EIN2, a Bifunctional Transducer of Ethylene and Stress Responses in *Arabidopsis*

Jose M. Alonso,* Takashi Hirayama,* Gregg Roman,* Saeid Nourizadeh, Joseph R. Ecker†

Ethylene regulates plant growth, development, and responsiveness to a variety of stresses. Cloning of the *Arabidopsis EIN2* gene identifies a central component of the ethylene signaling pathway. The amino-terminal integral membrane domain of EIN2 shows similarity to the disease-related Nramp family of metal-ion transporters. Expression of the EIN2 CEND is sufficient to constitutively activate ethylene responses and restores responsiveness to jasmonic acid and paraquat-induced oxygen radicals to mutant plants. EIN2 is thus recognized as a molecular link between previously distinct hormone response pathways. Plants may use a combinatorial mechanism for assessing various stresses by enlisting a common set of signaling molecules.

The plant hormone ethylene (C_2H_4) is a regulator of a variety of developmental and stress responses in plants including seed germination, cell elongation, cell fate, sex determination, fruit ripening, flower senescence, leaf abscission, defense against pathogens, and responses to mechanical trauma (*1*). The effect of ethylene on dark-grown seedlings (known as the triple response) has been studied extensively to investigate the role of ethylene in cell growth and stress responses (*2–4*). In *Arabidopsis*, the triple response consists of radial swelling of the hypocotyl, exaggeration of the apical hook, and inhibition of hypocotyl and root elongation (*2*, *4*). Mutant loci with an ethylene-insensitive phenotype include *etr1* (*2*), *etr2* (*5*), *ein2*, *ein3* (*3*, *6*), *ein5/ain1* (*4*, *7*), *ein4*, *ein6*, and *eir1* (*4*). Mutants that overproduce ethylene (*eto1*, *eto2*, and *eto3*), constitutively activate the ethylene signaling pathway (*ctr1*) (*8*), or fail to form an exaggerated apical hook (*hls1*) have also been identified (*3*). A genetic model has been proposed where many of these genes act in a linear pathway in which *EIN2* is a central component (*4*, *9*).

Cloning and characterization of genes in the ethylene pathway have provided insight into the molecular mechanisms that underlie the plant's response. Ethylene is perceived by a family of receptors (ETR1, ERS, ETR2, EIN4, and ERS2) that are similar to bacterial two-component histidine kinases (*10*). The receptors activate a Raf-like protein kinase, CTR1, which negatively regulates downstream ethylene signaling events (*8*). In animals, Raf kinases typically act through a mitogen-activated protein (MAP) kinase phosphorylation cascade (*11*). Thus, the ethylene signal transduction pathway has features of both the bacterial two-component system and eukaryotic MAP kinase pathways. A similar combination of signaling components is found in the osmolarity response system in yeast (*12*). Downstream components in the ethylene pathway include the EIN3/EIL (EIN3-LIKE) family of transcription factors (*13*). Genetic data indicate that EIN2 mediates an essential step in the signal propagation between the CTR1 and EIN3/EIL (*4*, *6*).

Mutations in the *EIN2* locus confer insensitivity to both exogenous and endogenous ethylene (Fig. 1) (*4*, *14*). Previously, five *ein2* mutant alleles had been identified (*3*, *4*), and by further mutant screens, we extended the number of alleles to 25. With the exception of *ein2-9*, all *ein2* alleles showed complete insensitivity to ethylene at the morphological, physiological, and molecular levels (Fig. 1). *ein2* mutants have also been recovered in screens for *Arabidopsis* mutants resistant to auxin transport inhibitors (*15*), cytokinins (*16*), or abscisic acid (*17*) and in screens for delayed senescence (*18*). It is intriguing, however, that none of the other ethylene-insensitive loci were recovered in these screens. In this regard, the unique characteristics of *EIN2* may be explained by the fact that *EIN2* is the only gene known whose loss-of-function mutations lead to complete ethylene insensitivity (*4*, *14*). Alternatively, it is possible that EIN2 mediates cross-talk between several hormone signaling pathways.

Cloning *EIN2*

We cloned the *EIN2* gene using a map-based approach (Fig. 2). The *ein2-1* allele was mapped to the top of chromosome 5, between the ATCTR1 and ubq6121 loci (*4*) (Fig. 2A). A yeast artificial chromosome (YAC) contig was assembled by hybridization of the markers to several libraries (Fig. 2A). Restriction fragment length polymorphisms were identified for several of the YACs and cosmids in the region (*19*) and used to position *EIN2* between yUP2G11L and ubq6121 (Fig. 2A).

Genomic clones from the *EIN2* region

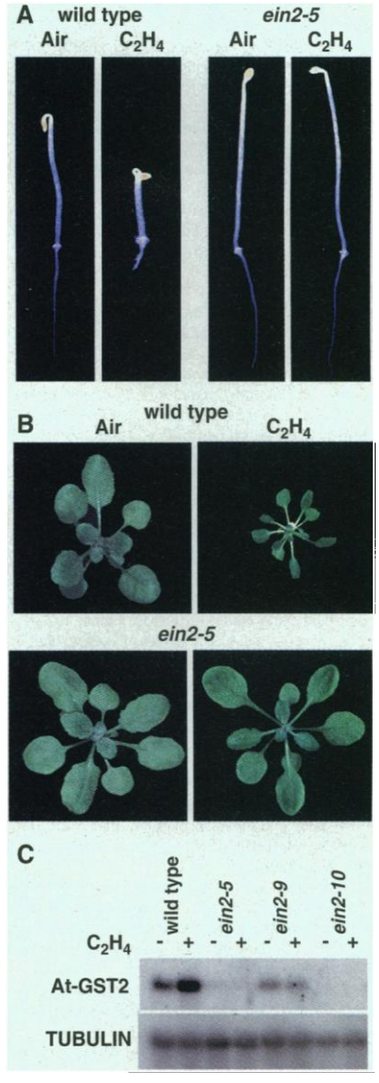

Fig. 1. Ethylene insensitivity of *ein2* mutant plants. (**A**) Comparison of 3-day-old etiolated wild-type and *ein2-5* seedlings grown in hydrocarbon-free air or 10 parts per million of ethylene. (**B**) Comparison of wild-type and *ein2-5* adult plants grown in hydrocarbon-free air or in the continuous presence of 1 ppm of ethylene. (**C**) Comparison of the ethylene-mediated induction of *At-GST2* in wild-type and *ein2-5* 3-day-old etiolated seedlings. Northern blots were performed with 20 μg of total RNA per lane and sequentially probed. Plant growth and ethylene treatment were carried out as described (*8*).

Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104–6018, USA.

*These authors contributed equally to this work and are listed alphabetically.

†To whom correspondence should be addressed. E-mail: jecker@atgenome.bio.upenn.edu

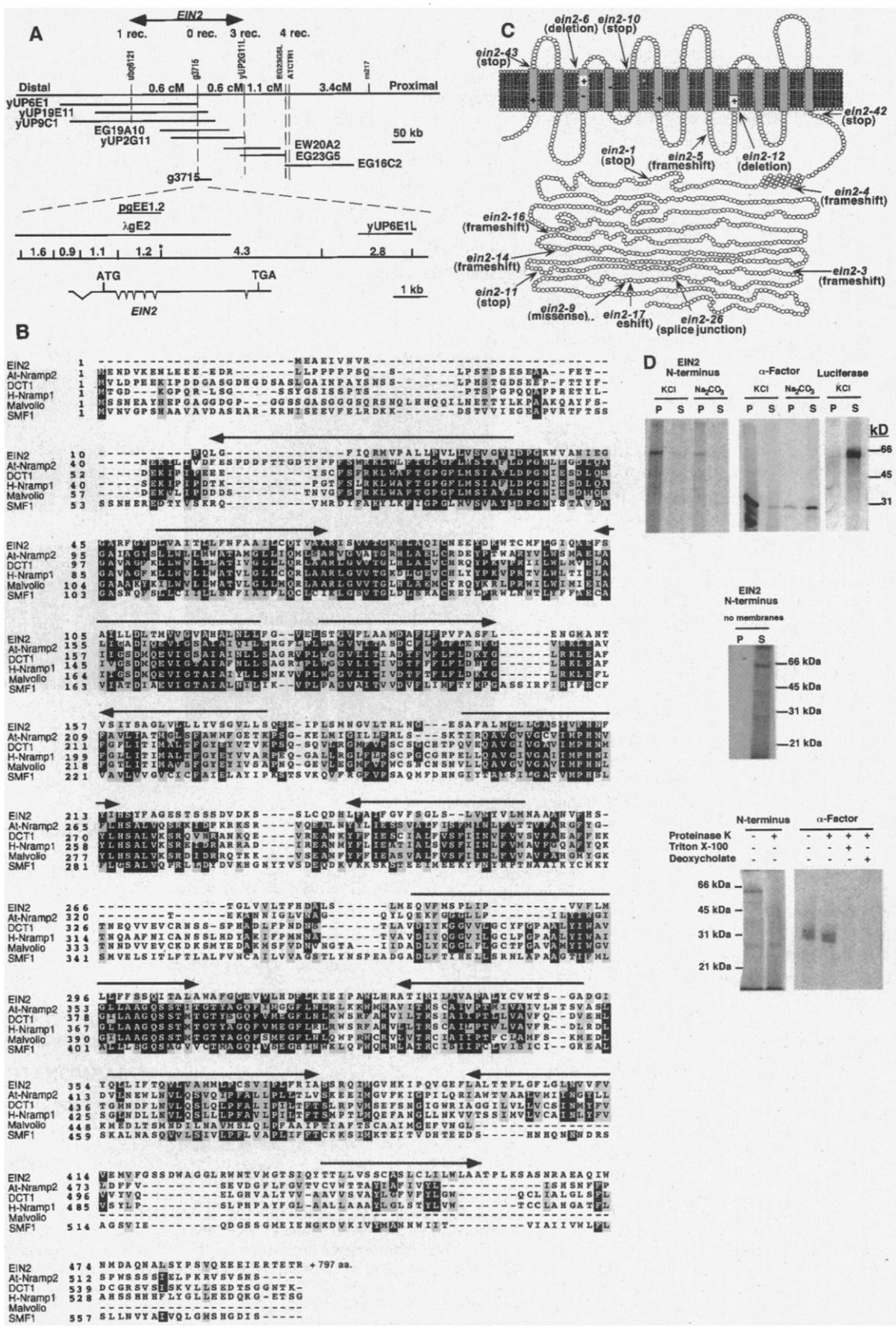

Fig. 2. Cloning, sequence, and membrane localization of *EIN2*. (**A**) Map of the *EIN2* gene relative to the chromosome walk. Molecular markers in the region and the number of recombinations between the marker and the *EIN2* locus are shown. The cosmid g3715 identified a polymorphism between the genomes of *ein2-12* and wild type. PgEE1.2 represents the Eco RI g3715 subclone that was absent in the *ein2-12* mutant genomic DNA. A genomic clone (λgE2) containing the *EIN2* gene was identified with this subclone as a probe. The *EIN2* gene (AF141202) is schematically represented; the seven introns are indicated by gaps. (**B**) Sequence alignments of the first 497 amino acids of EIN2 and full-length At-Nramp2 (AF141204), DCT1/mouse Nramp2 (AF008439), human Nramp1 (D50403), Malvolio (U23948), and yeast SMF1 (Z74864) proteins (*41*). The 12 putative transmembrane domains are highlighted by a line above the sequences, which shows the orientation relative to the membrane (arrows point inward). Identities and similarities among the different proteins are indicated by black and gray boxes, respectively. (**C**) Schematic representation of the predicted structure of EIN2 and identified changes in *ein2* mutant alleles. The predicted transmembrane helices are shown as cylinders through the lipid bilayer. Charged residues within the transmembrane regions are indicated (+ or −). The position of the putative coiled-coil is indicated by the coil after the 12th transmembrane domain. The positions of mutations in *ein2* alleles are indicated. (**D**) *EIN2* is an integral membrane protein. (Top and middle panels) A transcript encoding the NH_2-terminal 560 amino acids of EIN2 was translated in vitro in the presence or absence of canine pancreatic microsomes. Yeast α-factor and luciferase were used as controls. Translated proteins were extracted with 1 M potassium chloride or sodium carbonate (pH 11.5). Proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). (Bottom panel) In vitro–translated EIN2 NH_2-terminus and glycosylated α-factor were treated with Proteinase K (0.1 mg/ml) in the presence or absence of 0.5% Triton X-100 or deoxycholate (0.5 mg/ml) and then resolved by SDS-PAGE. P, pellet; S, supernatant.

were hybridized to *ein2* allele blots, and polymorphisms were identified in *ein2-6* and *ein2-12* (*19*). Using a polymorphic 1.2-kb Eco RI fragment as a probe, we identified genomic and cDNA clones (*20*). The pcE2.17 cDNA was 4747 bases in length with a single, large open reading frame encoding a predicted protein of 1294 amino acids (GenBank accession number AF141203) that spanned the polymorphic 1.2-kb Eco RI fragment (Fig. 2, A and B). The pcE2.17 cDNA detected a single 4.8-kb transcript, indicating that the cDNA was near full-length. Low-stringency hybridization of genomic DNA with the pcE2.17 cDNA indicated that *EIN2* is a single-copy gene in *Arabidopsis*. Mutations in *EIN2* cause phenotypes in roots, leaves, inflorescence stems, and flowers. Northern (RNA) analysis revealed that these tissues expressed the pcE2.17 transcript and that the steady-state mRNA level was unaffected by treatment with ethylene. Examination of the mRNA expression in *ein2* alleles revealed a reduction in the levels of this transcript, suggesting that pcE2.17 encodes the *EIN2* mRNA. Confirmation of the identity of *EIN2* was provided by examination of the sequences of *ein2* alleles (*21*) and by genetic complementation (*22*). Twelve of the sequenced *ein2* alleles predicted a premature termination of the protein (Fig. 2). In addition, the mutations in *ein2-6* and *ein2-12* result in in-frame deletions of 12 and 24 base pairs, respectively, although these mutations confer complete ethylene insensitivity. The least severe allele, *ein2-9*, contained a substitution of His-1143 to Pro (Fig. 2). For complementation testing, *ein2-5*:pcE2.17 plants were generated by in planta vacuum transformation (*22*). The ethylene insensitivity of *ein2-5* was abrogated by the ectopic expression of the *EIN2* cDNA; ethylene responsiveness was at least partially restored in 60 out of 100 independent transformed lines. We therefore conclude that pcE2.17 corresponds to the *EIN2* cDNA.

The Predicted Protein

Comparison of the *EIN2* cDNA (AF141203) and genomic (AF141202) sequences revealed that the coding region of *EIN2* is interrupted by seven introns (Fig. 2A). *EIN2* encodes a polypeptide of 1294 amino acids with a molecular mass of 141 kD and dimorphic structure. The NH_2-terminal 461 amino acids of EIN2 contain regions of extreme hydrophobicity, whereas the COOH-terminal 833 amino acids are predominately hydrophilic. The protein sequence predicts 12 transmembrane helices, all within the NH_2-terminal domain (Fig. 2, B and C). The NH_2 and COOH ends of the protein are predicted to lie on the same side of the membrane. The COILS algorithm (*23*) indicated an amphipathic helix in the *EIN2* COOH tail (Ser-485 to Leu-515) adjacent to the 12th transmembrane domain (Fig. 2C). The presence of a coiled-coil–forming helix suggests that this region of the COOH tail may be a site for protein-protein interactions.

EIN2 does not contain obvious consensus sequences for protein sorting, although its hydrophobic nature suggests that it is an integral membrane protein. We tested this possibility by in vitro translating EIN2 with canine pancreatic microsomes. Proteins translated in the presence of microsomes were treated under conditions that extract nonintegral membrane proteins, and then membrane fractions were sedimented by ultracentrifugation. An EIN2 protein fragment containing the first 560 amino acids was found to partition with the membrane fraction, whereas luciferase, a nonmembrane-associated protein, did not (Fig. 2D, top panel). Yeast α-factor was translocated into the lumen of intact microsomes (*24*) (Fig. 2D). After treatment of membranes under conditions that release the microsomal lumen contents, the EIN2 NH_2-terminus, but not the yeast α-factor, was found in the microsomal membrane

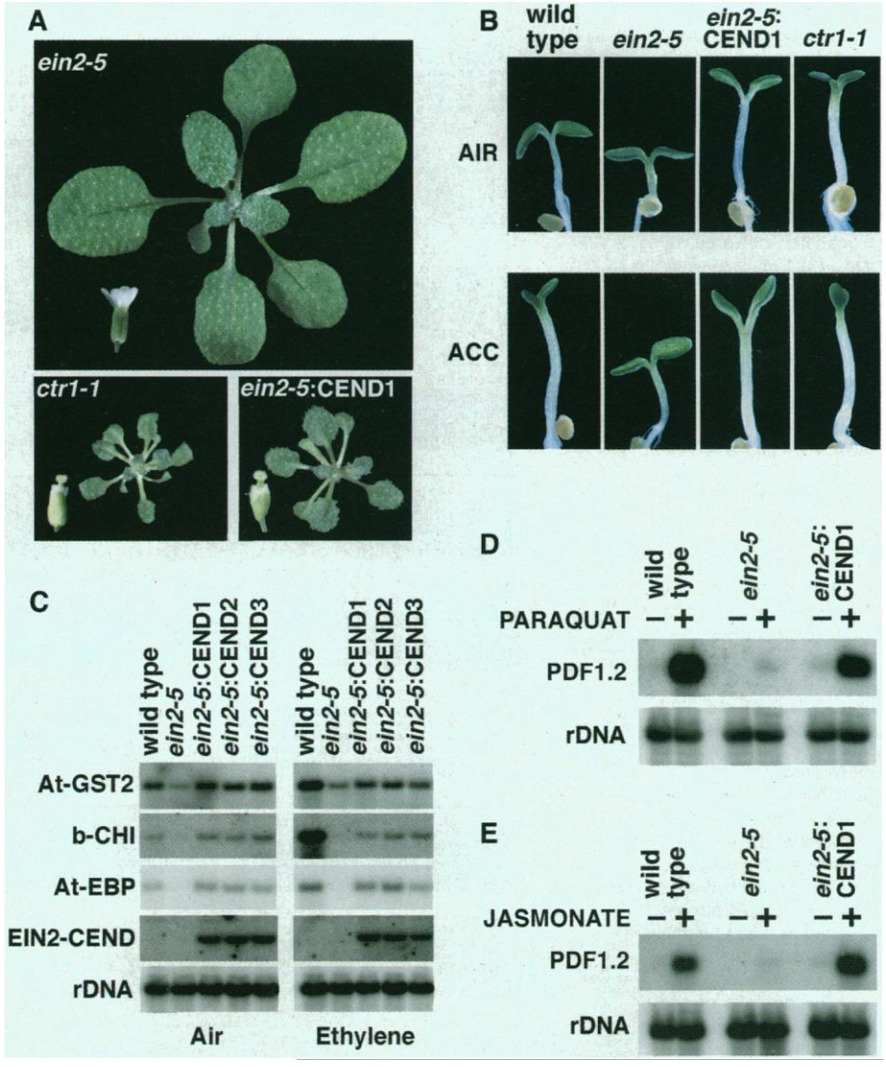

Fig. 3. Activation of ethylene responses by overexpression of the COOH end (CEND) of EIN2. (**A**) Comparison of *ein2-5*, *ctr1*, and *ein2-5*:CEND adult plants and flowers. Flowers of the same age from the first inflorescence are shown for the different backgrounds. (**B**) Comparison of 5-day-old seedlings germinated in constant light on water-agar plates (no salts or sugars added) containing no ACC (upper panels) or 50 μM 1-aminocyclopropane-1-carboxylic acid (lower panels). (**C**) Expression of ethylene-inducible genes in wild type, *ein2-5*, and three independent *ein2-5*:CEND transgenic lines. Northern blots were performed with 20 μg of total RNA per lane from adult plants treated for 48 hours with hydrocarbon-free air or 10 ppm of ethylene, and sequentially probed. Induction of *defensin* (*PDF1.2*) mRNA by paraquat (**D**) or methyl jasmonate (MeJA) (**E**), in wild-type, *ein2-5*, and *ein2-5*:CEND transgenic plants. Leaf samples were collected 48 hours after spraying with paraquat (25 μM) or MeJA [45 μM in 0.1% (v/v) ethanol]. Northern blots were performed with 20 μg of total RNA per lane and sequentially probed.

fraction. Thus, the hydrophobic portion of EIN2 protein behaves as an integral membrane protein. Consistent with the lack of a signal sequence or any consensus glycosylation sequence within the first 560 amino acids, the size of the EIN2 protein was not altered by the presence of membranes (Fig. 2D, top and middle panels). The topology of EIN2 within the microsomes was examined by protease digestion (Fig. 2D, bottom panel). The membrane-associated NH_2-terminus of EIN2 was not protected from proteinase digestion by the lipid bilayer. However, the lumen-localized glycosylated α-factor remained intact until the bilayer was disrupted (Fig. 2D, bottom panel). These results are consistent with a topology prediction where the NH_2-terminal portion of the EIN2 protein is integrated within the membrane. Attempts to determine the cellular location of endogenous EIN2 protein with either EIN2-specific antibodies, epitope-tagged EIN2 proteins, or EIN2::GFP (green fluorescent protein) fusion proteins were unsuccessful.

Database searches revealed that EIN2 contains sequence similarity (21% identity) to the Nramp family of proteins (Fig. 2B); the region of similarity between EIN2 and the Nramp proteins is restricted to the NH_2-terminal hydrophobic domain (EIN2 residues 1 to 480). Nramp-related proteins have been found in all organisms from bacteria to humans (*25*), and several of these function as transporters of a variety of divalent cations (*26*). Mutations in members of this family cause a variety of phenotypes, including hypersensitivity to bacterial infection (mouse Nramp1), anemia [mouse and rat Nramp2 (*25*)], and alteration of taste behavior [*Drosophila* Malvolio (*27*)]. In addition, we identified an *Arabidopsis* gene more closely related to mouse *Nramp1* than *EIN2* (Fig. 2B). Unlike mutations in *EIN2*, plants containing a transferred DNA (T-DNA) insertion mutation in *At-Nramp2* (*28*) showed normal responsiveness to ethylene.

Physiological studies indicated that ethylene perception may require a transition metal such as copper or zinc (*29*). Indeed, copper is needed for the reversible binding of ethylene to a protein receptor (*30*). Although a copper transporter necessary for ethylene receptor function has been recently identified (*31*), its similarity to Nramp proteins suggested that EIN2 may also function as a metal transporter. To test this hypothesis, we examined transport activity by expressing EIN2 (or truncated forms) in several heterologous systems, including *Xenopus* oocytes, yeast [*mif* mutant (*32*)], and baculovirus-infected insect cells. Although EIN2 expression was detected, no metal-transporting capacity was observed.

Ethylene Response Signaling

Overexpression of downstream positive regulators in the ethylene pathway, such as EIN3/EIL1 (*6*) or ERF1 (*13*), leads to constitutive ethylene response phenotypes. In contrast, none of the plants that expressed the full-length EIN2 protein or NH_2-terminal hydrophobic Nramp-related domain (amino acids 1 to 480) showed constitutive ethylene responses or hypersensitivity to ethylene. Transgenic plants that expressed the COOH end of EIN2 (CEND) in the *ein2-5* genetic background, however, showed constitutive ethylene response phenotypes as adults (Fig. 3A). *ein2-5*:CEND plants produced small rosettes, and their flowers showed morphological characteristics similar to those of the constitutive ethylene response mutant *ctr1* (Fig. 3A), as the gynoecium protruded through the petals before the bud opened and only the later flowers were fertile and produced viable seeds (*8*). Under long-day conditions, *ein2-5*:CEND transgenic plants flowered at the same time as wild type—1 week earlier than the parental *ein2-5* plants.

We further examined the basis of the phenotypes observed in *ein2-5*:CEND plants using another bioassay for ethylene response. When seeds are germinated and grown in the light in medium devoid of nutrients, ethylene can promote elongation in the hypocotyl (*33*). Under these growth conditions, the hypocotyl of ethylene-insensitive mutant seedlings was shorter than that of ethylene-treated wild-type or *ctr1* mutants (*33*) (Fig. 3B). The hypocotyl of *ein2-5*:CEND seedlings was longer than that of *ein2-5* seedlings and similar to that of *ctr1* seedlings (Fig. 3B). These results suggest that the phenotypes observed in *ein2-5*:CEND plants are mediated by constitutive activation of the ethylene response. Northern analysis revealed that *ein2-5*:CEND plants constitutively expressed mRNAs for all ethylene-regulated genes tested (*At-GST2*, *basic-chitinase*, and *At-EBP*), confirming the role of this domain of EIN2 in ethylene signaling at the molecular level. Moreover, gene expression was unaffected by ethylene treatment (Fig. 3C), indicating that the NH_2-terminus of EIN2 is necessary for regulation by the hormone. The fact that overexpression of CEND, but not full-length EIN2, confers constitutive ethylene responses also implies that the Nramp-like domain may control CEND function.

Components of both the ethylene and jasmonate signaling pathways are required for induction of the pathogen-responsive gene *PDF1.2* (*34*). Mutations that do not completely block the response to ethylene (such

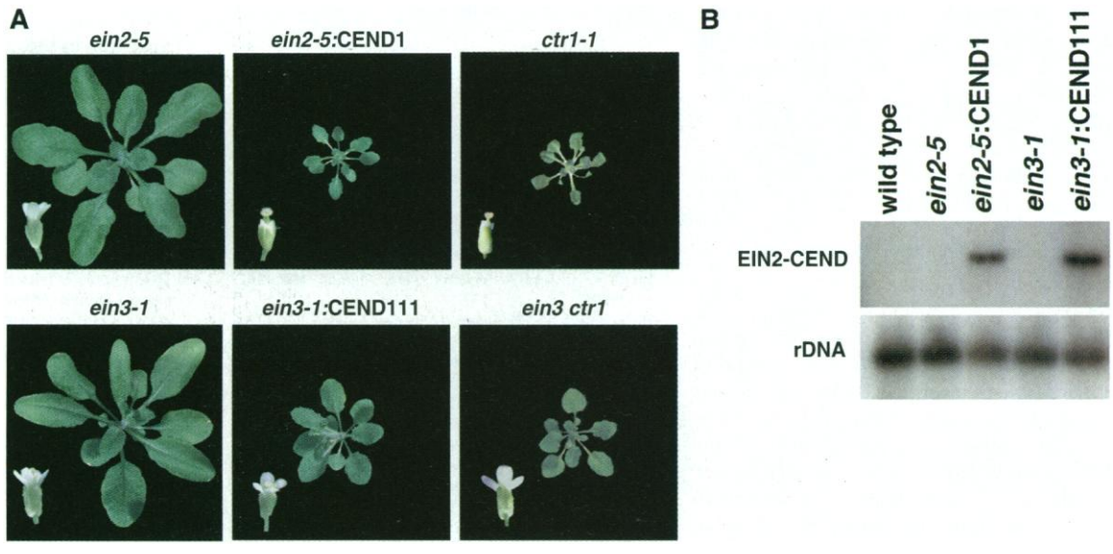

Fig. 4. Requirement of EIN3 for EIN2 CEND constitutive ethylene response phenotypes. **(A)** Phenotypes of 4-week-old plants and flowers of the indicated genotypes. **(B)** Comparison of the levels of mRNA expression of the *EIN2-CEND* transgene in the lines shown in (A) and (B).

as *etr1-3* or *ein3*), however, respond normally to jasmonate or the superoxide anion-producing compound paraquat (*34*). We tested whether the signal generated by the CEND of EIN2 was sufficient to alleviate the ethylene signaling requirement for the induction of PDF1.2 by jasmonate or paraquat. Whereas *ein2-5* plants were completely unresponsive, *ein2* plants overexpressing the EIN2 CEND were able to respond normally to treatment with paraquat (Fig. 3D) or jasmonate (Fig. 3E) and induce PDF1.2 gene expression. Thus, the signal generated by the CEND of EIN2 restores jasmonate and paraquat responsiveness in an ethylene-insensitive plant.

Although genetic studies demonstrate a requirement for an intact CEND sequence for ethylene-mediated activation of the triple response in etiolated seedlings, expression of EIN2 CEND domain in *ein2-5* was not sufficient to induce the triple response; the seedlings remained completely insensitive to ethylene (*35*). Thus, the Nramp-like domain of EIN2 is necessary for ethylene-mediated effects on skotomorphogenesis, seedling development in the absence of light. Overexpression of CEND may be unable to evoke the triple response in etiolated seedlings because of an insufficient threshold amount of active CEND or of a CEND-interacting protein in etiolated seedlings.

Order of Gene Action

Because EIN3 has been proposed to act after EIN2 in the ethylene pathway (*6*), we analyzed the effects of EIN2 CEND overexpression in *ein3-1* plants. From 100 independent *ein3*:CEND lines examined, two lines that showed the most severe *ctr1*-like phenotypes were characterized. Comparison of the effects of EIN2 CEND expression in *ein2-5* and *ein3-1* plants indicates that although the levels of transgene expression were similar (Fig. 4B), reduction in the rosette size was less profound in *ein3-1* and comparable to that observed in the *ein3/ctr1* double mutant (Fig. 4A). In addition, the *ctr1*-like flower morphology was suppressed in the *ein3*:CEND plants (Fig. 4A). Thus, ectopic expression of the COOH end of EIN2 is sufficient to activate downstream events of the pathway in an EIN3-dependent manner.

Comparisons to Other Pathways

Identification and analysis of *EIN2*, a regulator of ethylene signaling in *Arabidopsis*, has uncovered an unexpected feature in the ethylene response pathway. Whereas several of the previously identified components in this pathway have equivalents in other eukaryotic signaling cascades (that is, ETR1/SLN1 and CTR1/Raf-1), EIN2 has no such counterpart. At the amino acid sequence level, EIN2 seems to be structurally unique. Nevertheless, proteins with "functional equivalence" to EIN2 may be found in other regulatory cascades. Indeed, there appears to be a parallel between EIN2 and the yeast glucose sensors Snf3 and Rgt2 (*36*, *37*). Like EIN2, these proteins contain two distinct domains. Their hydrophobic NH_2-termini show similarity to glucose transporters but they are thought to act as glucose sensors, not transporters (*36*, *37*). They also possess hydrophilic COOH-termini that when overexpressed can partially activate the glucose response in the mutant yeast cells (*37*). Presumably like Snf3, EIN2 CEND may interact with downstream components in the pathway, while its hydrophobic NH_2-terminus may function in sensing an unknown signal. The requirement of the NH_2-terminal domain for ethylene responsiveness in etiolated seedlings and the sufficiency of its CEND for activation of the pathway imply that EIN2 acts as a bifunctional signal transducer. The similarity of the EIN2 NH_2-terminus to the Nramp proteins suggests that this domain may be involved in "sensing" a divalent cation, inferring the existence of a second messenger in the central part of the ethylene signal transduction pathway. Alternatively, this domain may simply anchor the protein to the membrane, bringing the CEND domain to the site of interaction with other ethylene pathway components, such as ETR1 and CTR1. The presence of a coiled-coil motif in the EIN2 CEND suggests that information transfer may involve interaction between proteins.

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22. Plant transformation was performed as described (*6*). Regions of the *EIN2* cDNA (pcE2.17) were subcloned into pRok2 [D. Baulcombe, G. Saunders, M. Bevan, M. Mayo, B. Harrison, *Nature* **321**, 446 (1986)]. Fragments of *EIN2* cDNA that were expressed under the control of the CaMV 35S promoter include the full-length cDNA (amino acids 1 to 1294), terminal deletions of the COOH end (amino acids 1 to 933, 1 to 854, and 1 to 476), an internal deletion (amino acids 89 to 228 removed), and the COOH end (CEND; amino acids 454 to 1294). From 100 *ein2*:CEND transgenic plants produced, 7 lines that expressed *EIN2* at a high level showed constitutive ethylene response phenotypes as adults.
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41. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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