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Plant Responses to Ethylene Gas Are Mediated by $SCF^{EBF1/EBF2}$-Dependent Proteolysis of EIN3 Transcription Factor

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Summary

Plants use ethylene gas as a signal to regulate myriad developmental processes and stress responses. The *Arabidopsis* EIN3 protein is a key transcription factor mediating ethylene-regulated gene expression and morphological responses. Here, we report that EIN3 protein levels rapidly increase in response to ethylene and this response requires several ethylene-signaling pathway components including the ethylene receptors (ETR1 and EIN4), CTR1, EIN2, EIN5, and EIN6. In the absence of ethylene, EIN3 is quickly degraded through a ubiquitin/proteasome pathway mediated by two F box proteins, EBF1 and EBF2. Plants containing mutations in either gene show enhanced ethylene response by stabilizing EIN3, whereas *efb1 efb2* double mutants show constitutive ethylene phenotypes. Plants overexpressing either F box gene display ethylene insensitivity and destabilization of EIN3 protein. These results reveal that a ubiquitin/proteasome pathway negatively regulates ethylene responses by targeting EIN3 for degradation, and pinpoint EIN3 regulation as the key step in the response to ethylene.

Introduction

As sessile organisms, plants have evolved elaborate mechanisms to respond to various biotic and abiotic stresses. One of these mechanisms is the release of a gaseous hormone, ethylene (C_2H_4). Ethylene can trigger a wide range of physiological and morphological responses, including inhibition of cell expansion, promotion of leaf and flower senescence, induction of fruit ripening and abscission, resistance to pathogen infection, and adaptation to stress conditions (Bleecker and Kende, 2000).

The most widely documented ethylene response is the so-called "triple response" of etiolated seedlings. In the presence of exogenous ethylene, dark-grown seedlings exhibit a short, thickened root and hypocotyl with exaggerated curvature of the apical hook (Abeles et al., 1992). The triple response phenotype has been extensively used in genetic screens in *Arabidopsis* to identify components of the ethylene signal transduction pathway. Several mutants that display an aberrant triple response have been isolated in *Arabidopsis* (Ecker, 1995). One class of mutants (e.g., *etr1*, *etr2*, *ein2*, *ein3*, *ein4*, *ein5*, and *ein6*) shows a reduction or absence of ethylene responsiveness in the presence of exogenous ethylene or its metabolic precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Bleecker et al., 1988; Guzman and Ecker, 1990; Roman et al., 1995; Sakai et al., 1998). A second class of mutants (*eto1, eto2, eto3*, and *ctr1*) exhibits a triple response in the absence of exogenous ethylene, either due to ethylene overproduction (*eto* mutants) or as a consequence of constitutive activation of ethylene-signaling pathway (*ctr1*) (Guzman and Ecker, 1990; Kieber et al., 1993).

Genetic and molecular characterization of these mutants has unraveled a largely linear ethylene signal transduction pathway (Stepanova and Ecker, 2000). Briefly, ethylene is perceived by a family of ER-associated receptors with sequence similarity to two-component histidine kinases (Chang et al., 1993; Chen et al., 2002). The ethylene receptors play a negative role, whereby ethylene binding acts to repress their activity (Hua and Meyerowitz, 1998). Accordingly, gain-of-function mutations in the receptors, which disrupt ethylene binding, result in constitutive activation of the receptors and produce an ethylene-insensitivity phenotype, such as found in *etr1-1*, *etr2-1*, and *ein4-1* mutants (Chang et al., 1993; Sakai et al., 1998; Hua et al., 1998). CTR1 is also a negative regulator of the pathway that acts downstream of the ethylene receptors. *CTR1* encodes a protein with similarity to the Raf family of Ser/Thr protein kinases, implying a mitogen-activated protein (MAP) kinase cascade in ethylene-signal transduction (Kieber et al., 1993). Recently, a MAPKK and MAPK cascade has been shown to be activated by ethylene and repressed by CTR1 (Ouaked et al., 2003). Overexpression of the *Medicago* MAPKK (SIMKK) in *Arabidopsis* evoked a constitutive triple response phenotype, suggesting that a MAPKK and MAPK cascade positively regulates downstream ethylene responses (Ouaked et al., 2003). Signal transmission from the MAP kinase cascade to the downstream components requires EIN2, an integral membrane protein with limited similarity to mammalian Nramp metal transporters (Alonso et al., 1999). Loss-of-function mutations in *EIN2* cause complete ethylene insensitivity, indicating that EIN2 is a positive component essential for ethylene responses. Ethylene signaling downstream of EIN2 is mediated by a nuclear protein, EIN3 (Chao et al., 1997). *ein3* mutants show a loss of ethylene-mediated effects including gene expression, triple response, cell growth inhibition, and reduced senescence (Chao et al., 1997). Conversely, overexpression of *EIN3* results in constitutive ethylene responses in both wild-type and *ein2* mutant backgrounds (Chao et al., 1997). These results demonstrate that EIN3 is both necessary and sufficient for the activation of the ethylene pathway. Biochemical studies revealed that EIN3 protein can bind to a specific sequence in the promoter of a target gene, *ERF1* (Solano et al., 1998). Although EIN3 has been shown to be an essential transcription factor mediating a diverse array of plant responses to ethylene, the mechanism of its activation by ethylene is unknown.

In this study, we show that EIN3 undergoes a post-translational mechanism of regulation by ethylene. The level of EIN3 protein is rapidly increased by ethylene

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treatment, and several known components of the ethylene-signaling pathway are required for ethylene regulation of EIN3 abundance. Furthermore, we show that EIN3 is a short-lived protein and is subject to ubiquitin/proteasome-dependent proteolysis mediated by two F box proteins, EBF1 and EBF2. The corresponding SCF complexes play a negative role in the ethylene-signaling pathway by targeting EIN3 for degradation, whereas ethylene acts to inhibit EIN3 proteolysis. These results provide new insights into the mechanism whereby plants rapidly respond to the stress hormone ethylene and define the critical role of the ubiquitin/proteasome pathway in the ethylene-response pathway.

Results

The Level of EIN3 Protein Is Controlled by Ethylene

EIN3 is a key transcription factor positively regulating gene expression in response to ethylene. We sought to investigate how EIN3 is regulated by ethylene. Overexpression of EIN3 (EIN3ox) in transgenic plants results in constitutive ethylene responses (Chao et al., 1997), implying that EIN3 abundance is important for its function. However, previous studies revealed that the level of *EIN3* RNA is not altered either by ethylene treatment, or in the *ein2* or *ctr1* mutant (Chao et al., 1997), suggesting a posttranscriptional mechanism of EIN3 regulation by ethylene. To determine whether the level of EIN3 protein is subject to ethylene regulation, we first monitored the levels of EIN3 protein in wild-type plants grown in growth medium supplemented with various concentrations of ACC, an ethylene biosynthetic precursor. As shown in Figure 1A, we observed a positive correlation between the levels of EIN3 protein and the severity of the seedling triple response phenotype. In the absence of ACC treatment, EIN3 protein was barely detectable. By comparison, the levels of EIN3 protein in plants treated with high concentrations of ACC (e.g., 10 and 50 μM) were dramatically elevated. To gain further insight into the kinetics of EIN3 induction by ethylene, we monitored the levels of EIN3 protein in plants treated with ethylene gas for different periods of time. In wild-type plants, the level of EIN3 markedly increased after 1 hr of hormone treatment (Figure 1B). No EIN3 protein was observed in the *ein3-1* mutant, confirming that our antibody specifically recognized EIN3 protein. The levels of transgenically overexpressed EIN3 protein in EIN3ox plants were also upregulated by ethylene treatment (Figure 1B). In agreement with this observation, EIN3ox seedlings displayed an extremely exaggerated triple response (with a very short root and hypocotyl) in the presence of ethylene (see Figures 5C and 5D). Taken together, we conclude that the levels of both endogenous and transgenically overexpressed EIN3 protein are increased by ethylene.

Components of the Ethylene-Signaling Pathway Are Required for EIN3 Accumulation

Genetic studies have identified several components of the ethylene-signaling pathway, including the ETR/ERS family of receptors, CTR1, EIN2, EIN5, and EIN6 (Stepanova and Ecker, 2000). We asked whether any of these signaling components are required for ethylene-induced EIN3 accumulation. Silver ion is a potent inhibitor of ethylene action that acts by interfering with ethylene perception (Abeles et al., 1992). We tested whether silver treatment perturbs EIN3 accumulation upon ethylene treatment. As shown in Figure 1C, the level of EIN3 was no longer increased by ethylene in wild-type seedlings treated with silver ion. This result indicates that perception of ethylene is required for EIN3 accumulation. We next monitored the levels of EIN3 protein in several ethylene-insensitive mutants, including two dominant receptor mutants, *etr1* and *ein4*, as well as three recessive mutants, *ein5*, *ein6*, and *ein2*. When compared with wild-type seedlings, *ein4*, *ein5*, or *ein6* mutants showed a significant delay in EIN3 accumulation in response to ethylene and also a reduction in maximal accumulation (Figures 1D and 1E). In *etr1*, EIN3 did not accumulate until 12 hr of treatment and the protein level was only slightly elevated (Figure 1D). However, in *ein2*, ethylene-induced EIN3 accumulation was completely blocked (Figure 1E). We were unable to detect any EIN3 protein in *ein2* seedlings even after three days of continuous hormone treatment (data not shown). These results demonstrate that an intact ethylene-signaling pathway consisting of the ETR/ERS receptors, EIN2, EIN5, and EIN6 is required for EIN3 accumulation, suggesting that these components function upstream of EIN3. Moreover, these results imply that the ethylene-insensitivity observed in these mutants (*etr1*, *ein2*, *ein4*, *ein5*, and *ein6*) might be the consequence of reduced EIN3 abundance.

In contrast to the ethylene-insensitive mutants, the *ctr1* and *eto* mutants display constitutively activated ethylene responses (Kieber et al., 1993). Consistent with its phenotype, *ctr1* expressed a higher level of EIN3 in comparison with wild-type when no exogenous ethylene was applied (Figure 1F). Interestingly, the level of EIN3 in *ctr1* remained inducible by ethylene. Given that *ctr1-1* produces a nonfunctional CTR1 protein (Huang et al., 2003), this result suggests that a parallel pathway, bypassing CTR1, might exist. We also observed elevated EIN3 protein levels in *eto1* and *eto2* mutants in the absence of exogenous ethylene (data not shown). Thus, the constitutive triple response mutants result in elevated levels of EIN3 protein.

EIN3 Protein Is Rapidly Degraded by a Proteasome-Mediated Pathway

As the level of EIN3 is low in the absence of ethylene, we asked whether EIN3 protein is unstable in this condition. After boosting EIN3 levels in wild-type seedlings by ethylene treatment, seedlings were placed in a stream of hydrocarbon-free air and level of EIN3 protein was monitored. As shown in Figure 2A, EIN3 levels dramatically decreased after 30 min and remained barely detectable for the subsequent 2 hr. Interestingly, after 4 hr in the air, EIN3 protein started to reaccumulate to a modest level. These results suggested that, in the absence of ethylene, EIN3 is a short-lived protein.

We next examined how ethylene evokes EIN3 protein accumulation. The rapid increase in EIN3 protein accumulation upon ethylene treatment could be attributed either to ethylene-induced de novo protein synthesis, or to ethylene-directed repression of constitutive EIN3

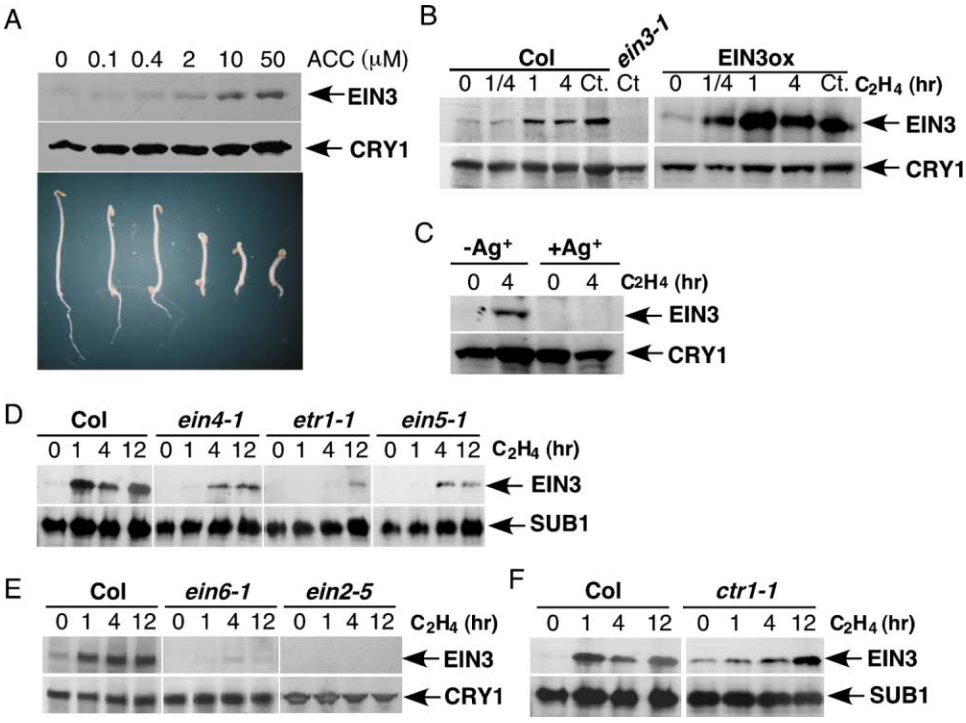

Figure 1. The Levels of EIN3 Protein Are Induced by Ethylene, and Several Ethylene Signaling Components Are Required for the Accumulation of EIN3 Protein

(A) The abundance of EIN3 protein correlates with the strength of triple response. Etiolated wild-type seedlings (Col) were grown on medium supplemented with various concentrations of ACC for 3 days. Total protein extracts were subjected to immunoblot with anti-EIN3 antibody. After stripping, the same membrane was reprobed with anti-CRY1 (*Arabidopsis* cryptochrome 1) or anti-SUB1 (*Arabidopsis* SHORT UNDER BLUE 1) antibody (in Figures 1D and 1F) as a loading control (middle image). Seedlings were photographed for triple response phenotype (bottom image).

(B) The levels of both endogenous and transgenically expressed EIN3 protein are induced by ethylene. Etiolated seedlings of Col and EIN3ox were grown in the air (indicated by time point 0) and subsequently treated with ethylene gas (10 ppm, all the same below) for the indicated amounts of time. Total protein extracts were subjected to immunoblot assays. Ct. indicates seedlings continuously grown in ethylene gas.

(C) EIN3 accumulation is abolished by Ag^+ treatment. Etiolated wild-type seedlings were grown on MS medium without or with 100 μM of $AgNO_3$ for 3 days and treated with air or ethylene for 4 hr.

(D and E) EIN3 accumulation is impaired in various ethylene-insensitive mutants.

(F) EIN3 is constitutively accumulated in *ctr1* mutant.

protein turnover, or to a combination of both processes. To address this issue, we blocked de novo translation initiation using cyclohexamide (CHX) and compared the levels of EIN3 protein in the presence or absence of ethylene. After 2 hr of hormone treatment, seedlings were incubated with CHX and kept in either ethylene or hydrocarbon-free air. As shown in Figure 2B, the levels of EIN3 remained constitutively high in the presence of ethylene but decreased rapidly in the absence of ethylene, indicating that de novo protein synthesis is not required for ethylene-mediated EIN3 protein accumulation. Thus, we conclude that ethylene acts to repress constitutive degradation of EIN3 protein, although we cannot rule out the possibility that translational regulation may play a minor role in ethylene-mediated EIN3 accumulation.

The ubiquitin/proteasome pathway is involved in the rapid degradation of many short-lived proteins that regulates numerous cellular processes (Hochstrasser, 1996). Because EIN3 turnover is rapid, we speculated that a proteasome-mediated pathway might be responsible for its degradation. To test this possibility, we treated *Arabidopsis* suspension cell cultures with proteasome-specific inhibitors, MG132 and MG115 (Lee and Goldberg, 1998). After 30 min of MG132 treatment, the levels of EIN3 protein markedly increased (Figure 2C). Likewise, MG115 treatment also enhanced EIN3 abundance (data not shown). As a control, treatment with a general cysteine protease inhibitor, Pefabloc, had no effect on EIN3 abundance (Figure 2C). We further demonstrated that the abundance of transgenically overexpressed EIN3 protein was similarly induced by treatment with MG132 or MG115, but not with Pefabloc (Figure 2D). These results indicate that EIN3 proteolysis is proteasome-dependent.

Ethylene or MG132 Treatment Promotes the Nuclear Accumulation of EIN3 Protein

EIN3 is localized in the nucleus in transiently transformed protoplast cells (Chao et al., 1997). To assess whether the nuclear localization of EIN3 is modulated by ethylene, we made transgenic *Arabidopsis* plants that express an EIN3-GFP fusion protein driven by the constitutive CaMV 35S promoter. Overexpression of EIN3-GFP was able to complement the *ein3* mutant (data not shown), indicating that this fusion protein was func-

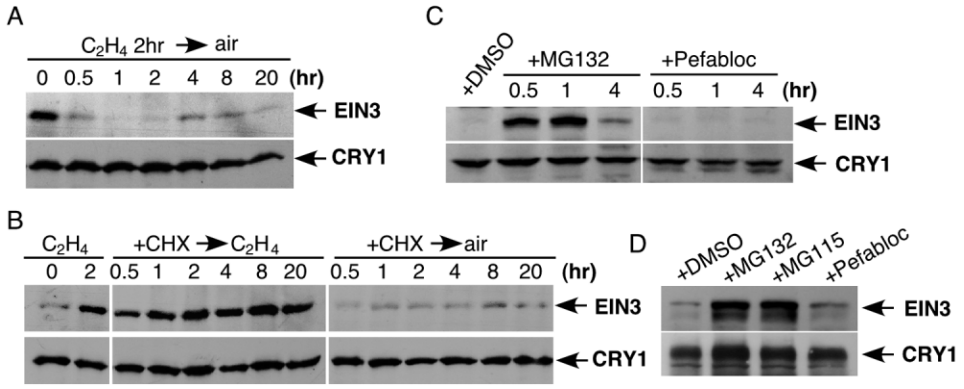

Figure 2. EIN3 Protein Is Rapidly Degraded through a Proteasome-Dependent Pathway

(A) EIN3 protein is unstable in the absence of ethylene. Etiolated wild-type seedlings grown in the air were treated with ethylene for 2 hr (indicated by time point 0) and subsequently moved back to hydrocarbon-free air for the indicated amounts of time. Total protein extracts were subjected to immunoblot assays.

(B) Ethylene treatment stabilizes EIN3 protein. Ethylene-pretreated wild-type seedlings were supplemented with 100 μM of cycloheximide (CHX) and then transferred into either ethylene or hydrocarbon-free air for the indicated amounts of time.

(C) EIN3 protein is stabilized by specific proteasome inhibitors. *Arabidopsis* suspension cells were treated with mock (1% DMSO), MG132 (50 μM), or Pefabloc SC (100 μM) for the indicated amounts of time before cells were harvested for immunoblot assays.

(D) Etiolated EIN3ox seedlings grown in the air were treated with mock (1% DMSO), MG132 (50 μM), MG115 (50 μM), or Pefabloc SC (100 μM) for 4 hr.

tional in plants. Immunoblot analysis indicated that the level of EIN3-GFP fusion protein was dramatically increased by ethylene (Figure 3A). We also observed that treatment with ACC substantially enhanced the abundance of EIN3-GFP in the nucleus (as visualized by DAPI staining; data not shown) (Figure 3B). To gain a more

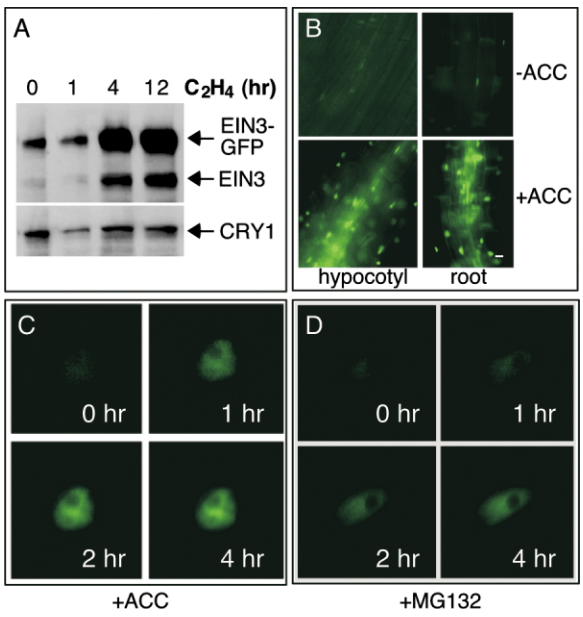

Figure 3. EIN3-GFP Fusion Protein Is Accumulated in the Nucleus upon Ethylene or MG132 Treatment

(A) The levels of EIN3-GFP fusion protein are induced by ethylene.

(B) EIN3-GFP is present in the nucleus (visualized by DAPI staining, data not shown) of both hypocotyl and root cells at low levels in the absence of ACC (−ACC). ACC treatment (+ACC) increases EIN3-GFP nuclear accumulation. Scale bar is equal to 20 μm.

(C) A time course study showing that the induction of EIN3-GFP nuclear accumulation by 50 μM of ACC.

(D) MG132 (50 μM) also induces EIN3-GFP nuclear accumulation.

dynamic view of how ethylene may trigger EIN3-GFP nuclear accumulation, we conducted a time-course study. As shown in Figure 3C, while barely detected in the absence of ACC treatment, EIN3-GFP accumulated in the nucleus after 1 hr of ACC treatment (but not in mock-treated cells, data not shown), and nuclear accumulation continued for at least 4 hr. We next asked whether inhibition of proteasome-mediated proteolysis by MG132 treatment is sufficient for EIN3-GFP to accumulate in the nucleus. Similar to ethylene, treatment of seedling with MG132 also led to rapid nuclear accumulation of EIN3-GFP in the absence of ethylene (Figure 3D). Taken together, we conclude that accumulation of the transcriptional regulator EIN3 in the nucleus is promoted by ethylene or by inhibition of proteasome function.

Two F Box Proteins, EBF1 and EBF2, Interact with EIN3

Several F box proteins have recently been identified as key regulators of plant hormone-signaling pathways (Frugis and Chua, 2002; Vierstra, 2003). Because EIN3 abundance is regulated by a proteasome-dependent pathway, we speculated that one or more specific F box proteins would mediate the EIN3 degradation process. While there are approximately 700 putative F box proteins in the *Arabidopsis* genome, only a few of them have been functionally characterized (Gagne et al., 2002). Using the EIN3 N-terminal domain as bait in yeast two-hybrid screen, we failed to identify any F box protein that could interact with EIN3. We then directly tested the possible interaction between EIN3 full-length protein and individual F box proteins. Using genome-wide microarray experiments, we narrowed the search for EIN3-interacting F box proteins by first testing those genes whose RNA levels were regulated by ethylene (Alonso et al., 2003; H.G. and J.R.E., unpublished data). From this gene list, two closely related candidate F box proteins were identified which were able to interact with EIN3 in yeast two-hybrid assays (Figures 4C and 4E).

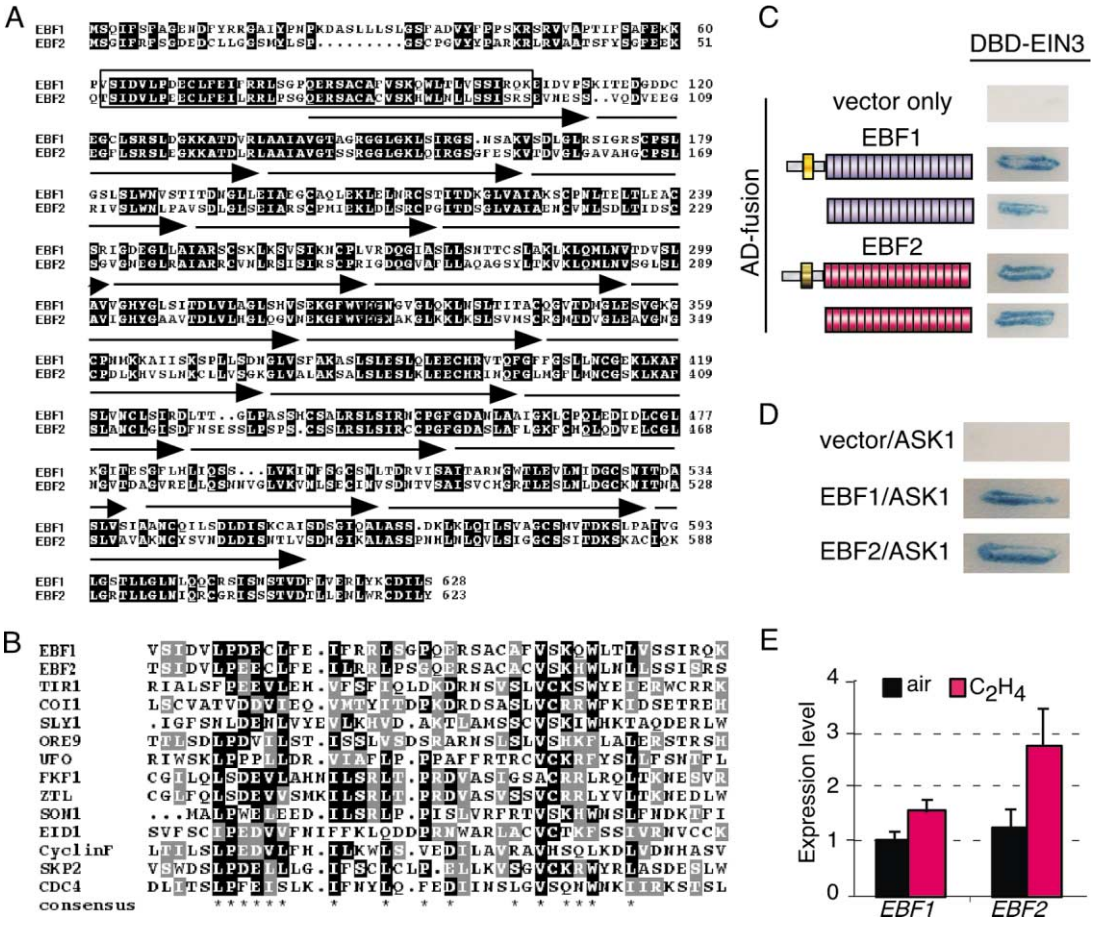

Figure 4. Two F Box Proteins Interact with EIN3

(A) Alignment of EBF1 and EBF2 amino acid sequences generated with ClustalW program. Identical amino acid residues in the two proteins are highlighted with black boxes. The putative F box motif sequences are boxed, and the 18 deduced leucine-rich repeats (LRRs) are indicated by arrows above the sequences.

(B) Alignment of F box motif sequences. Cyclin F and SKP2 are from human, CDC4 from yeast, and all others from *Arabidopsis*. Identities and similarities among the different proteins are highlighted by black and gray, respectively. Consensus residues are denoted by asterisks.

(C) The two F box proteins and their corresponding LRR domains interact with EIN3 in yeast two-hybrid assays.

(D) Both F box proteins interact with the ASK1 protein in yeast two-hybrid assays.

(E) The *EBF1* and *EBF2* genes are induced by ethylene. Etiolated wild-type seedlings were treated with air (air) or ethylene (C_2H_4) for 4 hr before RNA was extracted for analysis. Each treatment was performed in replicate. The means of normalized intensity were used to indicate relative expression levels.

We designated these two F box proteins as EBF1 and EBF2 (for *E*IN3-*b*inding *F* box protein *1* and *2*). EBF1 was previously named as FBL6 with no function assigned (Xiao and Jang, 2000). These two proteins share 57% identity in amino acid sequence (Figure 4A), and each contains a well-conserved F box motif in the amino terminus and 18 tandem leucine-rich repeats (LRRs) in the carboxyl terminus (Figures 4A and 4B). Because LRR domains in several F box proteins contribute to the substrate binding (Gagne et al., 2002), we tested whether the C-terminal LRR domains in EBF1 and EBF2 were sufficient for EIN3 interaction. As shown in Figure 4C, both LRR domains interacted with EIN3 protein in yeast two-hybrid assays. A truncated fragment comprising only the first or the last nine LRRs of EBF1 failed to interact with EIN3 (data not shown), suggesting that the entire LRR domain might be required for EIN3 binding. We also found that EIN3 C-terminal domain is required for EBF1/EBF2 binding (data not shown), providing an explanation for the failure of identifying these two F box proteins in the previous yeast two-hybrid screen. To examine whether EBF1 and EBF2 function as authentic F box proteins, which characteristically associate with Skp1 proteins (Deshaies, 1999), we tested the interaction between the two proteins and ASK1, an *Arabidopsis* Skp1 protein. Both F box proteins were able to interact with ASK1 in yeast two-hybrid assays (Figure 4D). Taken together, we identified two EIN3-interacting F box proteins as candidates for SCF-mediated targeting of EIN3 protein turnover.

ebf1 and *ebf2* Mutants Confer Enhanced Ethylene Responses and Stabilization of EIN3 Protein

To investigate the biological relevance of EBF1 and EBF2 in EIN3 regulation, we isolated mutants that harbor T-DNA insertion mutations in the two F box genes. Two

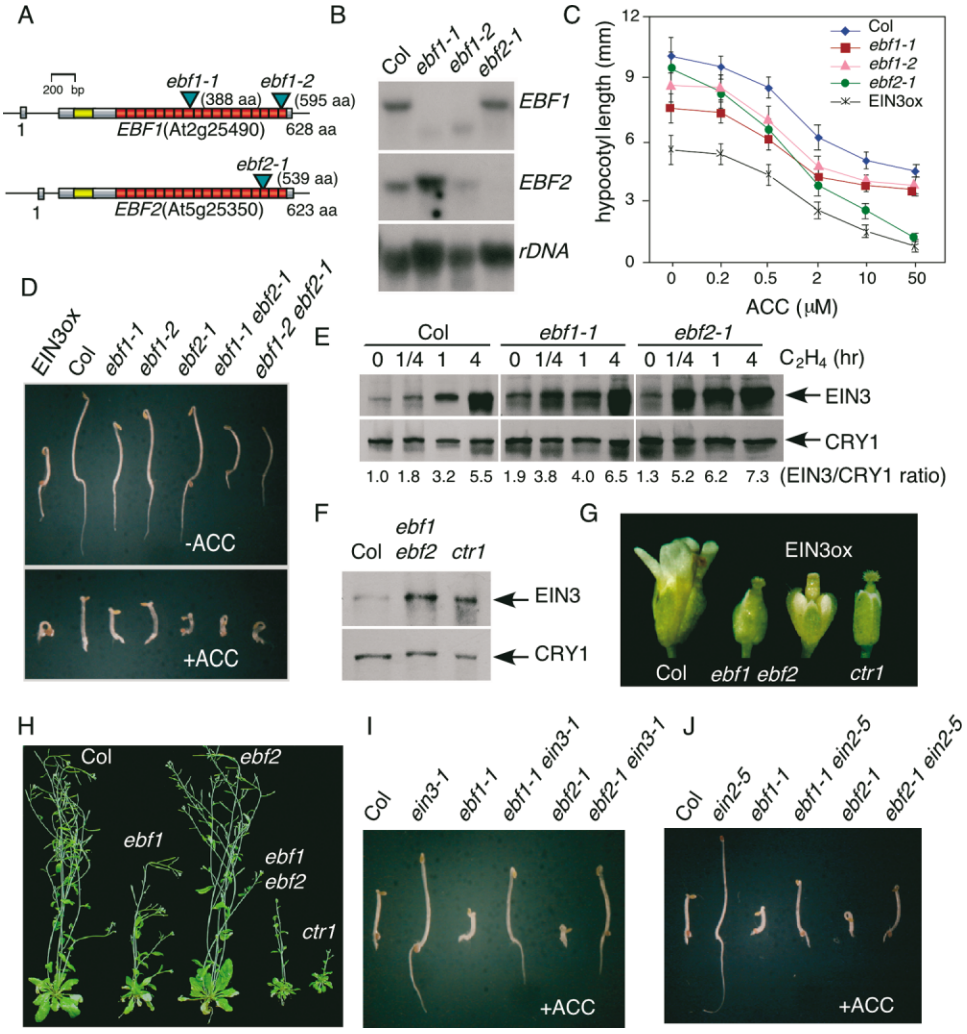

Figure 5. Mutations in the Two F Box Genes Result in Hypersensitivity to Ethylene and Elevated Accumulation of EIN3 Protein

(A) Schematic diagram of the *EBF1* and *EBF2* mutations. Coding regions are represented by boxes while noncoding regions are indicated by lines. F box and LRR motifs are colored with green and red, respectively. A triangle represents a T-DNA insertion event whose position is indicated.

(B) Northern blot analysis of Col, *ebf1-1*, *ebf1-2*, and *ebf2-1* seedlings.

(C) Dosage response of *EBF1* and *EBF2* mutants. Etiolated seedlings were grown on MS medium supplemented with various concentrations of ACC for three days. The length of hypocotyls from ten seedlings was measured, and the mean values and standard deviations were plotted.

(D) Phenotype of 3-day-old etiolated seedlings grown on MS medium supplemented without or with 10 μM of ACC.

(E) Immunoblot assays of EIN3 protein in the F box mutants. The relative intensity of the EIN3 protein was calculated by normalization of the intensity of EIN3 bands with the intensity of the corresponding CRY1 bands.

(F) Immunoblot assays of EIN3 protein in 2-week-old light-grown Col, *ebf1 ebf2*, and *ctr1* plants.

(G) Comparison of Col, *ebf1 ebf2*, EIN3ox, and *ctr1* flowers from 7-week-old plants.

(H) Phenotype of 7-week-old plants of the indicated genotypes.

(I and J) Phenotype of 3-day-old etiolated seedlings grown on MS medium supplemented with 10 μM of ACC.

homozygous *ebf1* mutants (*ebf1-1*, *ebf1-2*) and one homozygous *ebf2* mutant (*ebf2-1*) were obtained (Alonso et al. 2003, see Experimental Procedures). Each of these mutants contained a T-DNA insertion located within the LRR-encoding region (Figure 5A). Northern blot analysis showed that the *ebf1* mutants contained reduced levels of truncated versions of *EBF1* mRNA while the *ebf2* mutant contained no detectable *EBF2* mRNA (Figure 5B). We examined the dose response of these mutants when treated with different concentrations of ACC. Compared with wild-type, both *ebf1-1* and *ebf1-2* mutant alleles were hypersensitive to ACC treatment; although *ebf1-2* was generally less severe than *ebf1-1* (Figures 5C and 5D). *ebf2-1* also displayed hypersensitivity to ACC treatment, and a severe EIN3ox-like phenotype at the higher concentrations of ACC (Figures 5C and 5D). Therefore, both *ebf1* and *ebf2* mutants were found to be hypersensitive to ethylene. We next examined whether *ebf1* and *ebf2* mutations affect EIN3 accumulation. As shown in Figure 5E, the levels of EIN3 protein were higher in *ebf1-1* than in wild-type or *ebf2-1* in the absence of ethylene. Upon ethylene treatment, the

levels of EIN3 in either *ebf1-1* or *ebf2-1* were appreciably higher than those in wild-type. These data indicate that defects in either *EBF1* or *EBF2* lead to increased EIN3 accumulation, and a correspondingly enhanced ethylene response.

To test the genetic interaction of *ebf1* and *ebf2* mutants, we generated *ebf1 ebf2* double-mutant plants. *ebf1 ebf2* etiolated seedlings showed pronounced triple response phenotypes in the absence of exogenous ethylene (Figure 5D). Whereas *ebf1* and *ebf2* single-mutant adult plants were normal except that *ebf1* had modest dwarfism and reduced fertility (Figure 5H), *ebf1 ebf2* double mutants displayed phenotypes characteristic of *ctr1* mutants or plants overexpressing EIN3 (Figures 5G and 5H). *ebf1 ebf2* adult plants showed dwarfism, produced flowers with protruding gynoecium, and developed small size rosette leaves. Moreover, immunoblot analysis revealed that, in the absence of applied ethylene, the level of EIN3 protein was dramatically higher in *ebf1 ebf2* plants than in wild-type plants and similar to the level found in *ctr1* (Figure 5F). Interestingly, *ebf1 ebf2* plants also showed severely reduced fertility, a phenotype observed in EIN2-CENDox plants (Alonso et al.1999). These results reveal that a synergistic interaction exists between *ebf1* and *ebf2* mutants, suggesting a functional redundancy between two EBF proteins and confirming a role for SCF function in the response to ethylene.

To test whether the ethylene hypersensitivity caused by *ebf1* and *ebf2* mutations is the consequence of enhanced EIN3 stability, we generated double mutants between *ein3* and *ebf1* or *ebf2*. As shown in Figure 5I, *ein3-1* suppressed the ethylene phenotypes of both *ebf1-1* and *ebf2-1* mutants, indicating that the *ebf1* and *ebf2* mutants result in enhanced ethylene responsiveness by stabilizing EIN3 protein. Genetic studies revealed that EIN2 is an upstream component activating EIN3 function (Stepanova and Ecker, 2000). To examine the positions of EBF1 and EBF2 relative to other ethylene-signaling components, we generated double mutants between *ein2* and *ebf1* or *ebf2*. Phenotypic analysis revealed that *ebf1-1* and *ebf2-1* partially suppressed *ein2-5* (Figure 5J), suggesting that EBF1 and EBF2 might act downstream of EIN2, although we cannot exclude that these molecules could act in parallel pathways. Taken together, we conclude that EBF1 and EBF2 function as negative regulators of the ethylene-signaling pathway by destabilizing EIN3.

Overexpression of *EBF1* or *EBF2* Results in Reduced Sensitivity to Ethylene

To further define the function of EBF1 and EBF2 in the ethylene-signaling pathway, we constructed transgenic plants containing either F box gene under the control of the CaMV 35S promoter, allowing constitutive high-level (5- to 20-fold increase) expression of RNAs for each of these genes (data not shown). As exemplified by an elongated hypocotyl and opened hypocotyl hook, overexpression of *EBF1* caused partial insensitivity to ethylene, especially in aerial tissues (Figure 6A). Similarly, overexpression of *EBF2* resulted in strong ethylene insensitivity in both aerial and root tissues, comparable to the *ein3* mutant (Figure 6B). Furthermore, overexpression of *EBF2* greatly suppressed the phenotype of the constitutive ethylene overproduction mutant *eto2* (data not shown). To clarify whether the reduced ethylene sensitivity caused by overexpression of *EBF1* or *EBF2* arose from a defect in EIN3 protein accumulation, we monitored the levels of EIN3 in the *EBF1* and *EBF2* overexpression plants. Ethylene-dependent accumulation of EIN3 protein was greatly impaired in transgenic plants overexpressing *EBF1* or *EBF2* compared with wild-type (Figure 6C). Taken together, these results reveal that EIN3-dependent ethylene responses are regulated by two previously uncharacterized F box proteins, EBF1 and EBF2.

Discussion

EIN3 is a plant-specific DNA binding protein that is both necessary and sufficient for the activation of all known ethylene responses. Here, we show that EIN3 accumulation is regulated by ethylene gas at the protein level, and identify two genes (and their corresponding mutants) that function as negative regulators in the ethylene-signaling pathway. Several lines of evidence indicate that the level of EIN3 protein directly reflects the strength of the ethylene signal, and regulation of EIN3 abundance is a rate-limiting step in the ethylene-response pathway. First, the extent of ethylene response arising from different concentrations of ACC is positively correlated with the abundance of EIN3 protein. Second, the level of EIN3 protein is increased by ethylene, and the kinetics of EIN3 induction are comparable with those of the induction of ethylene-responsive genes (e.g., *ERF1*) (Solano et al., 1998). Third, elevated EIN3 levels in transgenic plants (EIN3ox) result in a constitutive ethylene response (Chao et al., 1997). Moreover, the level of EIN3ox protein can be further escalated by ethylene treatment, which consequently leads to even greater ethylene-response phenotypes. Fourth, all ethylene-insensitive mutants that we examined are impaired in the accumulation of EIN3 protein. Likewise, treatment with silver ion, a potent inhibitor of ethylene action, abolishes EIN3 accumulation. On the other hand, in the *ctr1* and *eto* mutants, which display constitutive ethylene response (Guzman and Ecker, 1990; Kieber et al., 1993), steady state EIN3 levels are greater than that of wild-type in the absence of ethylene. Fifth, inhibition of EIN3 proteolysis by mutations in the *EBF1*/*EBF2* genes increases EIN3 accumulation and consequently results in enhanced ethylene responses. Last, promotion of EIN3 degradation by overexpression of *EBF1*/*EBF2* leads to reduced EIN3 abundance and thereby ethylene insensitivity.

Genetic and molecular studies have unraveled a linear ethylene signal transduction pathway, in which EIN3 acts downstream of the ETR/ERS receptors, CTR1 and EIN2 (Stepanova and Ecker, 2000). Consistent with this notion, our biochemical studies show that ETR1, EIN4, CTR1, and EIN2 are all required for the regulation of EIN3 accumulation. In addition, we show that EIN5 and EIN6, whose positions in the ethylene-signaling pathway were previously obscure, are also required for EIN3 accumulation in response to ethylene. These results thus establish that both EIN5 and EIN6 act upstream of EIN3.

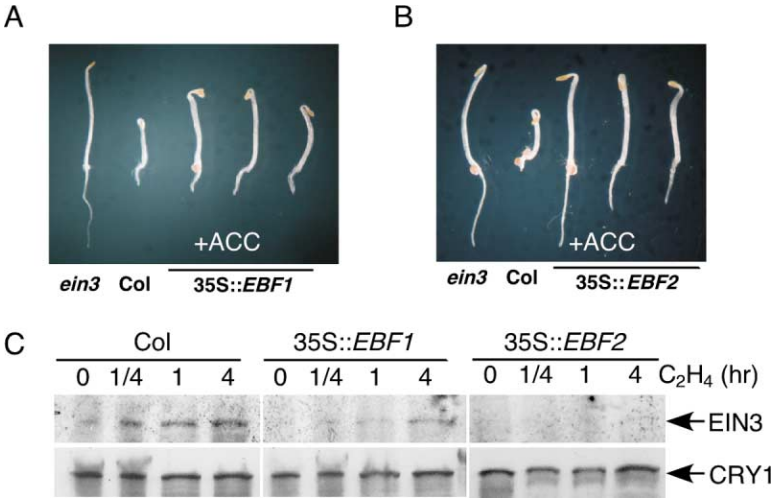

Figure 6. Overexpression of the F Box Genes Results in Ethylene Insensitivity and Reduced Accumulation of EIN3 Protein

(A and B) Phenotype of 3-day-old etiolated seedlings grown on MS medium supplemented with 10 μM of ACC. Three independent transgenic lines that overexpress *EBF1* (A) or *EBF2* (B) in Col background were presented.

(C) Immunoblot assays of EIN3 protein in transgenic overexpression lines.

With the notable exception of *ein2* null mutants, EIN3 can accumulate to some extent after long exposure to ethylene in all other ethylene-insensitive mutants examined (*etr1*, *ein4*, *ein5*, and *ein6*), suggesting that the ethylene signaling is not completely blocked in these plants. Furthermore, the level of EIN3 remains weakly responsive to ethylene in *ctr1-1* mutants, although an elevated basal level is observed in *ctr1-1* in the absence of ethylene. As *ctr1-1* was shown to be a null mutation (Huang et al., 2003), these results suggest the existence of a CTR1-independent response, which adds a new dimension to the linear ethylene pathway. This is consistent with the observation that *ctr1* seedlings respond to ethylene treatment (Larsen and Chang, 2001).

In the absence of ethylene, EIN3 is an unstable protein with a half-life shorter than 30 min. Several short-lived proteins are subject to proteasome-mediated protein degradation process in both animals and plants (Hellmann and Estelle, 2002; Hochstrasser, 1996). For instance, AUX/IAA proteins, a group of transcriptional repressors in auxin signaling, are degraded through a ubiquitin/proteasome pathway (Gray et al., 2001). Similarly, RGA/GAI/SLN proteins, negative components of the GA signaling pathway, are destroyed by a ubiquitin/proteasome pathway (Sasaki et al., 2003; McGinnis et al., 2003). Here, we demonstrate that EIN3 transcription factor, a positive regulator in the ethylene-signaling pathway, is targeted by the ubiquitin/proteasome pathway for degradation. Both endogenous and transgenically overexpressed EIN3 or an EIN3-GFP fusion protein can be stabilized by treatment with proteasome inhibitors (MG132 and MG115), as well as by ethylene treatment. In addition to increasing abundance, exposure to ethylene and MG132 treatment leads to nuclear localization of EIN3 protein. Thus, inhibition of proteasome function is reminiscent of the effects of exogenous ethylene treatment on EIN3 regulation, suggesting that ethylene induces EIN3 accumulation in the nucleus via interfering with the proteasome-mediated EIN3 degradation process. One possibility is that ethylene inhibits the function of proteolytic machinery that specifically targets EIN3 for destruction. Alternatively, ethylene might modulate EIN3 in a posttranslational manner so that EIN3 becomes less accessible or more resistant to the degradation process. It is well known that ubiquitination of a target protein is often preceded by protein phosphorylation (Deshaies, 1999). As many ethylene-signaling components are protein kinases (Gamble et al., 1998; Huang et al., 2003; Ouaked et al., 2003), it is conceivable that EIN3 may be phosphorylated, which could trigger EIN3 ubiquitination/degradation.

We have identified two F box proteins (EBF1 and EBF2) that interact with EIN3, and demonstrated the involvement of these two proteins in EIN3 regulation as well as in the ethylene-response pathway. Loss-of-function mutations in the two F box genes (*ebf1* and *ebf2*) resulted in increased EIN3 accumulation, and consequently, enhanced response to ethylene. *ebf1 ebf2* double mutants showed a wide range of *ctr1*-like phenotypes including the constitutive triple response in etiolated seedlings, protruding gynoecium, small size rosette and dwarfism in adult plants. Genetic studies revealed that *ein3* suppresses the monogenic *ebf1* and *ebf2* mutants (this study) as well as the *ebf1 ebf2* double mutants (Potuschak et al., 2003 [this issue of *Cell*]), indicating that the function of these two F box proteins is dependent upon the presence of EIN3. Moreover, transgenic overexpression of either F box gene leads to reduced EIN3 abundance and a corresponding decrease in sensitivity to ethylene. While monogenic *ebf1* and *ebf2* mutants slightly suppress *ein2* (this study), *ebf1 ebf2* double-mutant plants show strong suppression (or bypass) of *ein2* (Potuschak et al., 2003), suggesting that EBF1/EBF2 acts downstream of or parallel with EIN2. Together, these results demonstrate that EBF1 and EBF2 play a negatively regulatory role in the ethylene-signaling pathway by targeting EIN3 for degradation.

Based on these results, we propose a model for EIN3 regulation by ethylene (Figure 7). In the absence of ethylene, the ER-associated ethylene receptors (e.g., ETR1) are active, and constitutively activate the Raf-like kinase CTR1. CTR1 then represses a MAPKK and MAPK cascade (Ouaked et al., 2003), and further inhibits the function of downstream components including EIN2, EIN5, and EIN6 (Wang et al., 2002). As a result, EIN3 is targeted and ubiquitinated by the SCF complex containing one

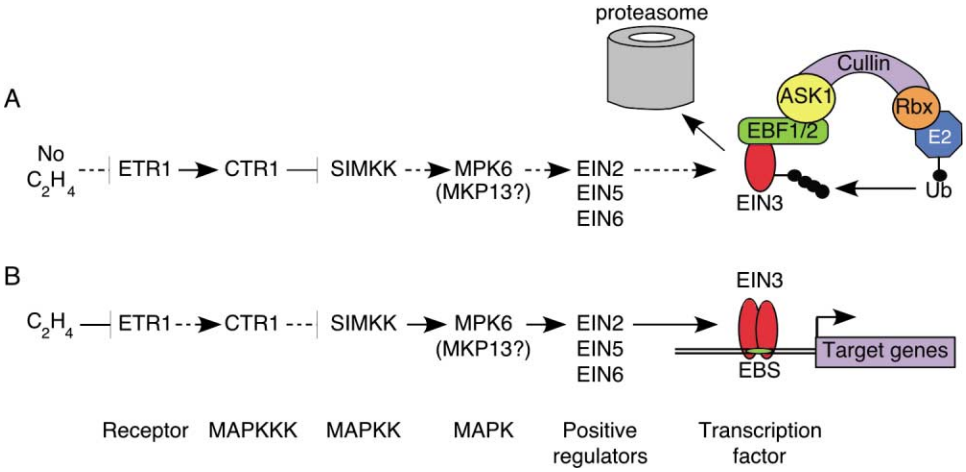

Figure 7. A Proposed Model on How EIN3 Is Regulated by Ethylene

(A) In the absence of ethylene, the ETR/ERS receptors and CTR1 are active, thereby repress a MAPKK/MAPK cascade and inhibit the function of downstream components including EIN2, EIN5, and EIN6. As a result, EIN3 is targeted and ubiquitinated by a SCF complex containing one of the two F box proteins, EBF1 and EBF2. The ubiquitinated form of EIN3 protein is thus recruited to the 26S proteasome for degradation.

(B) In the presence of ethylene, the ETR/ERS receptors are bound with and inactivated by ethylene. CTR1 is inactive and the repression on the downstream pathway is released. Thus, the signal is transmitted through MAPKK/MAPK, EIN2, EIN5, and EIN6 to EIN3 transcription factor. As a result, EIN3 protein accumulates in the nucleus and binds to EIN3 binding site (EBS), which in turn activates gene expression.

Arrows and bars represent positive and negative regulations, respectively. The solid and dotted lines indicate that the signal is on and off, respectively.

of the two F box proteins, EBF1 and EBF2. The ubiquitinated form of EIN3 protein is thus recruited to the 26S proteasome for degradation (Figure 7A). In the presence of ethylene, the ETR/ERS receptors are inactivated by ethylene binding. Without a positive regulatory signal from the receptors, CTR1 is inactive. Thus, the downstream positive regulators in the pathway are derepressed, and the signal is transmitted through EIN2, EIN5, and EIN6 to EIN3 transcription factor. As a result, EIN3 protein becomes "resistant" to the SCF/proteasome-mediated proteolysis, accumulates in the nucleus, and binds to EIN3 binding site (EBS) and activates gene expression (Figure 7B). Interestingly, the RNA levels of both *EBF1* and *EBF2* genes are induced by ethylene (Figure 4E), suggesting that a negative-feedback mechanism may exist to allow fine tuning of EIN3 protein level.

This study (and the accompanying work of Potuschak et al., 2003) reveals that the gaseous hormone ethylene exerts its effect on plant growth by controlling the abundance of EIN3 transcription factor. In addition, a link has been established between the ethylene-signaling pathway and the ubiquitin/proteasome pathway, which has recently become the paradigm for plant hormone signaling. A ubiquitin/proteasome pathway has been demonstrated in auxin, GA, and JA signaling, and implicated in the ABA, SA, cytokinin, and brassinosteroid responses (reviewed by Frugis and Chua, 2002; Vierstra, 2003). It is worth noting that the ubiquitin/proteasome pathway positively regulates the auxin, GA, and JA signaling pathway by targeting negative regulators for degradation (Gray et al., 1999; Sasaki et al., 2003; Xie et al., 1998). The corresponding hormone acts to promote the repressors' degradation. In contrast, the ubiquitin/proteasome pathway mediated by EBF1/EBF2 negatively regulates the ethylene-signaling pathway by targeting EIN3 transcription factor for degradation. Ethylene acts to stabilize EIN3 protein by preventing its degradation process. A similar regulatory mechanism might also occur in the ABA signaling pathway, mediated by the bZIP transcription factor ABI5 (Lopez-Molina et al., 2001), and in the brassinosteroid-signaling pathway, mediated by two nuclear proteins BES1/BZR1 (He et al., 2002; Yin et al., 2002). In both of these cases, the corresponding hormone has been shown to alter the stability of these positive regulators, although no F box proteins or other SCF components have been identified that target these proteins for degradation. In this regard, ethylene is able to trigger an EIN3-mediated response rapidly by blocking protein turnover, rather than by initiating the more time-consuming route of de novo transcription/translation. This "jump-start" feature of the ethylene-response pathway might be vital for the role of ethylene as an endogenous stress hormone, especially in the rapid response to wounding or various plant pathogens.

Experimental Procedures

Plant Growth Conditions, Drug Treatments, and RNA Blot Analysis

With the exception of *eto2* in Landsberg *erecta* (L*er*) background, the ecotype Columbia (Col-0) was the parent strain for all mutant and transgenic lines used in this study. *Arabidopsis* seeds were surface-sterilized and plated on the surface of MS medium (4.3 g MS salts, 10 g sucrose, [pH 5.7], 8 g bactoagar per liter). After 3–4 days in the light at 4°C, the plates were wrapped in foil and kept in a 24°C incubator before the phenotypes of seedlings were analyzed. For propagation, seedlings from plates were transferred to soil (Promix-HP) and grown to maturity at 22°C under a 16 hr light/8 hr dark cycle.

Ethylene treatment of *Arabidopsis* seedlings grown on plates was performed in containers by flowing through hydrocarbon-free air supplemented with 10 ppm (parts per million) ethylene or were treated with hydrocarbon-free air alone (Kieber et al., 1993). For

drug treatments, *Arabidopsis* suspension cell cultures were treated with MG132 (50 μM), MG115 (50 μM), Pefabloc SC (100 μM), or DMSO (0.1%) for a time course study. Alternatively, etiolated seedlings were germinated for three days on a disc of Whatman paper resting on the surface of MS medium. MG132 (50 μM), MG115 (50 μM), Pefabloc SC (100 μM), or DMSO (0.1%) was added to the paper discs for 4 hr. Cycloheximide (100 μM) was added to the paper discs and ethylene or air was applied for different amounts of time.

Total RNA extractions and Northern blot analysis were performed as described (Chao et al., 1997).

Antibody Preparation and Immunoblot Assays

The coding region corresponding to residues 349–581 of *EIN3* protein was PCR-amplified, expressed, purified from E. *coli*, and used to raise polyclonal antibodies in rabbits. Immunoblot assays were performed as described (Lin et al., 1995) with minor modifications. Protein samples were prepared by homogenizing the liquid nitrogen ground tissues in 2× SDS-PAGE sample buffer and boiling the homogenate for 5 min. After centrifugation, the protein extracts were fractionated by 4%–12% gradient Tris-Glycine Novex precast gels (Invitrogen), blotted onto a nitrocellulose filter. The blot was probed first with anti-EIN3 antibody, and was subsequently stripped with 0.2 N glycine [pH 2.5] for 3 times and reprobed with either anti-CRY1 antibody (Lin et al., 1995) or anti-SUB1 antibody (Guo et al., 2001).

Yeast Two-Hybrid Assays

The cDNA sequences of the *EIN3*, *ASK1*, F box genes (Yamada et al., 2003), and their derivatives were cloned into pAS2 or pACT2 vector (Clontech). Yeast transformation, growth conditions, and filter-lift assays for β-galactosidase activity were performed according to the manufacturer's instructions (Clontech).

Isolation of T-DNA Insertion Lines and Genetic Analysis

To identify knockouts in the *EBF1* and *EBF2* genes, we first searched the database of Salk T-DNA collections (Alonso et al., 2003) and identified one mutant line, *ebf1-1*. We next screened a pooled genomic DNA collection containing approximately 80,000 T-DNA tagged lines by a PCR-based method (Alonso et al., 2003) and two additional mutants (*ebf1-2* and *ebf2-1*) were identified. Plants homozygous for the insertions were identified by PCR-based genotyping. Cosegregation between phenotype of *ebf1* or *ebf2* mutant and the corresponding T-DNA insertion was established in the segregation population of backcross lines. Double mutants were constructed by genetic crosses and homozygous lines were identified by PCR-based genotyping.

Construction of Transgenic *Arabidopsis* Plants

EIN3 cDNA was cloned into the binary vector CHF3-GFP (Yin et al., 2002). The resulting 35S::EIN3-GFP construct was introduced into Agrobacterium strain C58 and subsequently transformed into *Arabidopsis* wild-type (Col-0) and *ein3-1* plants (Bechtold and Pelletier, 1998). Kanamycin-resistant T1 plants were selected by plating seeds on MS medium supplemented with 1% sucrose and 50 μg/ml kanamycin. The triple-response phenotype was scored in T2 seedlings originated from individual kanamycin-resistant T1 plants. Transgenic seedlings that expressed a functional EIN3-GFP fusion protein were mounted on glass slides using PBS as a mounting medium. All images were collected on a fluorescence microscope (Olympus BX60) and the color of the images was artificially added in Photoshop 5.0 (Adobe Systems). For the time course experiment, after the first cell image was taken, 50 μM of ACC or MG132 was applied to the slide and the same cell was followed for 4 hr.

The binary vector pKYLX7 was modified by inserting a loxP site in the MCS region (H. Li and J.R.E., unpublished data). The *EBF1* and *EBF2* full-length cDNA sequences were cloned into pUNI15 vector at NdeI/BamHI site (a gift from Dr. Stephen Elledge). An in vitro plasmid fusion reaction, catalyzed by Cre recombinase, was carried out between pUNI15 (containing F box cDNA sequence) and the modified pKYLX7. The resulting constructs that harbor the F box coding regions driven by CaMV 35S promoter were introduced into Agrobacterium strain C58 and subsequently transformed into *Arabidopsis* plants. Transgenic T1 plants were identified by Kanamycin selection. The triple response phenotype was scored in T2 seedlings originated from individual transgenic T1 plants. Homozygous T3 seedlings were subjected to ethylene treatment and immunoblot assays.

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Accession Numbers

AY01933 and AY485830 are the accession numbers for the EBF1 and EBF2 cDNA clones, respectively.