & Somerville, C. R. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 555–578.
12. Chang, C., Kwok, S. F., Bleecker, A. B. & Meyerowitz, E. M. (1993) *Science* **262,** 539–544.
13. Hua, J., Chang, C., Sun, Q. & Meyerowitz, E. M. (1995) *Science* **269,** 1712–1714.
14. Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. & Ecker, J. R. (1999) *Science* **284,** 2148–2152.
15. Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. & Ecker, J. (1997) *Cell* **89,** 1133–1144.
16. Solano, R., Stepanova, A., Chao, Q. & Ecker, J. R. (1998) *Genes Dev.* **12,** 3703–3714.
17. Bell, C. & Ecker, J. (1994) *Genomics* **19,** 137–144.
18. Doyle, J. J. & Doyle, J. L. (1987) *Phytochem. Bull.* **19,** 11–15.
19. Wisman, E., Hartmann, U., Sagasser, M., Baumann, E., Palme, K., Hahlbrock, K., Saedler, H. & Weisshaar, B. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 12432–12437.
20. *Arabidopsis* Genome Initiative (2000) *Nature* **408,** 796–815.
21. Kosugi, S. & Ohashi, Y. (2000) *Nucleic Acids Res.* **28,** 960–967.
22. Thomma, B. P., Eggermont, K., Tierens, K. F. & Broekaert, W. F. (1999) *Plant Physiol.* **121,** 1093–1102.
23. Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J. & Estelle, M. (1998) *Genes Dev.* **12,** 198–207.
24. Chang, C. & Shockey, J. A. (1999) *Curr. Opin. Plant Biol.* **2,** 352–358.
25. Rodriguez, F. I., Esch, J. J., Hall, A. E., Binder, B. M., Schaller, G. E. & Bleecker, A. B. (1999) *Science* **283,** 996–998.
26. Schaller, G. & Bleecker, A. (1995) *Science* **270,** 1809–1811.
27. Wang, K. L. C., Li, H. & Ecker, J. R. (2002) *Plant Cell* **14,** S131–S151.
28. del Pozo, J. C. & Estelle, M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 15342–15347.
29. Luschnig, C., Gaxiola, R. A., Grisafi, P. & Fink, G. R. (1998) *Genes Dev.* **12,** 2175–2187.
30. Utsuno, K., Shikanai, T., Yamada, P. & Hashimoto, T. (1998) *Plant Cell Physiol.* **39,** 1111–1118.
31. Müller, A., Guan, C., Gälweiler, L., Tänzler, P., Huijser, P., Marchant., A., Parry G., Bennett, M., Wisman, E. & Palme K. (1998) *EMBO J.* **17,** 6903–6911.
32. Chen, R., Hilson, P., Sedbrook, J., Rosen, E., Caspar, T. & Masson, P. H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15112–15117.