Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1

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Response to the gaseous plant hormone ethylene in *Arabidopsis* requires the EIN3/EIL family of nuclear proteins. The biochemical function(s) of EIN3/EIL proteins, however, has remained unknown. In this study, we show that EIN3 and EILs comprise a family of novel sequence-specific DNA-binding proteins that regulate gene expression by binding directly to a primary ethylene response element (PERE) related to the tomato E4-element. Moreover, we identified an immediate target of EIN3, *ETHYLENE-RESPONSE-FACTOR1* (*ERF1*), which contains this element in its promoter. EIN3 is necessary and sufficient for *ERF1* expression, and, like *EIN3*-overexpression in transgenic plants, constitutive expression of *ERF1* results in the activation of a variety of ethylene response genes and phenotypes. Evidence is also provided that ERF1 acts downstream of EIN3 and all other components of the ethylene signaling pathway. The results demonstrate that the nuclear proteins EIN3 and ERF1 act sequentially in a cascade of transcriptional regulation initiated by ethylene gas.

[*Key Words*: Plants; *Arabidopsis*; EIN3; *ERF1*; transcription]

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The plant hormone ethylene regulates a variety of stress responses and developmental adaptations in plants. This gaseous molecule is well known for its participation in physiological processes as diverse as fruit ripening, senescence, abscission, germination, cell elongation, sex determination, pathogen defense response, wounding, nodulation, and determination of cell fate (Abeles et al. 1992; Tanimoto et al. 1995; O'Donnell et al. 1996; Penninckx et al. 1996; Penmetsa and Cook 1997). Control of these processes by ethylene involves complex regulation of both ethylene biosynthesis and the ability of cells to perceive and respond to the hormone in an appropriate manner. Understanding the molecular events that lead to this diversity of plant responses is essential to elucidate how this gas modulates such functions.

The morphological changes evoked by continuous exposure of *Arabidopsis* seedlings to ethylene, the triple response, have allowed the identification of a number of components of the ethylene response pathway. Several classes of mutants impaired in their response to the hormone have been identified. Mutants that display a constitutive triple response phenotype may result either from ethylene overproduction (*eto1*, *eto2*, and *eto3*), or constitutive activation of the pathway (*ctr1*). Insensitive mutants are defective in their ability to perceive or respond to ethylene and include *etr1*, *etr2*, *ein2*, *ein3*, *ein4*, *ein5/ain1*, *ein6*, and *ein7* mutants (Ecker 1995; McGrath and Ecker 1998; Sakai et al. 1998; Solano and Ecker 1998). On the basis of epistasis analysis, a genetic framework for the action of these genes has been established (Roman et al. 1995; Sakai et al. 1998). *ETR1*, *ETR2*, and *EIN4* genes act upstream of *CTR1*, whereas *EIN2*, *EIN3*, *EIN5/AIN1*, *EIN6*, and *EIN7* genes act downstream of *CTR1*.

Several of the early-acting ethylene signaling pathway genes have been cloned and characterized. *ETR1*, *ETR2*, and *EIN4* encode members of a family of membrane proteins with significant similarity to two-component histidine kinase receptors found in bacteria and fungi (Chang et al. 1993; Hua et al. 1998; Sakai et al. 1998). Expression of ETR1 in yeast cells allows them to bind ethylene, consistent with its suggested role as an ethylene receptor (Schaller and Bleecker 1995; Chang 1996). The *ETR1* family also includes two members (*ERS1* and *ERS2*) that were cloned by sequence homology. When mutations that confer dominant ethylene insensitivity to *ETR1* are introduced into these two genes, the resulting transgenic plants are ethylene insensitive, suggesting that ERS1 and ERS2 may also be ethylene receptors (Hua et al. 1995,1998). The *CTR1* gene encodes a protein with similarity to the Raf-family of protein kinases, implicating a MAP-kinase cascade in the ethylene response pathway (Kieber et al. 1993). Coupling of bacterial-type re-

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ceptor and Raf-like protein kinases in the osmosensing pathway in yeast is provided by phosphorelay proteins (Posas et al. 1996). While several proteins with both structural and functional similarity to response regulators have been identified in *Arabidopsis* (Imamura et al. 1998), the ethylene receptors ETR1 and ERS1 can interact physically with CTR1 (Clark et al. 1998); thus bypassing an absolute requirement for such intermediates.

Less is understood about the downstream components of the ethylene signaling pathway. Cloning and characterization of the *EIN3* gene revealed that it encodes a nuclear-localized protein (Chao et al. 1997). Although sequence analysis failed to uncover homology to previously described proteins, EIN3 shares amino acid sequence similarity, conserved structural features, and genetic function with three EIN3-LIKE (EIL) proteins. Genetic studies revealed that *EIL1* and *EIL2* are able to functionally complement the *ein3* mutation, suggesting their participation in the ethylene signaling pathway. High-level expression of *EIN3* or *EIL1* in transgenic wild-type or *ein2* mutant plants conferred constitutive ethylene response phenotypes in all stages of development, indicating their sufficiency for activation of the pathway in the absence of ethylene. However, the function(s) of the EIN3/EIL family of proteins remains unknown. Further analysis of their biochemical activities has been hampered by the absence of candidate target genes.

Among the different classes of ethylene-responsive genes, the most extensively studied are those whose expression is activated by ethylene in response to pathogen attack. This class includes basic-chitinases, β-1,3-glucanases, defensins, and other pathogenesis-related (PR) proteins (Boller et al. 1983; Felix and Meins 1986; Broglie et al. 1989; Ohme-Takagi and Shinshi 1990; Samac et al. 1990; Eyal et al. 1993; Penninckx et al. 1996). Analysis of the promoters of several of these genes revealed a common *cis*-acting ethylene response element called the GCC box. This element was shown to be necessary and sufficient for ethylene regulation in a variety of plant species (Eyal et al. 1993; Hart et al. 1993; Meller et al. 1993; Ohme-Takagi and Shinshi 1995; Sessa et al. 1995; Shinshi et al. 1995; Sato et al. 1996). Efforts to isolate *trans*-acting factors in tobacco that bind the GCC box identified a family of proteins termed Ethylene-Responsive-Element-Binding-Proteins (EREBPs) (Ohme-Takagi and Shinshi 1995). These novel DNA-binding proteins interact in vitro with the GCC box through a domain homologous to that previously observed in the floral homeotic protein APETALA2 (Ecker 1995; Weigel 1995). In *Arabidopsis*, >30 genes belonging to this family have been identified by several groups (Wilson et al. 1996; Buttner and Singh 1997; Okamuro et al. 1997) and as a result of the *Arabidopsis* Genome Initiative (Bevan et al. 1997; Ecker 1998). On the basis of the available genomic sequence data, 125 members of this family have been estimated to exist in the *Arabidopsis* genome (Riechmann and Meyerowitz 1998). The expression of several members of this family has been reported to be regulated by ethephon, an ethylene-releasing compound (Ohme-Takagi and Shinshi 1995; Buttner and Singh 1997), raising the tantalizing possibility that one or more EREBPs may constitute an intermediate step(s) between EIN3/EILs and ethylene-inducible target genes such as *basic-chitinase*. However, evidence for the direct involvement of EREBPs in the ethylene signaling pathway in *Arabidopsis* is still lacking.

Here we report the cloning and characterization of *ETHYLENE-RESPONSE-FACTOR1* (*ERF1*), an early ethylene responsive gene that encodes a GCC-box-binding protein. *EIN3* expression is both necessary and sufficient for *ERF1* transcription and, like *EIN3* overexpression in transgenic plants, constitutive expression of *ERF1* results in activation of a variety of ethylene response genes and phenotypes. Moreover, we demonstrate that EIN3 and EILs are novel sequence-specific DNA-binding proteins that bind a primary ethylene response element in the promoter of *ERF1*. Consistent with the biochemical studies, genetic analysis revealed that *ERF1* acts downstream of *EIN3* and all previously identified components in the ethylene gas signaling pathway.

Results

Cloning and characterization of ERF1

To identify targets of the EIN3/EILs proteins and to examine the role of EREBPs in the ethylene signaling pathway, a PCR-based approach was used to isolate members of the EREBP family in *Arabidopsis*. By use of oligonucleotides complementary to the tobacco EREBP1 sequence (Ohme-Takagi and Shinshi 1995), a 597-bp fragment was amplified from tobacco genomic DNA, and this fragment was used to screen an *Arabidopsis* cDNA library under low stringency. Among the positive clones, two classes of cDNAs showed high homology to the tobacco *EREBP1/2* and *EREBP3/4* genes. One of these genes, *ERF1* (Fig. 1A) showed rapid induction in response to ethylene (Fig. 1B). More importantly, *ERF1* mRNA began to accumulate after 15 min of hormone treatment of plants. Induction of *ERF1* mRNA was also dependent on the presence of functional EIN3, as no expression was detected in *ein3-1* mutants (Fig. 1B). To compare the kinetics of *ERF1* induction with that of another known ethylene-inducible gene, the same blot was hybridized with *PDF1.2*, a member of the defensin gene family (Penninckx et al. 1996). Consistent with the hypothesis that ERF1 may be a regulator of these genes, maximal *ERF1* expression occurred earlier than that of *PDF1.2* (Fig. 1B). Consequently, we focused our studies on this gene as it fits the criteria for a candidate target of EIN3.

It has been demonstrated previously that overexpression of the ethylene pathway gene *EIN3* causes activation of all known ethylene response phenotypes (Chao et al. 1997). Although the level was somewhat lower than that achieved by exogenous ethylene treatment, *ERF1* mRNA showed constitutive high-level expression in *35S::EIN3*-expressing transgenic plants (Fig. 1C), indicating that EIN3 is sufficient for *ERF1* expression. Taken together, the results indicate that EIN3 is both necessary and sufficient for expression of the early ethylene response gene *ERF1*.

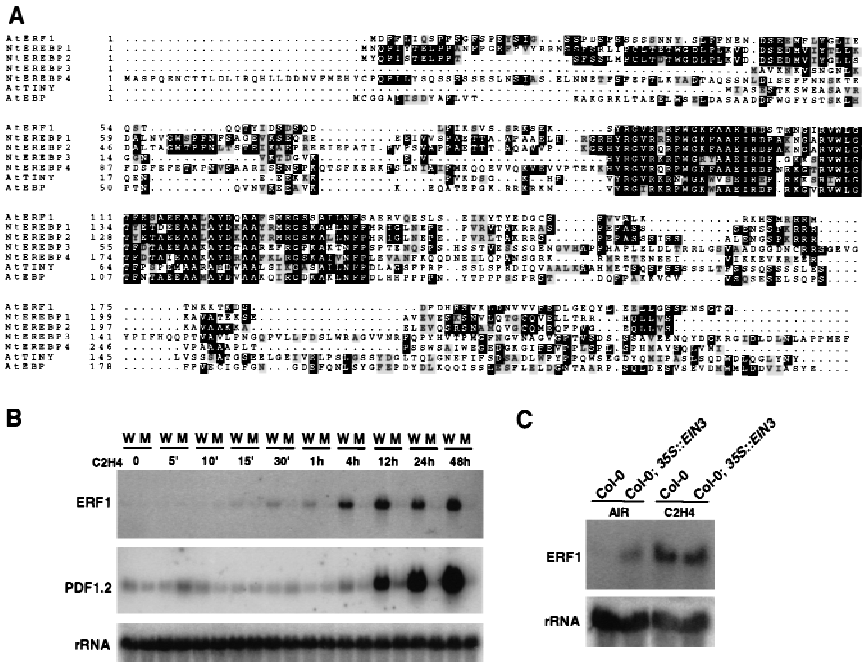

Figure 1. Cloning and ethylene inducibility of *ERF1*. (*A*) Sequence alignment of ERF1 and EREBP proteins from tobacco (EREBP1, EREBP2, EREBP3, and EREPB4; Ohme-Takagi and Shinshi 1995) and *Arabidopsis* [AtEBP (Buttner and Singh 1997) and AtTINY (Wilson et al. 1996)]. (*B*) RNA blot analysis of the induction of *ERF1* mRNA expression by ethylene gas and comparison with the expression of *PDF1.2*. Total RNA was isolated from 4-week-old wild-type Col-0 (W) or *ein3-1* (M) plants grown in air and exposed to ethylene gas for different times (0–48 hr). (*C*) RNA blot analysis of the expression of *ERF1* in *EIN3*-overexpressing plants. Thirty micrograms of total RNA were loaded per lane in *B* and 60 μg in *C*.

ERF1 was mapped to chromosome III, in the ABI3 contig, by PCR amplification of YAC pools with *ERF1*-specific primers. Other than *EIN3*, none of the known ethylene signaling mutants map to this region.

Sequence-specific binding of EIN3 in the promoter of ERF1

To test whether the nuclear protein EIN3 is capable of DNA binding, we performed electrophoretic mobility shift assays (EMSAs) with in vitro-translated EIN3 protein and the 5′ promoter region of the *ERF1* gene. A 6-kb fragment containing the *ERF1* promoter was isolated from genomic sequences (BAC F11F14) and subcloned into pBluescript. Five overlapping fragments that covered ~1.4-kb upstream of the *ERF1* translation initiation site were amplified and radioactively labeled by PCR. As shown in Figure 2A, a slower migrating band was observed when one of the fragments (–1238 to –950) was incubated with reticulocyte lysates containing EIN3. The binding of EIN3 to the –1238 to –950 fragment was not competed by a 500-fold excess of poly[d(IC)] or poly[d(AT)], demonstrating the specificity of the EIN3–DNA interaction. To delimit further the EIN3 target site, this fragment was subdivided, and each subfragment was subjected to binding experiments. Only one of the subfragments (–1213 to –1179) was recognized specifically by EIN3, confirming further its sequence specificity (Fig. 2B).

To demonstrate that EIN3 was in fact the protein present in the mobility-shifted band, a series of carboxy-terminal truncated EIN3 derivatives was generated and subjected to binding and EMSA with the 36-bp EIN3-binding fragment described above. A mobility shift that correlated with the molecular weight of each of the truncated proteins was observed, confirming the presence of EIN3 in the protein–DNA complex (Fig. 2B). The smallest protein that retained DNA-binding capacity was EIN3Δ269, delimiting the EIN3 DNA-binding domain to amino acids 1–359. In addition, a mutant version of EIN3 that contained the amino acid substitution encoded by the *ein3-3* allele (Lys^{245} to Asn), was generated by in vitro translation of the corresponding mRNA. The amino acid substitution in the *ein3-3* mutant lies within the basic domain III of the EIN3 protein (Chao et al. 1997). Interestingly, the mutant EIN3-3 protein was unable to recognize the 36-bp target site (Fig. 2B).

Further experiments demonstrated that the source of the EIN3 protein does not affect its ability to bind DNA. As with the in vitro-translated protein, baculovirus-expressed EIN3 was also able to recognize the 36-bp fragment containing the target sequence (data not shown).

To examine whether other members of the EIN3/EIL family are also able to bind DNA, we performed EMSA experiments with in vitro-translated EIL1, EIL2, and EIL3 proteins, and a DNA fragment containing the EIN3-binding site or a mutant version. Consistent with the ability of EIL1 and EIL2, but not EIL3, to complement the *ein3-1* mutation in transgenic plants (Chao et al. 1997), both EIL1 and EIL2, but not EIL3, were able to recognize specifically the EIN3-binding site (Fig. 2C).

To define more precisely the sequence requirements of EIN3 binding, scanning mutagenesis of the 36-bp fragment was performed. As shown in Figure 2D, all mutations affecting the affinity of EIN3 for its target site resided within a 28-bp sequence with a palindromic structure. Single mutations within the core of this (imperfect) palindrome completely abolished binding, and mutations that affected both distal ends of the palindromic repeats greatly reduced the interaction with EIN3. Inter-

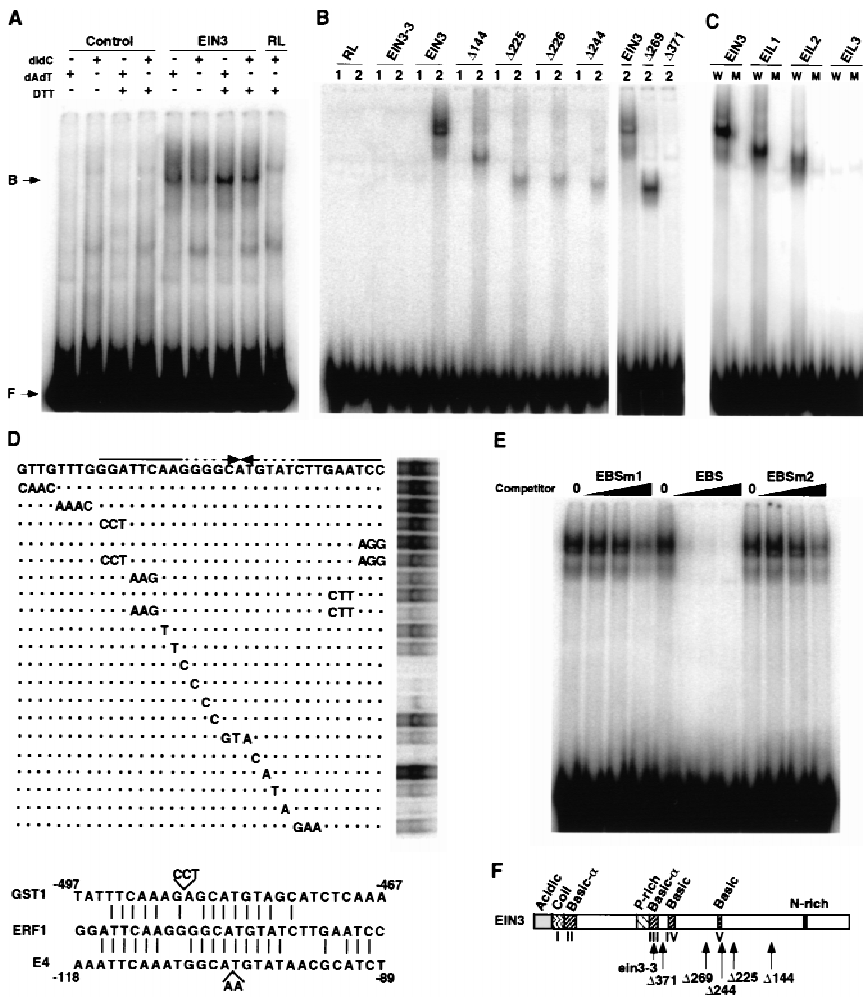

Figure 2. EIN3 is a sequence-specific DNA-binding protein. (*A*) EMSA of in vitro-translated EIN3 protein binding to the −1238 to −950 fragment of the *ERF1* promoter. A control protein (control) or mock-translated reticulocyte lysates (RL) were used in the indicated lanes. (*B*) EMSA of EIN3-3 mutant protein, EIN3, and several carboxy-terminal deletion derivatives bound to fragments −1238 to −1204 (1) and −1213 to −1178 (2) of the *ERF1* promoter. (*C*) Binding of EIL proteins to the EIN3 target site in the *ERF1* promoter. EMSA was performed with in vitro-translated EIN3, EIL1, EIL2, and EIL3 proteins and the wild-type EIN3-binding site (W) or a mutant version (M) corresponding to the mutant G17 to C in Fig. 2D. (*D*; *top*) Scan mutagenesis of the EBS. Wild-type EBS is shown with the palindromic repeats indicated by arrows. Base changes in the mutants tested are indicated on the lines below. Dashed lines indicate mismatches. Dots indicate similar bases as in the wild-type EBS. (*D; bottom*) Sequence alignment of the EBS and a fragment of the promoters of the *E4* and *GST1* genes (including the ERE). (*E*) Competition of the EIN3–EBS complex formation by addition of an excess of unlabeled EBS or two mutant versions, EBSm1 and EBSm2 (see Materials and Methods). No competitor was added in the lanes labeled as 0. Black wedges represent increasing amounts of competitor (20, 60, and 200 ng). One nanogram of labeled EBS was used per lane. (*F*) Summary of EIN3 structural features and mutants used in EMSA experiments (adapted from Chao et al. 1997).

estingly, the EIN3-binding site shows significant similarity to sequences present in the promoter regions required for ethylene responsiveness in the tomato *E4* (Montgomery et al. 1993) and *LEACO1* genes (Blume and Grierson 1997) and in the carnation *GST1* gene (Itzhaki et al. 1994; Fig. 2D, bottom). In *GST1*, a 197-bp promoter fragment containing this sequence was also sufficient to confer ethylene responsiveness to a minimal CaMV 35S promoter in transient assays (Itzhaki et al. 1994).

To examine further the specificity of the binding to its target site, competition experiments were performed with an excess of unlabeled EIN3-binding site (EBS), or two mutated versions (EBSm1 and EBSm2) not recognized by EIN3 (see Materials and Methods). As shown in Figure 2E, the formation of the EIN3–EBS complex was more efficiently competed by an excess of unlabeled EBS than by any of the EBS mutant versions, further supporting the sequence specificity of the EIN3–EBS interaction.

EIN3 recognizes its target as a homodimer

The presence of palindromic repeats in the EBS suggested that EIN3 interacts with its target as a dimer. To address this question, full-length EIN3 and several carboxy-terminal deletion derivatives were translated in vitro, alone or in pair-wise combinations. The resulting translation or cotranslation products were tested for DNA binding to the EBS. As shown in Figure 3A, in addition to the bands corresponding to the full-size EIN3 and deletion derivatives bound to DNA, a band of intermediate mobility appeared when the cotranslation products were used. The intermediate band corresponds to the mobility shift for a heterodimer, indicating that these proteins bind to the EBS as dimers.

Additional evidence that EIN3 has the capacity to form dimers came from screening for EIN3-interacting proteins by use of the yeast two-hybrid system (Fields and Song 1990; Durfee et al. 1993). Consistent with EIN3 being a transcriptional activator, fusion of the full-size protein with the GAL4 DNA-binding domain (BD) activated transcription of the *lacZ* reporter gene (not shown), indicating that EIN3 possesses activation domains that are functional in yeast. To avoid this activation of the reporter gene, an EIN3 derivative containing amino acids 53–257, fused to the GAL4-BD, was used as a bait. As a prey, the GAL4 activation domain was fused to an *Ara-*

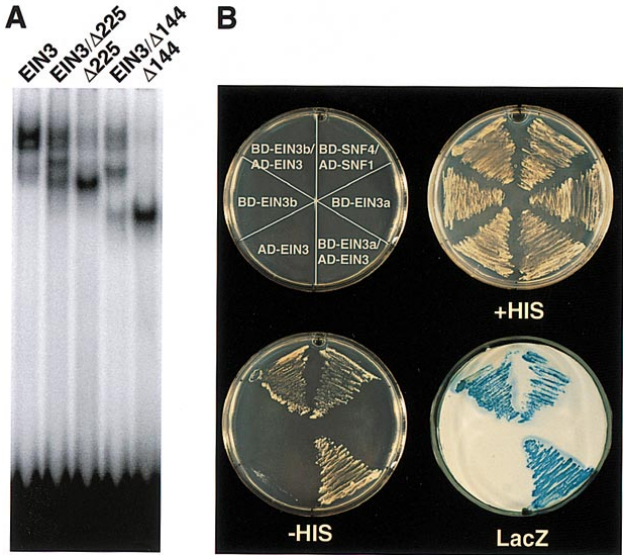

Figure 3. EIN3 homodimerization. (*A*) EMSA of full-size EIN3 and deletion derivatives binding to the EBS. Proteins were translated in vitro alone or in pair-wise combinations. (See Fig. 2F for description of EIN3 deletion derivatives.) (*B*) EIN3–EIN3 interactions assayed by the yeast two-hybrid system. Yeast cells transformed with the indicated constructs in the top left plate, were grown on synthetic complete (SC) medium (+HIS) or in SC medium without histidine (–HIS) and with 50 mM 3-aminotriazole (3-AT, Sigma) to repress basal activity of the *his3* reporter gene. β-Galactosidase activity of the colonies grown in –HIS medium (LacZ) was determined by the filter-lift assay. SNF4/SNF1 were used as a positive control. Colonies from two independent transformation experiments are shown (BD-EIN3a and BD-EIN3b).

bidopsis cDNA library constructed with mRNA from etiolated seedlings (Kim et al. 1997). Yeast strain Y190 transformed with the bait construct was subsequently transformed with the prey and 4×10^6 independent transformants were screened for positive interactions, as described by Kim et al. (1997). Twenty-six independent positive clones were obtained, the plasmids were recovered, and the cDNAs were sequenced. Among them, six different clones corresponded to *EIN3*. All positives were retested by direct transformation of yeast with both the original bait and the recovered prey. Figure 3B shows an example of the interaction with one of these positives that included amino acids 113–628 of EIN3. Because the bait contains EIN3 residues 53–257, the dimerization domain most likely resides between amino acids 113 and 257. These results also indicate that interaction of the EIN3 DNA-binding domain with DNA is not required for protein dimerization.

To assess whether EIN3 and EILs are capable of forming heterodimers, DNA-binding experiments with all combinations of cotranslated EIN3/EILs proteins were performed. While mobility-shifted bands corresponding in position to homodimeric forms of EIN3, EIL1, and EIL2 were observed, DNA–protein complexes with intermediate mobility were not seen, suggesting that these proteins do not form heterodimers (data not shown).

ERF1 is a GCC-box-binding protein

The results described above are consistent with EIN3 being a transcriptional activator that is both necessary and sufficient for *ERF1* expression. ERF1, in turn, may be expected to direct the expression of target genes containing the GCC element. However, at least one EREBP, involved in the regulation of cold and drought response, is known to bind a DNA sequence unrelated to the GCC element (i.e., C-box/DRE element; Stockinger et al. 1997). To determine whether ERF1 contains a functional DNA-binding domain able to interact with the GCC box in a sequence-specific manner, we performed DNA-binding experiments with in vitro-translated ERF1 protein. Radiolabeled promoter fragments of the ethylene-regulated *Arabidopsis basic-chitinase* (Samac et al. 1990) and bean *chitinase5B* genes (Broglie et al. 1989) were incubated with ERF1 and analyzed by EMSA. To examine the specificity of the interaction, we also used a mutated version of the promoter fragments in which the cytosines of the GCC box were substituted with thymines (see Materials and Methods). As shown in Figure 4, ERF1 was able to bind specifically the promoter fragments containing the GCC element, whereas no binding was observed to the mutant sequences. The lower band in each lane containing ERF1 and the wild-type element likely corresponds to a truncated form of ERF1 as two major bands were obtained as products of the in vitro translation of *ERF1* mRNA (not shown).

Downstream activation of ethylene responses by ERF1

To evaluate further the role of ERF1 in the ethylene signaling pathway, transgenic plants constitutively expressing *ERF1* mRNA under the control of the CaMV 35S promoter were constructed. T2 segregants of these transgenic lines were examined for ethylene response phenotypes. Of a total of 26 independent lines, plants

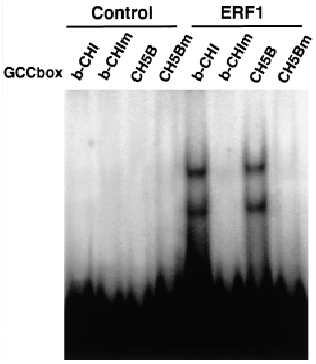

Figure 4. ERF1 is a GCC box DNA-binding protein. EMSAs were performed using in vitro-translated ERF1 protein and promoter fragments from the *Arabidopsis basic-chitinase* (*b-CHI*) and bean *chitinase5B* (*CH5B*) genes. DNA fragments containing the GCC box or mutated versions (*b-CHIm* and *CH5Bm*) of these same elements were incubated with mock translated rabbit reticulocyte lysates (control) or those containing ERF1 protein.

from 9 lines displayed phenotypes similar to those observed in the constitutive ethylene response mutant *ctr1*, in *EIN3*- or *EIL1*-overexpressing plants, or in wild-type plants exposed to ethylene (Kieber et al. 1993; Chao et al. 1997). Etiolated *35S::ERF1* seedlings grown in hydrocarbon-free air showed inhibition of root and hypocotyl elongation, typical of treatment with ethylene (Fig. 5). However, the apical hook did not display exaggerated curvature typical of an ethylene response. The cotyledons of *ERF1*-expressing seedlings were still apressed, and many were still encapsulated in the seed coat. Consistent with this phenotype, *HOOKLESS1*, an ethylene response gene required for apical hook curvature (Lehman et al. 1996), was not expressed in *ERF1*-overexpressing plants (data not shown). Expression of only a partial seedling triple response phenotype in these lines is consistent with a role for *ERF1* in mediating a subset of the ethylene responses. ERF1 may act along with

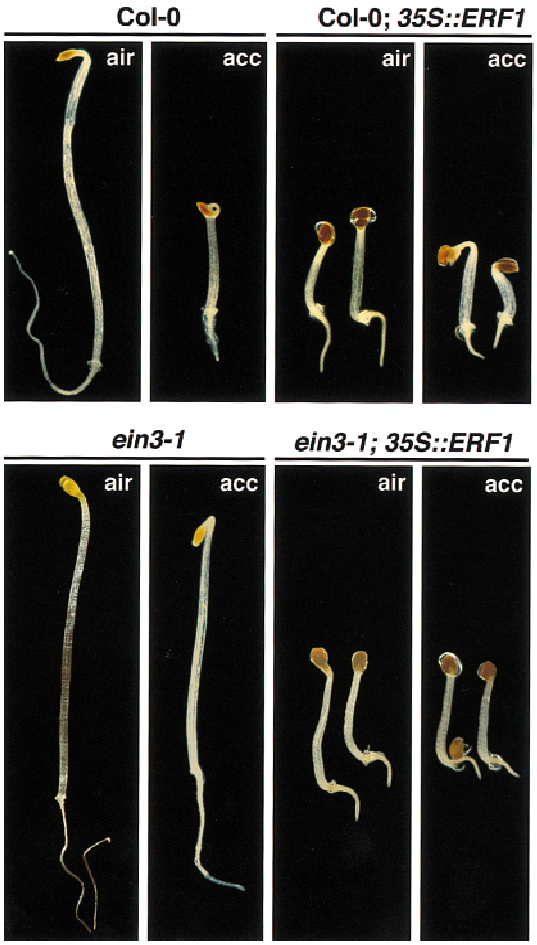

Figure 5. Constitutive activation of ethylene response phenotypes in *35S::ERF1*-expressing seedlings. Three-day-old etiolated seedlings overexpressing *ERF1* in wild-type (Col-0) and *ein3* mutant backgrounds grown in agar plates with or without 10 μM acc (1-aminocyclopropane-D-carboxylic acid). Untransformed wild-type and *ein3* mutant plants are also shown for comparison.

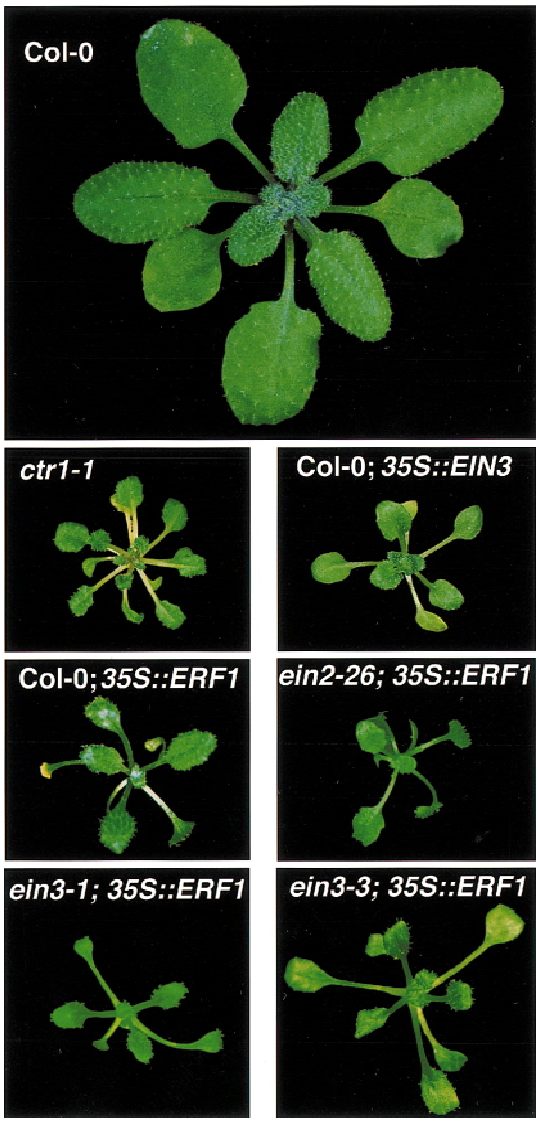

Figure 6. ERF1 acts downstream of EIN2 and EIN3 in the ethylene signaling pathway. Transgenic plants overexpressing *ERF1* in wild-type (Col-0), *ein2*, *ein3-1*, and *ein3-3* mutant backgrounds grown in continuous flowthrough cambers with hydrocarbon-free air for 5 weeks. Untransformed wild-type, *ctr1-1*, and *EIN3*-overexpressing plants are shown for comparison.

other proteins (EREBPs and others) to fully mediate the various seedling responses to ethylene.

As adults, *35S::ERF1* transgenic plants showed an extreme dwarf phenotype similar to that of the constitutive ethylene response mutant *ctr1* and EIN3/EIL1-overexpressing transgenic plants (Fig. 6). As in the case of the quadruple ethylene receptor knockout mutant (Hua and Meyerowitz 1998), plants from several *ERF1*-expressing lines showed extreme inhibition of cell enlargement and ultimately the plants wilted and died before bolting (data not shown).

To determine whether the ethylene morphology displayed by these plants was the consequence of ethylene overproduction, or due to constitutive activation of the

signaling pathway, the *35S::ERF1* gene was also introduced into several mutant backgrounds (*ein2-5*, *ein2-17*, *ein2-26*, *ein3-1*, *ein3-3*, and *ein5-1*) that suppress phenotypes resulting from ethylene overproduction (Roman et al. 1995). In all cases, the transgenic plants displayed a morphology indistinguishable from *35S::ERF1*-expressing wild-type plants (Figs. 5 and 6; data not shown). These results indicate that the observed morphology evoked by expression of *ERF1* was not a consequence of ethylene production but rather, like CaMV *35S::EIN3* expression (Chao et al. 1997), results from constitutive activation of the response pathway. Moreover, as there was an absence of the requirement for functional EIN2, EIN3, or EIN5 proteins for the constitutive activation phenotype, these results provide strong evidence for the downstream location of ERF1.

To confirm whether the observed morphology in the *35S::ERF1* lines was due to activation of ethylene responses, the expression of several ethylene-regulated genes was examined. As expected, no accumulation of two ethylene-inducible messages, *basic-chitinase* and *PDF1.2*, was detected in wild-type plants in the absence of the hormone, or in ethylene-insensitive mutants (*ein2*, *ein3*, or *ein5*). In contrast, high-level constitutive expression of both transcripts was observed in *35S::ERF1* transgenic plants regardless of the background (Fig. 7A). In the case of *ein5-1*, the lower expression of *ERF1* in one of the two transgenic lines correlated with the lower expression of *PDF1.2* and *basic-chitinase* and with the weaker constitutive ethylene response phenotype.

The effect of *35S::ERF1* expression on a chitinase promoter–reporter fusion, *CH5B::GUS* (Broglie et al. 1989), was also examined. This well-characterized ethylene-responsive reporter gene has been shown to be a reliable marker for ethylene-evoked transcription in bean (Broglie et al. 1989) and *Arabidopsis* (Chen and Bleecker 1995). F_1 plants derived from crosses between plants carrying the *CH5B::GUS* reporter gene and *ERF1*-overexpressing lines or wild-type plants were stained for GUS activity. As revealed by the intense staining in seedlings, high-level GUS activity was observed in the presence of *35S::ERF1*, whereas no staining was detected in the control plants (absence of *35S::ERF1*; Fig. 7B). Moreover, introduction of the *35S::ERF1* construct into an ethylene-repressible enhancer-trap reporter line also resulted in inhibition of GUS expression in the absence of ethylene (R. Solano, R. McGrath, and J.R. Ecker, unpubl.). These results confirm that *ERF1* expression is sufficient to promote (or repress) transcription of ethylene-regulated target genes in a variety of tissues.

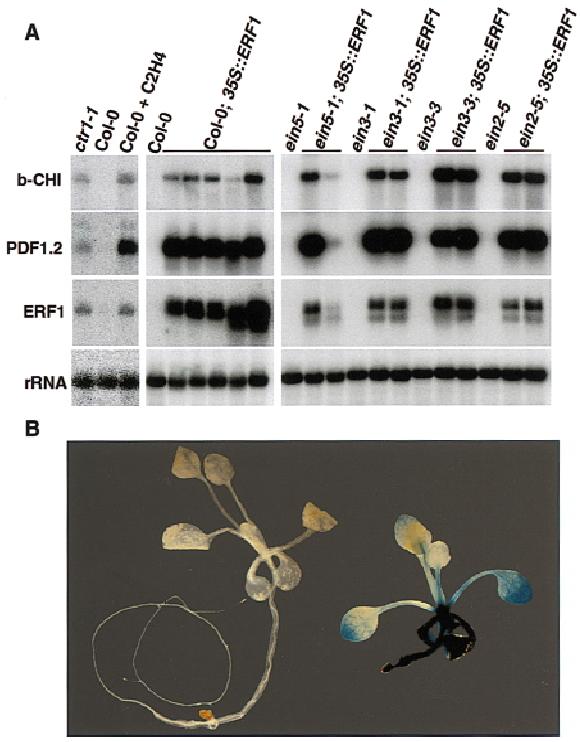

Figure 7. Transcriptional activation of ethylene-responsive genes by ERF1 (*A*) RNA blot analysis of the expression of ethylene-inducible genes in transgenic lines overexpressing *ERF1* in wild-type (Col-0) and ethylene-insensitive mutant backgrounds. Five independent transgenic lines in Col-0 and two independent lines in each of the mutants are shown. Five micrograms of total RNA from 5-week-old plants grown in air were loaded per lane in the *middle* and *right* panels, and 50 µg in the *left* panel. The same blot was probed with *ERF1*, a loading control probe (rDNA), and ethylene-inducible genes *PDF1.2* and *basic-chitinase*. (*B*) Constitutive activation of the ethylene-inducible *CH5B–GUS* reporter gene in *ERF1*-overexpressing transgenic plants. Three-week-old plants grown on agar plates were assayed for GUS activity.

Discussion

Cloning of *EIN3* and related *EIL* genes identified a new family of proteins that are necessary and sufficient for signaling events initiated by ethylene (Chao et al. 1997). In this study, we show that EIN3 and EILs proteins comprise a novel family of sequence-specific DNA-binding proteins and define a primary ethylene response element (PERE) present in the promoters of several ethylene-regulated genes that is similar to the tomato E4 ethylene response element. EIN3 was both necessary and sufficient for the activation of ethylene responsive target genes and in particular for *ERF1,* a novel gene encoding an AP2/EREBP-type DNA-binding protein. Evidence is provided that sequences in the promoter of *ERF1* serve as an immediate target of EIN3 binding and that constitutive expression of *ERF1* activates the transcription of downstream effector genes, such as *basic-chitinase* and *PDF1.2*, to achieve the ethylene response (Fig. 8).

ERF1 is a downstream component in the ethylene signaling pathway

Previous efforts to understand the hormonal regulation of ethylene-regulated genes in several plant systems led to the identification of two kinds of ethylene response elements (EREs). One type of ERE was found to be responsible for ethylene-regulated expression of genes in-

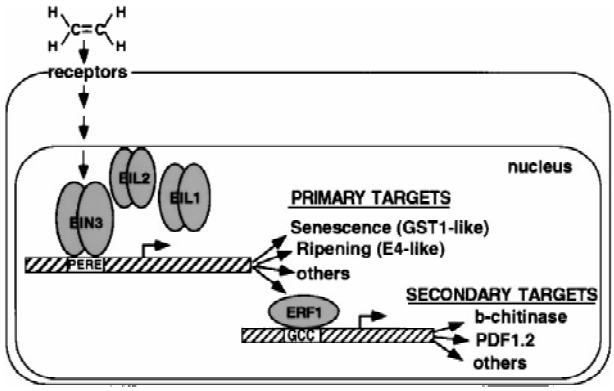

Figure 8. Nuclear events in the ethylene gas signaling pathway. Model depicts the transcriptional regulatory cascade that mediates ethylene responses. Binding of ethylene (C_2H_4) to membrane receptors activates EIN3, and most likely EIL1 and EIL2, through a signaling cascade described elsewhere (Chao et al. 1997). EIN3 directs the expression of *ERF1* and other primary target genes by binding directly, as a dimer to the E4-like PERE present in their promoters. ERF1 and probably other EREBPs bind to the GCC box (SERE) and activate the expression of secondary ethylene response genes such as *basic-chitinase* and defensin (*PDF1.2*). Although we favor this simple model, the results presented in this work do not exclude other more complicated models that may involve the existence of an intermediate between EIN3 and ERF1 or the existence of EIN3-interacting proteins that modulate EIN3 activity.

duced during ripening and senescence (Montgomery et al. 1993; Itzhaki et al. 1994). A second element, the GCC box, was identified as being necessary for ethylene inducibility in response to pathogen attack (for review, see Deikman 1997). On the basis of their ability to bind to the GCC element, a family of DNA-binding proteins (EREBPs) was identified in tobacco (Ohme-Takagi and Shinshi 1995). The fact that these genes were themselves transcriptionally activated by treatment with ethylene suggested that they might represent an intermediate step between the EIN3/EILs proteins and downstream effector genes such as *basic-chitinase*.

To identify targets of EIN3, members of the *Arabidopsis* EREBP family were cloned and characterized. *ERF1* was rapidly induced in response to ethylene gas, with mRNA levels increasing within 15 min of ethylene treatment. *ERF1* was constitutively expressed in the ethylene pathway mutant *ctr1*, and ethylene induction of *ERF1* was completely dependent on a functional EIN3 protein, as no expression was detected in the *ein3-1* mutant. Moreover, transgenic plants overexpressing *EIN3* showed high-level expression of *ERF1* mRNA. These results indicate that EIN3 is both necessary and sufficient for *ERF1* expression and are consistent with *ERF1* being a direct target of EIN3. The level of *ERF1* mRNA expression in *EIN3*-overexpressing plants was somewhat lower than in *ctr1* mutants or in ethylene treated wild-type plants. This indicates that although EIN3 is sufficient for *ERF1* expression, other factors may be required for full ethylene-dependent *ERF1* induction. In this regard, we have identified a DNA-binding protein that interacts with EIN3, using *EIN3* as a bait in the two-hybrid screen (R. Solano, Q. Chao, and J. Ecker, unpubl.). This protein also binds to the *ERF1* promoter in a sequence specific-manner, suggesting that it may be a partner of EIN3 and could be needed for full *ERF1* expression in response to ethylene.

Thus far, loss-of-function mutations have not been reported for any member of the EREBP family. This finding, together with the large number of these genes present in *Arabidopsis* (125 estimated; Riechmann and Meyerowitz 1998), suggests a functional redundancy among members of this family. In the case of functionally redundant genes, loss-of-function alleles may not show a phenotype. A clear example of this is provided by studies of the ethylene receptors in *Arabidopsis* (Hua and Meyerowitz 1998). Implication in the ethylene signaling pathway of each of the five ETR1-related genes was made through the identification (or creation by site-specific mutagenesis) of dominant mutations (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). While single loss-of-function mutations in these genes do not exhibit defects in ethylene response, triple and quadruple mutants display constitutive ethylene response phenotypes, revealing that ethylene responses are negatively regulated by the receptors (Hua and Meyerowitz 1998).

For this reason, we have used a gain-of-function strategy to address the in vivo function of ERF1. Gain-of-function mutations obtained by insertional mutagenesis of T-DNA or transposon elements carrying a CaMV 35S promoter (enhancer-trap/gene-trap) have proven to be a powerful tool for assessing the in vivo function of a gene. Constitutive *ERF1* expression resulted in seedling and adult phenotypes very similar to those displayed by loss-of-function *ctr1* mutants, plants overexpressing *EIN3* or *EIL1*, or plants grown in ethylene (Kieber et al. 1993; Chao et al. 1997). Some significant differences, however, can be observed between *EIN3*- and *ERF1*-overexpressing plants. While *ERF1* overexpression causes inhibition of hypocotyl and root cell elongation, seedlings lack an exaggerated apical hook. Consistent with this observation, *HOOKLESS1*, which contains a GCC element in its promoter, is not induced in *ERF1* transgenic plants, suggesting that ERF1 is responsible for the activation of a subset of the ethylene-responsive GCC-box-containing target genes. Perhaps other EREBP family members may be responsible for activation of these target genes. Indeed, a transposon-induced gain-of-function mutant (*tiny*) that constitutively expresses an EREBP displays seedling phenotypes reminiscent of a partial ethylene response (Wilson et al. 1996), suggesting that TINY may be a partner of ERF1 in ethylene signaling. Alternatively, ERF1 may act in concert with other transcription factors in the activation of some promoters. In fact, interactions between EREBPs and bZIP transcription factors, as well as synergistic effects of their DNA target sites, the GCC box and G box, have been described previously (Hart et al. 1993; Sessa et al. 1995; Buttner and Singh 1997).

Another member of the *Arabidopsis* AP2/EREBP family (*AtEBP*) has been reported to be regulated by ethylene (Buttner and Singh 1997). Like *ERF1*, *AtEBP* is constitu-

tively expressed in *ctr1* mutants. However, unlike *ERF1*, *AtEBP* is probably not a direct target of EIN3 as its expression in response to ethylene can be blocked by cycloheximide (A. Stepanova and J.R. Ecker, unpubl.). This observation may suggest a regulatory cascade among members of the EREBP family in which AtEBP acts downstream of ERF1. Consistent with this idea, several of the EREBP genes contain GCC box elements in their promoters, suggesting that their expression might be autoregulated or controlled by other EREBPs. The existence of a transcriptional regulatory cascade is not constrained to EREBPs involved in ethylene signaling as it has also been inferred in the case of RAP genes (AP2/EREBP family members), which are not obviously involved in the response to ethylene (Okamuro et al. 1997).

EIN3 is a novel type of DNA-binding protein that regulates the expression of ERF1

Although EIN3 and EILs proteins do not share similarity with any known proteins, their nuclear location suggested their putative role as transcription factors (Chao et al. 1997). DNA-binding assays with in vitro-translated and baculovirus-expressed EIN3 protein demonstrated that EIN3 is able to bind to specific sequences in the *ERF1* promoter. EMSA experiments with truncated forms of the protein confirmed the presence of EIN3 in the DNA–protein complex. A mutant protein corresponding to the *ein3-3* allele of *EIN3* was unable to recognize the target sequence. This mutation consists of a Lys to Asn substitution in the basic domain III, which may form part of the DNA-binding motif.

Two additional proteins that belong to the EIN3/EIL family, EIL1 and EIL2, were also able to recognize specifically the EIN3 target in the promoter of *ERF1*. Consistent with this result, EIN3 can be replaced functionally by EIL1 or EIL2 as overexpression of either of these genes in transgenic plants can complement the *ein3-1* mutation (Chao et al. 1997). Deletion analysis of EIN3 has allowed us to define its DNA-binding domain within the amino-terminal half of the protein. This region is the most conserved among all four members of the family and does not contain any previously known DNA-binding motif. Defining of the EIN3/EILs DNA-binding domain will require further structural analysis of these proteins. Nevertheless, these four proteins, along with a fifth more recently identified homolog (EIL4; R. Solano and J.R. Ecker, unpubl.), possess several predicted α-helices, two of them rich in basic amino acids (Chao et al. 1997), that are good candidates to form a DNA-interaction surface.

Scanning mutagenesis of the DNA fragment containing the target site allowed us to determine the sequence requirements for the interaction. The defined target site includes two inverted repeats and is recognized by the protein as a dimer. Interestingly, the EIN3-binding site shares significant identity with sequences within the promoter region of the carnation *GST1* gene that has been defined as necessary and sufficient for ethylene responsiveness. The conserved sequences are also present in the promoter regions required for ethylene responsiveness in the tomato *E4* and *LEACO1* genes (Montgomery et al. 1993; Itzhaki et al. 1994; Blume and Grierson 1997). This observation suggests that the EIN3 target site represents a PERE conserved in different species in which there are also orthologs of EIN3 (H. Klee; D. Cook, pers. comm.). Consistent with this hypothesis, one of the genes that contains this element (*E4*) has been previously proposed to be a primary ethylene response gene (Lincoln et al. 1987). The GCC element may be a secondary ethylene response element (SERE) present only in a subset of the ethylene-regulated genes (e.g., pathogenesis-related genes, *HOOKLESS1* and some *EREBPs*) that may be regulated by a subgroup of the EREBP family of proteins. Experiments to test the functionality of the EIN3 target site as an autonomous ethylene response element are underway.

Cascades of transcription factors are a common theme in gene regulation, present in virtually all organisms from bacteria to humans, and involved in the regulation of processes as diverse as nitrogen fixation, embryogenesis, cell differentiation, response to extracellular signals or circadian rhythmicity (Kranz and Foster-Hartnett 1990; Jackle and Sauer 1993; Brun et al. 1996; Hwang et al. 1997; Allada et al. 1998; Darlington et al. 1998; Gekakis et al. 1998; Rutila et al. 1998). Rapid EIN3-dependent induction of *ERF1* expression in response to ethylene, binding of EIN3 to the E4-like element in the *ERF1* promoter and constitutive expression of *ERF1* in *EIN3*-overexpressing plants provide three pieces of evidence supporting the hypothesis that *ERF1* is an immediate target of EIN3. Binding of ERF1 to the GCC element in the promoters of ethylene-regulated genes, and constitutive activation of ethylene response genes and phenotypes in both etiolated seedling and adult plants in the ERF1 gain-of-function experiments provide additional evidence that *ERF1* is a downstream ethylene signaling pathway gene.

The sequential action of EIN3 and ERF1 DNA-binding proteins adds a new level of complexity in the regulatory hierarchy of the ethylene-signaling pathway. Moreover, the strong similarity of the target site of EIN3 to an ERE previously identified in the promoters of ethylene response genes from a variety of species suggests that there is broad commonality among the downstream ethylene signaling events in plants. The existence of this hierarchy of transcription factors in the signaling pathway for ethylene may serve to amplify the input signal and/or provide a means to finely regulate the complex plant response to this gaseous plant growth regulator/stress signal.

Materials and methods

Strains and growth conditions

The *Arabidopsis* ecotype Columbia (Col-0) was the parent strain of all mutants and transgenic plants used in this work. Triple response screens were performed as described previously (Guzman and Ecker 1990). Plant growth in air and ethylene was carried out as described previously (Kieber et al. 1993).

Nucleic acid analysis

Total RNA extractions and Northern analysis were performed as described (Reuber and Ausubel 1996; Chao et al. 1997). β-Glucuronidase activity was assayed by incubation of the plants with the substrate of the enzyme (X-Gluc, 1 mg/ml) in sodium phosphate buffer for 18 hr. cDNA clones corresponding to *ERF1* were isolated by hybridization of a size-selected cDNA library in λZAPII (Kieber et al. 1993). The probe, corresponding to a fragment of the tobacco *EREBP1* gene, was obtained by PCR amplification with the following primers: EREBP1f, 5′-CACGCCATAGACATAATAC-3′, and EREBP1r, 5′-GCTACGATTCCTGTTCCTTCAG-3′.

ERF1 genomic sequences were isolated by hybridization of two BAC genomic libraries (TAMU and IGF; Choi et al. 1995; R. Wing, unpubl.; T. Altmann, unpubl.). The map position of *ERF1* was obtained by PCR amplification of YAC pools with specific primers. PCR highlighted two YAC clones (CIC12H5 and CIC12H6) both located in the ABI3 contig.

Synthesis of proteins, DNA-binding reactions, and EMSA

Full-length ERF1, EIN3, and the EIN3 deletion derivatives were generated by in vitro translation (or cotranslation in the dimerization experiments) by use of the flexi-rabbit reticulocyte system (Promega) as described (Solano et al. 1997). PCR and Klenow labeling of promoter fragments and oligonucleotides, DNA-binding reactions and EMSAs were performed as described (Solano et al. 1995). In the competition experiments, the appropriate amount (see Fig. 2, legend) of the competitor (cold EBS, EBSm1, or EBSm2) was included in the reaction before the protein was added. Once the protein was added to the reaction, the mixture was incubated for 30 min on ice and then loaded onto the acrylamide gel.

Sequences of the mutant versions of EBS used in competition experiments were: EBSm1, 5′-GTTGTTTGGGATTCTTCGGGCATGTATCTTGAATCC-3′; EBSm2, 5′-GTTGTTTGGGATTCAAGCCCCATGTATCTTGAATCC-3′.

The promoter fragments of the *Arabidopsis* and bean basic-chitinase genes containing the GCC box were obtained by Klenow filling of the following overlapping primers:

b-CHIforward, 5′-GTTGATCACGAACCCGCCGCCTCATATTCATAATTA-3′; b-CHImutant, 5′-GTTGATCACGAACCCGTTGTTTCATATTCATAATTA-3′; b-CHIreverse, 5′TTTAACTTTAATTATGAATATGA-3′; CH5Bforward, 5′-CTTCACGCTTGGGAAGCCGCCGGGGTGGGCCCGCAG-3′; CH5Bmutant, 5′-CTTCACGCTTGGGAAGTTGTTGGGGTGGGCCCGCAG-3′; and CH5Breverse, 5′-AAACCTTTCTGCGGGCCCACCCC-3′.

Plant transformation

A 0.8-kb *Bam*HI–*Kpn*I fragment of *ERF1* cDNA was cloned into *Bam*HI–*Kpn*I-digested pROK2 (Baulcombe et al. 1989). The C58 strain of *Agrobacterium tumefaciens* containing the above construct was used to transform *Arabidopsis* ecotype Col-0 and the ethylene insensitive mutants *ein2-5*, *ein2-17*, *ein2-26*, *ein3-1*, *ein3-3*, and *ein5-1* by *in planta* vacuum infiltration (Bechtold et al. 1993). Kanamycin-resistant T1 plants were selected by plating seeds on Murashige and Skoog medium supplemented with 100 μg/ml kanamycin and transferring kan^R seedlings to soil.

Yeast transformation and two-hybrid screening

Yeast strain Y190 was transformed by the PEG/lithium acetate method as described previously (Gietz et al. 1992). Growth conditions, screening procedures, and filter-lift assay for β-galactosidase activity were performed as described previously (Kim et al. 1997).

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Note

GenBank accession numbers for the *ERF1* cDNA and genomic sequences reported in this paper are AF076277 and AF076278, respectively.

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