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Ethylene-Induced Stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 Is Mediated by Proteasomal Degradation of EIN3 Binding F-Box 1 and 2 That Requires EIN2 in *Arabidopsis* [C][W]

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Plant responses to ethylene are mediated by regulation of EBF1/2-dependent degradation of the ETHYLENE INSENSITIVE3 (EIN3) transcription factor. Here, we report that the level of EIL1 protein is upregulated by ethylene through an EBF1/2-dependent pathway. Genetic analysis revealed that EIL1 and EIN3 cooperatively but differentially regulate a wide array of ethylene responses, with EIL1 mainly inhibiting leaf expansion and stem elongation in adult plants and EIN3 largely regulating a multitude of ethylene responses in seedlings. When *EBF1* and *EBF2* are disrupted, EIL1 and EIN3 constitutively accumulate in the nucleus and remain unresponsive to exogenous ethylene application. Further study revealed that the levels of EBF1 and EBF2 proteins are downregulated by ethylene and upregulated by silver ion and MG132, suggesting that ethylene stabilizes EIN3/EIL1 by promoting EBF1 and EBF2 proteasomal degradation. Also, we found that EIN2 is indispensable for mediating ethylene-induced EIN3/EIL1 accumulation and EBF1/2 degradation, whereas MKK9 is not required for ethylene signal transduction, contrary to a previous report. Together, our studies demonstrate that ethylene similarly regulates EIN3 and EIL1, the two master transcription factors coordinating myriad ethylene responses, and clarify that EIN2 but not MKK9 is required for ethylene-induced EIN3/EIL1 stabilization. Our results also reveal that EBF1 and EBF2 act as essential ethylene signal transducers that by themselves are subject to proteasomal degradation.

INTRODUCTION

Ethylene, a simple gaseous phytohormone, triggers a wide range of physiological and morphological responses in plants, such as inhibition of cell expansion, promotion of leaf and flower senescence, induction of fruit ripening and abscission, and resistance to pathogens and insect attack (Abeles et al., 1992; Bleecker and Kende, 2000). One of the most widely documented ethylene responses is the triple response of etiolated seedlings. For instance, in the presence of ethylene or its biosynthetic precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), dark-grown *Arabidopsis thaliana* seedlings develop a short, thickened root and hypocotyl with exaggerated curvature of the apical hook (Abeles et al., 1992; Ecker, 1995; Roman and Ecker, 1995).

Based on this highly reproducible and specific phenotype, a number of ethylene response mutants that show an aberrant triple response phenotype have been identified in *Arabidopsis* (Bleecker et al., 1988; Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995; Lehman et al., 1996; Hua et al., 1998; Sakai et al., 1998; Larsen and Chang, 2001; Larsen and Cancel, 2003; Resnick et al., 2006). Genetic and molecular characterization of those mutants has defined a largely linear ethylene signal transduction pathway from hormone perception at the endoplasmic reticulum membrane to transcriptional regulation in the nucleus (Chen et al., 2005). In *Arabidopsis*, ethylene is perceived by a family of membrane-associated receptors that are similar to bacterial two-component His kinases (Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998; Chen et al., 2002). The ethylene receptors are negative regulators of the signaling pathway, and ethylene binding leads to functional inactivation of the receptors (Hua and Meyerowitz, 1998). One of the inactivation mechanisms is 26S proteasome-mediated protein degradation of the receptors, at least for ETR2 (Chen et al., 2007). In the absence of ethylene, the active receptors recruit CTR1, a Raf-like Ser/Thr kinase, to associate with the membrane and thus become activated (Kieber et al., 1993; Gao et al., 2003). This subsequently represses the downstream signaling pathway mediated by ETHYLENE INSENSITIVE2 (EIN2) and EIN3. EIN2 is a

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membrane-spanning protein whose biochemical function is unknown, although genetic studies indicate that it is absolutely required for ethylene signaling (Alonso et al., 1999). Recently, it has been shown that EIN2 is recognized by two F-box proteins, EIN2-TARGETING PROTEIN1 (ETP1) and ETP2, for proteasomal degradation (Qiao et al., 2009). EIN2 was also reported to interact with several components of the COP9 signalosome (CSN) (Christians et al., 2008), a deneddylation enzyme required to sustain optimal activity of SCF-type (for Skp1, Cul1, F-box protein, and Rbx1) E3 ligase (Lyapina et al., 2001; Cope et al., 2002). Mutation in a putative CSN subunit gene (*EER5*) indeed leads to hypersensitivity to ethylene (Christians et al., 2008), implying that EIN2 might modulate CSN function to optimize the activity of SCF ligases in ethylene signaling.

Ethylene signaling downstream of EIN2 is mediated by EIN3, a plant-specific transcription factor (Chao et al., 1997; Solano et al., 1998). EIN3 is a short-lived protein that is degraded by a ubiquitin/26S proteasome pathway in the absence of ethylene. Upon ethylene treatment, EIN3 protein is quickly stabilized and accumulates in the nucleus (Guo and Ecker, 2003; Potuschak et al., 2003; Yanagisawa et al., 2003; Gagne et al., 2004). Genetic and biochemical studies revealed that EIN3 proteolysis is mediated by two F-box proteins, EBF1 and EBF2 (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). It has been shown that EBF1 and EBF2 have distinct roles in regulating EIN3 stability (Guo and Ecker, 2003; Gagne et al., 2004; Binder et al., 2007). EBF1 exerts its effect primarily in the absence of ethylene and during the initial phase of the response of etiolated seedlings to ethylene, whereas EBF2 plays a more prominent role during the later stages of the response and the resumption of seedling growth following ethylene removal (Binder et al., 2007). Interestingly, the expression of *EBF2* is transcriptionally induced by EIN3 that directly binds to the promoter of *EBF2*, thus allowing a negative feedback regulation to desensitize ethylene signaling (Konishi and Yanagisawa, 2008).

An important question remaining to be answered is how EBF1/EBF2-mediated EIN3 proteolysis is repressed by ethylene. Two alternative models have been suggested in which the ethylene signal either directly modulates EIN3 or inhibits the function of $SCF^{EBF1/EBF2}$ (Guo and Ecker, 2003; Kepinski and Leyser, 2003; Potuschak et al., 2003; Gagne et al., 2004). A recent study reported that the MKK9, one of the 10 *Arabidopsis* mitogen-activated protein kinase (MAPK) kinases, and its associated MAPKs, MPK3 and MPK6, are activated by ethylene and the MAPK cascade acts positively in the ethylene signaling pathway downstream of CTR1 (Yoo et al., 2008). MKK9-activated MPK3/6 was reported to phosphorylate EIN3 and stabilize it, and accordingly, the *mkk9* mutant displays a partial ethylene insensitivity phenotype (Yoo et al., 2008). The authors concluded that the MKK9-MPK3/6 module acts downstream of receptors/CTR1 and bypasses EIN2 to modulate EIN3 directly (Yoo et al., 2008), supporting the first model. Differing from this scenario, however, two independent studies demonstrated that the same MPK3/6, together with their upstream MKK9 or MKK4/5, function in the ethylene biosynthesis pathway by augmenting the protein stability of ACS2 and ACS6 as well as inducing their gene expression (Liu and Zhang, 2004; Joo et al., 2008; Xu et al., 2008). These studies failed to detect MPK6 activation induced by ACC, a precursor of ethylene biosynthesis (Liu and Zhang, 2004), which was reinforced by another report that ethylene signaling lies downstream of MPK6 activation (Bethke et al., 2009). Therefore, the role of the MKK9-MPK3/6 module in the ethylene signaling pathway is somewhat controversial and awaits clarification (Ecker, 1994).

There are five EIN3 homologs (EIL1 to EIL5) in the *Arabidopsis* genome, among which EIN3 and EIL1 are the most closely related. Overexpression of *EIL1* complements the *ein3* mutation and results in constitutive activation of the ethylene response pathway (Chao et al., 1997). Although the *eil1* mutant shows modest ethylene insensitivity, the *ein3 eil1* double mutant displays strong ethylene insensitivity phenotypes in terms of the triple response and the defense response (Alonso et al., 2003a) and represses the constitutive ethylene response phenotype of the *ebf1 ebf2* double mutant (Binder et al., 2007). Collectively, these genetic studies indicate that EIL1 is another positive regulator of ethylene responses, although the precise functions and activation mode of EIL1 in the ethylene signaling pathway have not been examined.

In this study, we investigated the role of EIL1 in several developmental responses to ethylene and found that EIL1 is a critical nuclear regulator that works cooperatively with, but distinctly from, EIN3 to regulate myriad ethylene responses. We also explored the regulatory mechanism by which ethylene enhances EIN3 and EIL1 stability and discovered that ethylene stabilizes EIN3/EIL1 by inducing EBF1/EBF2 proteasomal degradation. Furthermore, we examined the requirement of EIN2 and MKK9 for ethylene signal transmission and concluded that EIN2 but not MKK9 is indispensible for ethylene-induced EIN3/EIL1 stabilization.

RESULTS

EIL1 Is a Nuclear Protein That Is Stabilized by Ethylene and MG132

EIL1 is the closest homolog of EIN3 in the *Arabidopsis* genome. Genetic analysis revealed EIL1 as a positive regulator of ethylene responses (Chao et al., 1997; Alonso et al., 2003a; Binder et al., 2007). To determine how EIL1 is regulated by ethylene, we first monitored the level of EIL1 protein using an anti-EIL1 antibody. The protein levels of EIL1 increased in wild-type Columbia-0 (Col-0) plants after 1 h of hormone treatment, whereas no EIL1 protein was detected in the *ein2* mutant (Figure 1A). We also found that EIL1 protein overaccumulated in *eto1* (an ethylene overproduction mutant) or *ctr1* (a constitutive ethylene response mutant) compared with wild-type Col-0 (see Supplemental Figure 1A online). Conversely, application of silver ion blocked the ethylene-induced EIL1 accumulation (see Supplemental Figure 1B online). These results indicate that the levels of EIL1 protein are increased by ethylene.

We next examined whether the ubiquitin/proteasome pathway is involved in the ethylene-induced EIL1 accumulation. We treated *Arabidopsis* suspension cell cultures derived from wild-type hypocotyls with a 26S proteasome inhibitor, MG132 (Lee and Goldberg, 1998). After 4 h of MG132 treatment, the levels of

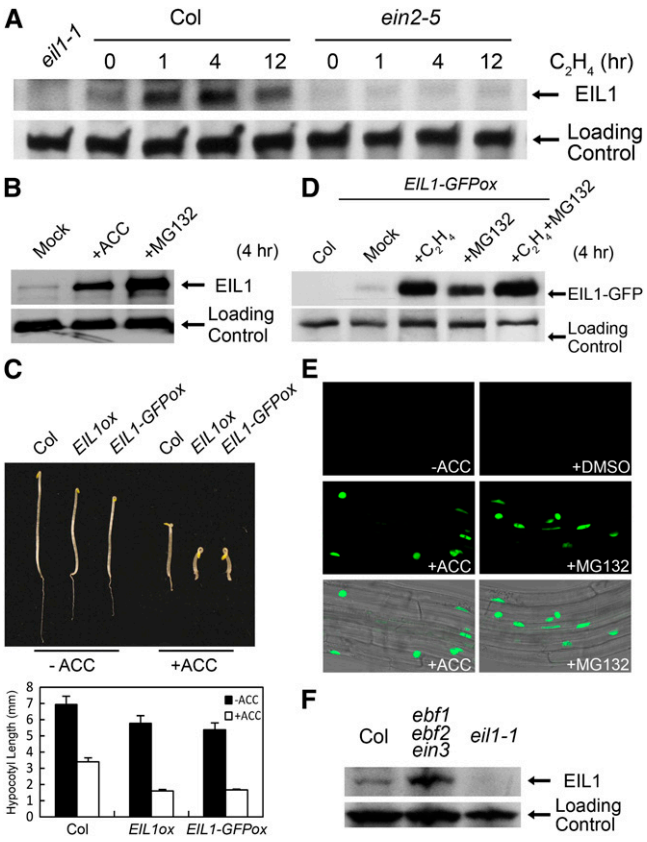

Figure 1. EIL1 Is a Nuclear Protein That Is Stabilized by Ethylene and the Proteasome Inhibitor MG132.

(A) Ethylene treatment stabilizes EIL1 protein in wild-type Col-0 but not in *ein2* mutant. Etiolated seedlings grown on MS medium for 4 d were treated with ethylene (C_2H_4, 20 ppm) gas for 0, 1, 4, and 12 h before tissues were harvested for immunoblot assays using anti-EIL1 antibody. A nonspecific band was used as a loading control.

(B) MG132 treatment stabilizes EIL1 protein. Suspension cell cultures derived from wild-type Col-0 were treated with mock (0.5% DMSO), ACC (100 μM), or MG132 (50 μM) for 4 h before tissues were harvested for immunoblot assays.

(C) Overexpression of EIL1 (*EIL1ox*) or EIL1-GFP (*EIL1-GFPox*) caused ethylene hypersensitivity. **(Top)** Three-day-old etiolated seedlings grown on MS medium supplemented without or with 10 μM ACC. **(Bottom)** Quantification of hypocotyl lengths in the top panel. Each bar represents the average length (±SD) of at least 20 seedlings. Experiments were repeated three times with similar results.

(D) Ethylene and MG132 treatments stabilize EIL1-GFP protein. Six-day-old light-grown *EIL1-GFPox* seedlings (Col seedlings used for control) were treated with mock (0.5% DMSO), ethylene (20 ppm), and MG132 (50 μM) alone or in combination for 4 h before tissues were harvested for immunoblot assays.

(E) ACC and MG132 treatments promote EIL1-GFP protein accumulation in the nucleus. Four-day-old etiolated seedlings were treated with 100 μM ACC (MS medium used for control) or 50 μM MG132 (0.5% DMSO in MS medium used for control) for 4 h before tissues were used for detecting GFP inflorescence (the top four panels show ×40 GFP images; the bottom two show differential interference contrast and GFP merged).

(F) EIL1 protein overaccumulates in the *ein3 ebf1 ebf2* triple mutant. Proteins were extracted from 2-week-old light-grown seedlings and were subjected to immunoblots with anti-EIL1 antibody.

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EIL1 protein markedly increased (Figure 1B), suggesting the involvement of the 26S proteasome in EIL1 proteolysis.

The EIL1 protein sequence contains a number of predicted nuclear localization signals (Chao et al., 1997). To determine whether EIL1 is a nuclear protein, it was C-terminally tagged with green fluorescent protein (GFP) and expressed in *Arabidopsis* plants under the control of cauliflower mosaic virus 35S promoter. Overexpression of EIL1 in the wild-type background (*EIL1ox*) gave rise to a hypersensitive response to ethylene, manifested by remarkably shorter hypocotyls and roots than the wild type had upon ACC treatment (Figure 1C). Overexpression of EIL1-GFP (*EIL1-GFPox*) resulted in an ethylene hypersensitive phenotype similar to that of *EIL1ox* (Figure 1C), confirming the functionality of this fusion protein in plants. We examined the GFP fluorescence in *EIL1-GFPox* and found that ACC or MG132 treatment substantially enhanced the abundance of EIL1-GFP in the nucleus (Figures 1D and 1E). Therefore, EIL1 is a nuclear protein, and its accumulation in the nucleus is enhanced by ethylene or proteasome inhibition.

EIL1 and *EIN3* Mutations Differentially Suppress *ebf1/ebf2* Single and Double Mutants

Both EIN3 and EIL1 were previously shown to interact with EBF1/EBF2 in the yeast two-hybrid assays (Potuschak et al., 2003). To assess whether EIL1 proteolysis is also mediated by the $SCF^{EBF1/EBF2}$ complex that degrades EIN3, we examined EIL1 protein accumulation in plants lacking EBF1 and EBF2 functions. Given the difficulty to obtain homozygous lines of the *ebf1-1 ebf2-1* double mutant because they were severely growth arrested in early seedling stages (see below), we compared EIL1 protein levels in wild-type Col-0 and the *ein3-1 ebf1-1 ebf2-1* triple mutant, which was partially fertile and able to set enough seeds for experimental analysis. As shown in Figure 1F, the level of EIL1 protein was increased in the triple mutant when compared with the wild type, suggesting that like EIN3, EIL1 is subject to $SCF^{EBF1/EBF2}$-mediated degradation.

It has been reported that EBF1 and EBF2 have overlapping but distinct roles in ethylene signaling (Binder et al., 2007). To investigate whether the functional differences between EBF1 and EBF2 arise from their specific dependences on EIN3 and EIL1, we tested the genetic interactions between EBF1/EBF2 and EIN3/EIL1. We generated various combinations of the double and triple mutants. The *eil1-1* mutant largely suppressed the hypersensitivity phenotype of the etiolated *ebf1-1* seedlings but had only a marginal effect on the *ebf2-1* seedlings. Conversely, the *ein3* mutation suppressed the phenotypes of *ebf1-1* and *ebf2-1* equally well (Figures 2A and 2B). Soil-grown *ebf1-1* plants are modestly dwarfed (Guo and Ecker, 2003; Figures 2C and 2D). We found that *eil1-1* almost completely suppressed the dwarfism phenotype of *ebf1-1*, whereas *ein3-1* had little effect (Figures 2C and 2D), suggesting that EIL1 plays a predominant role in inhibiting EBF1-induced stem elongation.

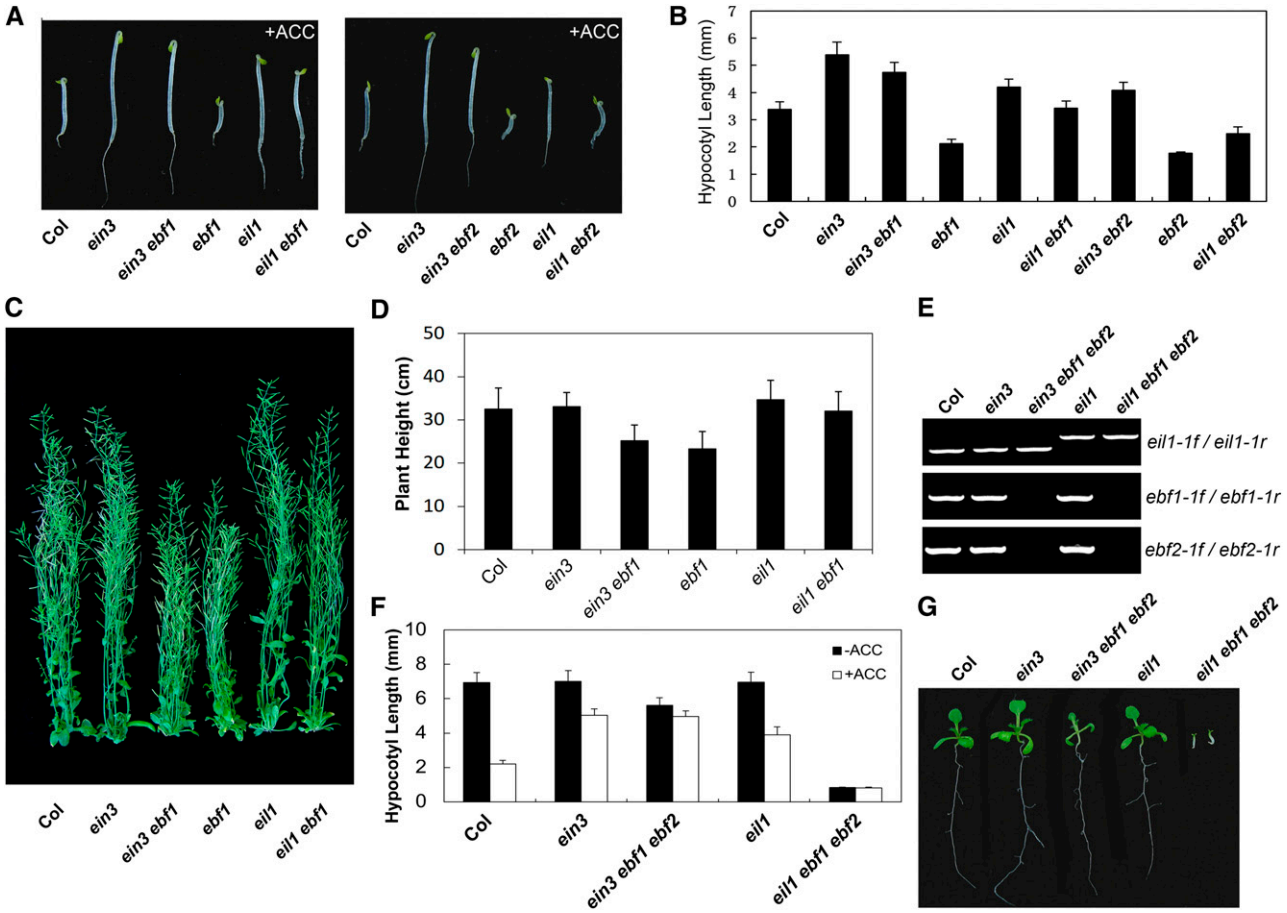

Figure 2. *EIL1* Mutation Partially Suppresses Phenotypes of the *ebf1*/*ebf2* Single and Double Mutants.

(A) The triple response phenotype of 3-d-old etiolated seedlings grown on MS medium alone or supplemented with 10 μM ACC.
(B) Graphical quantification of hypocotyl lengths in **(A)**.
(C) Fifty-day-old soil-grown plants, showing that the semidwarf phenotype of the *ebf1* mutant is rescued by the *eil1*, but not the *ein3*, mutation.
(D) Graphical quantification of plant height in **(C)**. Each bar represents the average length (±SD) of at least 10 plants. *eil1 ebf1* shows a statistically significant difference (Student's *t* test) compared with *ebf1* ($P < 0.001$), whereas *ein3 ebf1* does not ($P > 0.1$).
(E) Genomic DNA analyses of *eil1 ebf1 ebf2* triple mutant by PCR. *eil1-1* contains a transposon insertion, and the amplification band is ~200 bp larger than that of the wild type. Both *ebf1-1* and *ebf2-1* contain T-DNA insertion, and no band is amplified under the amplification conditions. The primers used are labeled on the right. The detailed primer sequence information is provided in Supplemental Table 1 online.
(F) Graphical quantification of hypocotyl lengths of 3-d-old etiolated seedlings grown on MS medium supplemented without or with 10 μM ACC.
(G) Ten-day-old light-grown seedlings. The *eil1 ebf1 ebf2* triple mutant was growth arrested at the seedling stage.
Each bar in **(B)** and **(F)** represents the average length (±SD) of at least 20 plants. Experiments were repeated three times with similar results.
[See online article for color version of this figure.]

We had previously obtained the *ebf1-1 ebf2-1* double mutant that shows severe growth defects and complete infertility (Guo and Ecker, 2003). After a few generations, it became increasingly difficult to obtain homozygous *ebf1-1 ebf2-1* progeny from the self-pollinated heterozygous parents. A strong growth arrest phenotype was also observed in another allele of the *ebf1 ebf2* double mutant (*ebf1-3 ebf2-3*) that dies at its seedling stage (Gagne et al., 2004). We found that loss of EIN3 function rescued the seedling lethality of the *ebf1-1 ebf2-1* mutant, suggesting that overaccumulation of EIN3 contributes to the severe growth arrest phenotype observed in the *ebf1 ebf2* mutants. To determine whether EIL1 also contributes to the defects caused by the *EBF1/EBF2* mutations, we generated the *eil1 ebf1 ebf2* triple mutant (Figure 2E). Three-day-old etiolated seedlings of this mutant displayed a strong constitutive triple response phenotype with an extremely short and swollen hypocotyl and virtually no primary root when grown on Murashige and Skoog (MS) medium. Treatment with ACC had no additional effect on the phenotype of the triple mutant (Figure 2F). Light-grown *eil1 ebf1 ebf2* seedlings were severely stunted with tiny cotyledons after germination (Figure 2G). The cotyledons gradually became yellow and the mutant plants died before true leaves emerged. Therefore, unlike *ein3*, *eil1* is unable to suppress the growth arrest phenotype of the *ebf1 ebf2* mutant, implying EIN3 (but not

EIL1) functions as a master regulator that restrains seedling growth and development when it accumulates excessively. Taken together, these results demonstrate that EIN3 and EIL1 play distinct roles in suppressing the functions of EBF1 and EBF2.

EIL1 Acts Cooperatively with EIN3 in the Regulation of EBF1/EBF2-Mediated Ethylene Responses

We noticed that although *ein3 ebf1 ebf2* was able to finish its life cycle, this triple mutant differed from the wild type by displaying numerous ethylene response phenotypes, including reduced hypocotyl length, a compact rosette with small and epinastic leaves, a dwarfed and bushy stature, and a reduction of apical dominance and seed fertility (see Supplemental Figure 2 online). This constitutive ethylene response phenotype can be explained by the elevated accumulation of EIL1 protein in the mutant (Figure 1F). In agreement with this possibility, we found that inactivation of one copy of *EIL1* suppressed numerous ethylene-related phenotypes of the *ein3 ebf1 ebf2* triple mutant, including rosette size, leaf epinasty, plant height, apical dominance, and infertility (see Supplemental Figure 2 online). Loss of both copies of *EIL1* (i.e., in the *ein3 ebf1 ebf2 eil1* quadruple mutant) completely suppressed *ein3 ebf1 ebf2* mutant phenotypes, such that the quadruple mutant was phenotypically indistinguishable from the *ein3 eil1* double mutant (see Supplemental Figure 2 online). Combined with the observation that the phenotype of *eil1* mutant is nearly wild-type, the remarkable suppression of *ein3 ebf1 ebf2* by *eil1* reveals that EIL1 dose-dependent regulation of a wide array of ethylene responses is unmasked when EBF1 and EBF2 are genetically inactivated.

Taken together, these studies demonstrate that EIL1 plays a pivotal role in regulating a subset of ethylene responses (rosette size, leaf epinasty, plant stature, and fertility), particularly manifested in the absence of the EBF1/EBF2 and EIN3 functions. In addition, the nearly complete suppression of the *ebf1 ebf2* defects by loss of *EIN3* and *EIL1* functions led us to speculate that EIN3 and EIL1 are the primary targets of EBF1/EBF2 and that both transcription factors work cooperatively to regulate myriad ethylene responses. It is also postulated that the excessive accumulation of EIN3/EIL1 proteins (e.g., in the case of *ebf1 ebf2* mutations) is responsible for the severe growth arrest and various developmental defects throughout the plant life cycle.

Loss of EBF1/EBF2 Functions Leads to Nuclear Accumulation of EIL1 and Nonresponse to Exogenous Ethylene

From the above studies, we observed that the *ein3 ebf1 ebf2* mutant exhibited almost complete insensitivity to the exogenously applied ethylene (see Supplemental Figures 2A and 2B online), implying that the endogenous EIL1 protein accumulating in the triple mutant is not responsive to ethylene. This was in contrast with the ethylene-hypersensitive phenotype of *EIL1ox* and *EIL1-GFPox* (Figure 1D), in which EBF1/EBF2 are present. To assess further whether loss of EBF1/EBF2 eliminates the sensitivity of EIL1 to ethylene, we made transgenic *Arabidopsis* plants that expressed an EIL1-GFP fusion protein driven by the cauliflower mosaic virus 35S promoter in the *ein3 ebf1 ebf2* mutant (*OE/tm*). Of 45 independent transgenic lines obtained, 39 transformants displayed reduced stature and lower fertility when compared with the *ein3 ebf1 ebf2* parent line (Figure 3A). In addition, these *OE/tm* plants produced flowers with stunted petals and protruding gynoecia (Figure 3B), morphological characteristics of the *ctr1* mutant, the *etr1 ers1* double mutant, and EIN2-CEND–overexpressing plants (Kieber et al., 1993; Alonso et al., 1999; Hall and Bleecker, 2003; Qu et al., 2007). In support of this observation, we found that in the petals of the *OE/tm* plants, EIL1-GFP was constantly detectable in the nucleus even without hormone treatment (Figure 3C). Consistent with this, the expression of ethylene-responsive genes *ACO2* and *ERF1* was induced in the *OE/tm* plants (Figure 3D), indicative of a constitutively activated ethylene signaling pathway arising from EIL1-GFP overaccumulation.

Because *EIL1-GFPox* plants exhibited an ethylene hypersensitivity phenotype (Figure 1C), which could be explained by further accumulation of EIL1-GFP protein in the nucleus upon ACC treatment (Figures 1D and 1E), we next determined whether this is the case for the *OE/tm* plants. Two representative *OE/tm* lines (*OE2/tm* and *OE3/tm*) that displayed a moderately and strongly activated ethylene response phenotype in seedlings, respectively, were selected for further analysis (Figure 3E). However, measurements of hypocotyl length revealed that the ethylene response phenotype of both plants was not exacerbated by ACC treatment (Figure 3E). Accordingly, we detected comparable accumulation of EIL1-GFP in the nuclei of the *OE/tm* hypocotyls with or without ACC application (Figure 3F). Collectively, these results indicate that, when EBF1 and EBF2 are functionally disrupted, EIL1 protein is unable to be further stabilized and activated by the addition of ethylene. In other words, EBF1/EBF2 are required for the transmission of the ethylene signal to regulate EIL1 accumulation and function.

EBF1/EBF2 Are Indispensable Ethylene Signaling Components That Act Genetically Downstream of CTR1 and EIN2

We also noted that *eil1 ebf1 ebf2* was not responsive to exogenous ethylene (Figure 2F), in contrast with the ethylene hypersensivity of the *EIN3ox* plants (Guo and Ecker, 2003), suggesting that ethylene-induced EIN3 accumulation might also rely on the presence of EBF1/EBF2. As *ebf1 ebf2* and *eil1 ebf1 ebf2* mutants are seedling-lethal, we generated transgenic plants expressing estradiol-inducible EIN3-FLAG in the *ein3 eil1 ebf1 ebf2* quadruple mutant (*iE/qm*) to test this possibility. The *iE/qm* seedlings displayed an estradiol concentration-dependent constitutive ethylene response phenotype (i.e., application of higher concentrations of estradiol resulted in shorter hypocotyls and roots) (Figure 4A). However, when grown in the presence of both estradiol and ACC, the transgenic etiolated seedlings were indistinguishable from those on medium with estradiol alone (Figures 4B and 4C), indicative of their complete insensitivity to ethylene application. Immunoblot analysis showed that the accumulation of EIN3-FLAG fusion protein was induced in *iE/qm* transgenic plants by estradiol in a dose-dependent manner, but ACC treatment did not further increase its protein accumulation

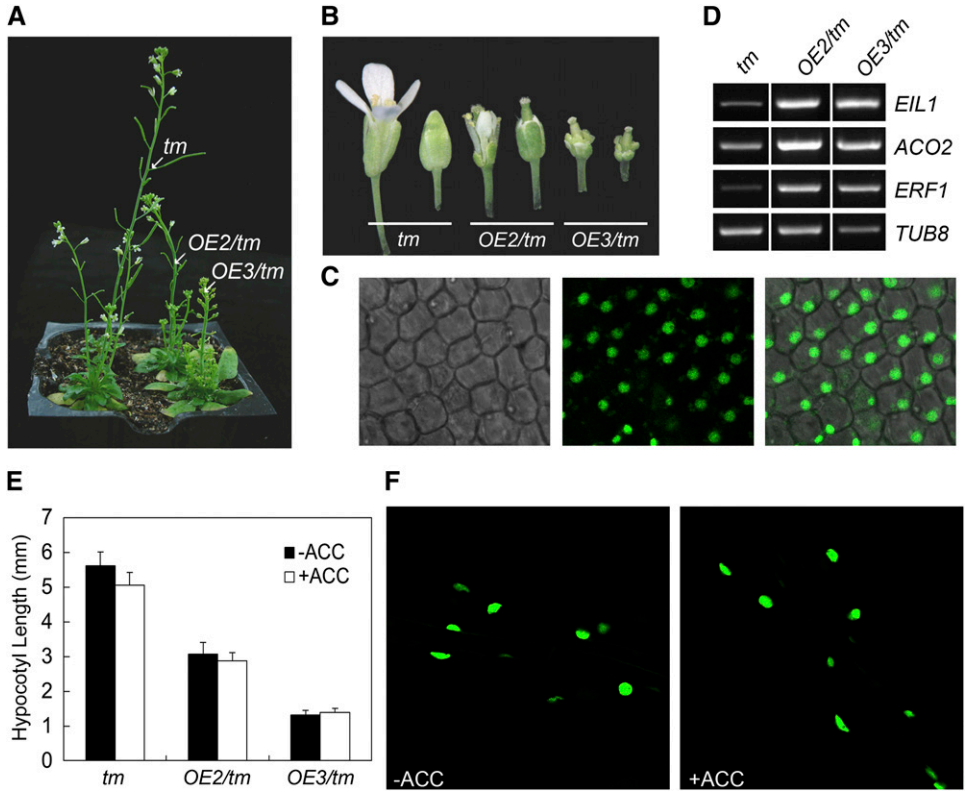

Figure 3. A Functional EIL1-GFP Protein in the *ein3 ebf1 ebf2* Triple Mutant Is Not Responsive to Exogenous Ethylene.

(A) Forty-five-day-old transgenic plants overexpressing EIL1-GFP in *ein3 ebf1 ebf2* (*OE2/tm* and *OE3/tm*). The *ein3 ebf1 ebf2* triple mutant (*tm*) is shown for comparison.

(B) Flowers of the *ein3 ebf1 ebf2* triple mutant (*tm*) and *OE/tm* transgenic plants, showing the stunted sepal/petal and protruding gynoecium in *OE/tm*.

(C) Constitutive accumulation of the EIL1-GFP fusion protein in the nuclei of the *OE2/tm* petals (from left to right, ×40 DIC, ×40 GFP, and DIC and GFP merged).

(D) RT-PCR analysis of *EIL1* and ethylene marker gene expression. RNA extracted from 45-d-old soil-grown plants was used.

(E) Graphical quantification of hypocotyl lengths of *OE/tm* etiolated seedlings grown on MS medium without or with 10 μM ACC for 3 d. Each bar represents the average length (±SD) of at least 20 seedlings.

(F) Constitutive accumulation of EIL1-GFP in the nuclei of the *OE3/tm* hypocotyl tissue without or with ACC treatment.

regardless of inducer concentration (Figure 4D). We also found that the expression levels of *ERF1* (a direct target gene of EIN3) were similarly induced by estradiol in a concentration-dependent manner but remained unaffected by ACC treatment (Figure 4E). Taken together, these results indicate that the EBF1 and EBF2 proteins are indispensable for ethylene-induced EIN3 accumulation and activation. It is thus conceivable that, once EIN3/EIL1 proteins accumulate by means of removing EBF1/EBF2, they are sufficient to activate gene expression and ethylene responses, while ethylene is not able to further enhance their stability and activity.

By demonstrating EBF1/EBF2 as essential ethylene signal transducers regulating EIN3/EIL1 stability, we next studied the genetic relationships between EBF1/EBF2 and the signaling components upstream of EIN3/EIL1 (e.g., CTR1 and EIN2). Since strong alleles of the *ebf1 ebf2* double mutant are seedling-lethal, whereas the *ebf1 ebf2 ein3* triple mutant is viable, we generated *ein2 ein3 ebf1 ebf2* and *ctr1 ein3 ebf1 ebf2* quadruple mutants. Interestingly, these two quadruple mutants showed nearly identical etiolated seedling and adult plant phenotypes to *ein3 ebf1 ebf2* but clearly differed from *ctr1* or *ein2* (Figure 5). In addition, these two quadruple mutants were not responsive to ethylene treatment (Figures 5A and 5B). These data suggest that EBF1 and EBF2 are ethylene signaling pathway components that act genetically downstream of CTR1 and EIN2 but upstream of EIN3/EIL1.

The Protein Levels of EBF1 and EBF2 Are Downregulated by Ethylene but Upregulated by MG132

The data presented above indicate that EBF1 and EBF2 are essential signaling components necessary for ethylene responsiveness. We next investigated how EBF1 and EBF2 are regulated by ethylene. Our initial effort to produce polyclonal antibodies that could detect the endogenous EBF1 or EBF2 protein in plant tissues proved to be unsuccessful. Therefore, we generated transgenic *Arabidopsis* plants (*35S:EBF1-TAP* and *35S:EBF2-TAP*) overexpressing the EBF1 or EBF2 protein fused

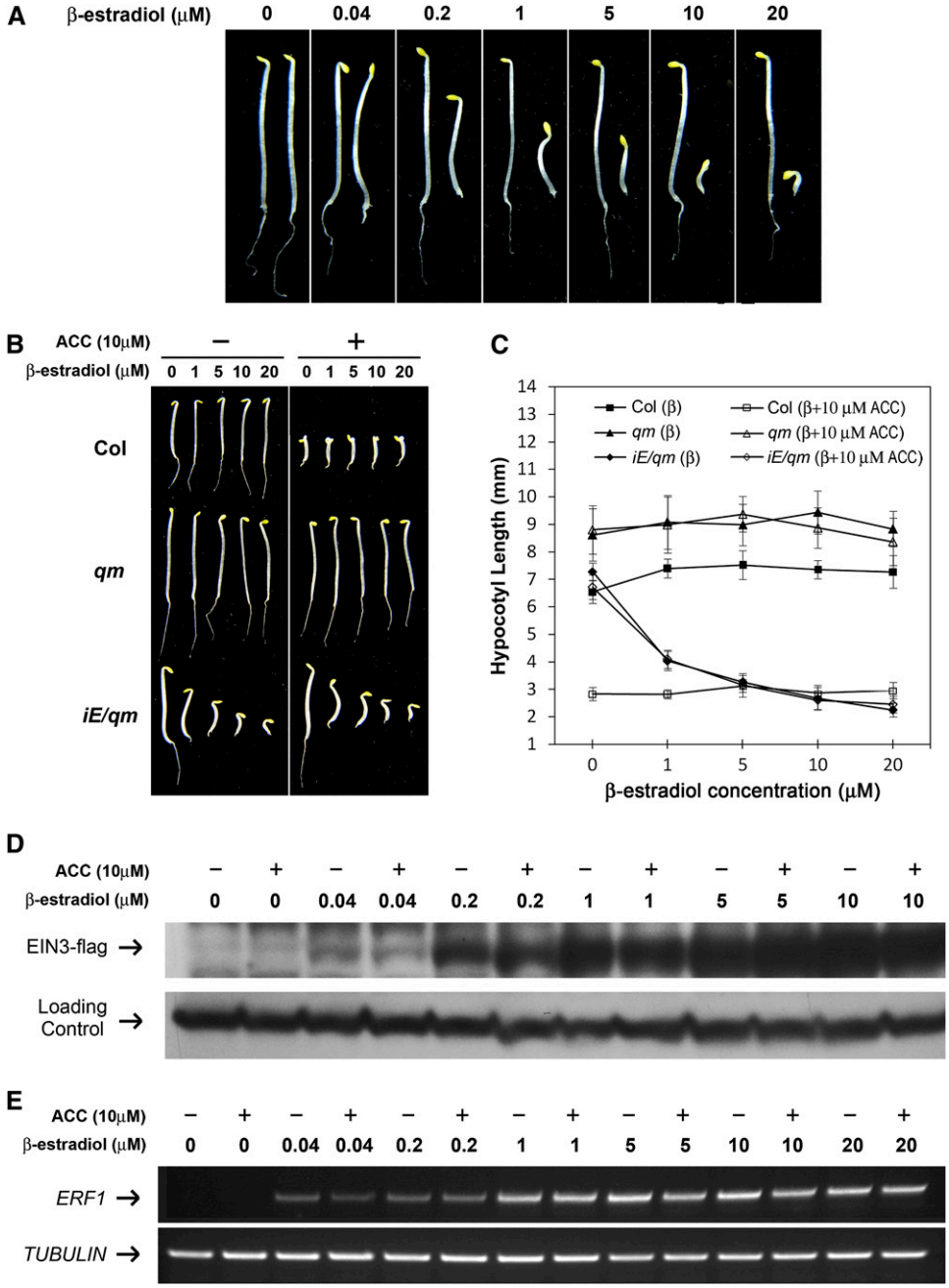

Figure 4. A Functional EIN3-FLAG Protein in the *ein3 eil1 ebf1 ebf2* Quadruple Mutant Is Not Responsive to Exogenous Ethylene.

(A) The triple response phenotype of 3-d-old *ein3 eil1 ebf1 ebf2* (*qm*, left seedling in each image) and estradiol-inducible *EIN3-FLAG* in the *qm* background (*iE/qm*, right seedling in each image) etiolated seedlings grown on MS medium supplemented with increased concentrations of estradiol.
(B) Response to ACC (10 μM) of 3-d-old Col, *qm*, and *iE/qm* etiolated seedlings grown on MS medium supplemented with variable concentrations of estradiol.
(C) Graphical quantification of hypocotyl lengths in response to various concentrations of estradiol with or without ACC (10 μM). Each bar represents the average length (±SD) of at least 20 seedlings.
(D) ACC treatment does not enhance the accumulation of EIN3-3FLAG fusion protein. Proteins were extracted from 6-d-old light-grown seedlings treated with indicated concentrations of estradiol for 8 h, treated with or without 100 μM ACC for another 4 h, and subjected to immunoblots using anti-FLAG antibody.
(E) RT-PCR analysis of *ERF1* expression in *iE/qm* transgenic plants. Total RNA was prepared from 6-d-old light-grown transgenic plants treated with indicated concentrations of estradiol for 8 h and then treated with or without 100 μM ACC for another 4 h.
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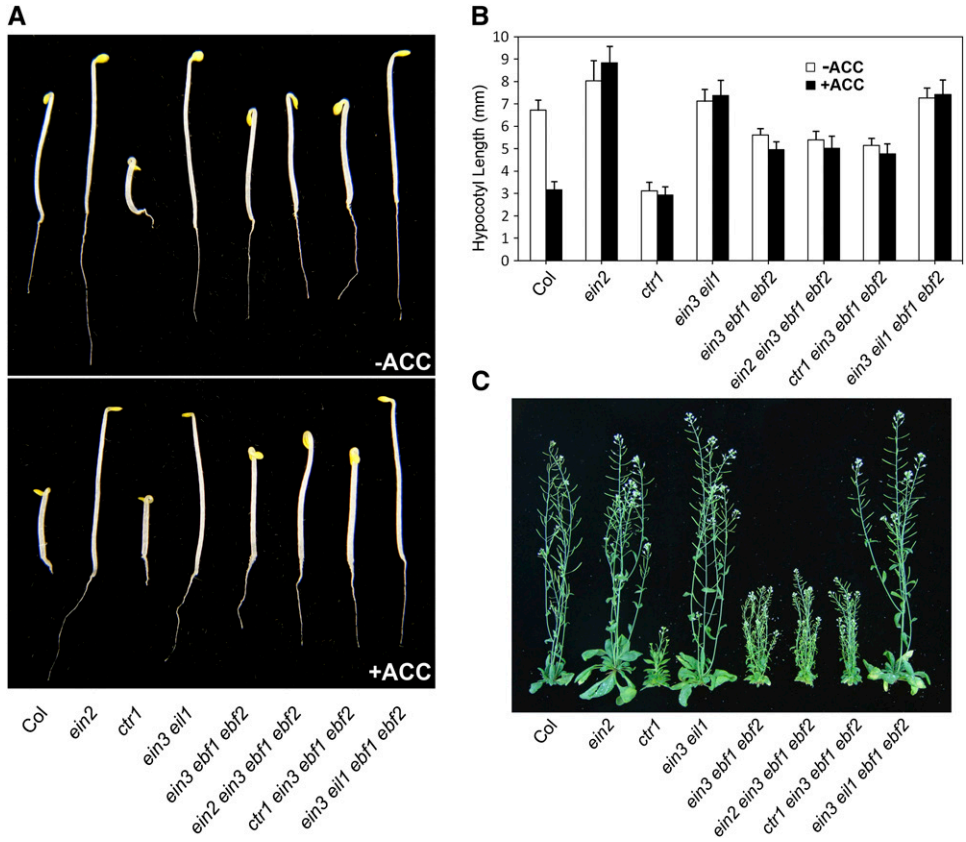

Figure 5. EBF1/EBF2 Genetically Act Downstream of CTR1 and EIN2.

(A) The triple response phenotype of 3-d-old etiolated seedlings grown on MS medium with or without 10 μM ACC.
(B) Graphical quantification of hypocotyl lengths of various mutants in **(A)**. Each bar represents the average lengths (±SD) of at least 20 seedlings.
(C) Sixty-day-old representative plants grown on soil, showing that neither the *ein2* nor the *ctr1* mutation was able to suppress the phenotype of *ein3 ebf1 ebf2*, which was fully suppressed by the *eil1* mutation.
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with the tandem affinity purification (TAP) tag (Rubio et al., 2005). Etiolated *35S:EBF1-TAP* and *35S:EBF2-TAP* seedlings displayed an ethylene insensitivity phenotype (Figure 6A), indicating that these fusion proteins are functional in planta. Immunoblot analysis with anti-MYC (part of the TAP tag) antibody showed that the protein levels of EBF1-TAP markedly decreased upon ACC treatment (Figure 6B). By contrast, treatment with silver ion (Ag^+) promoted a dramatic accumulation of EBF1-TAP and reversed the ACC-induced EBF1-TAP degradation (Figure 6B). Similar regulations on EBF2-TAP were also observed, although ACC-induced EBF2-TAP proteolysis was less evident than it was for EBF1-TAP (Figure 6C). This could be due to an increased effect of endogenously produced ethylene on EBF2-TAP proteolysis, as the basal level of EBF2-TAP was much lower than that of EBF1-TAP (Figure 6C). Thus, ethylene promotes EBF1/EBF2 protein degradation, providing a possible mechanism by which ethylene induces EIN3/EIL1 accumulation.

Since the proteolysis of EBF1 was rapid (within 1 h;Figure 6B), we then investigated whether proteasome-mediated proteolysis is involved in EBF1/EBF2 proteolysis. Treatment with the proteasome inhibitor MG132 dramatically enhanced EBF1 and EBF2 protein levels and blocked the ACC-induced EBF1/EBF2 protein degradation (Figures 6B and 6C).

We also generated the transgenic *Arabidopsis* plants *35S:EBF1-GFP* and *35S:EBF2-GFP* expressing the EBF1 or EBF2 protein fused with GFP and confirmed their functionality in planta (Figure 6A). The basal GFP fluorescence in the transgenic plants grown on MS medium was hardly detectable (Figure 6D). However, treatments with Ag^+ and MG132, but not ACC, caused remarkable accumulation of EBF1-GFP and EBF2-GFP in the nucleus (Figure 6D). Taken together, these results indicate that ethylene promotes proteasomal degradation of EBF1 and EBF2 proteins.

EIN2 Is Required for Ethylene-Induced EIN3 Stabilization and EBF1/EBF2 Degradation

Our previous data and current results demonstrate that EIN2 is required for ethylene-induced EIN3 and EIL1 accumulation, as no EIN3 or EIL1 protein is detectable in *ein2* (Guo and Ecker, 2003; Figure 1A). However, a recent study reported that ethylene bypasses EIN2 to activate a MKK9-MPK3/MPK6 cascade that

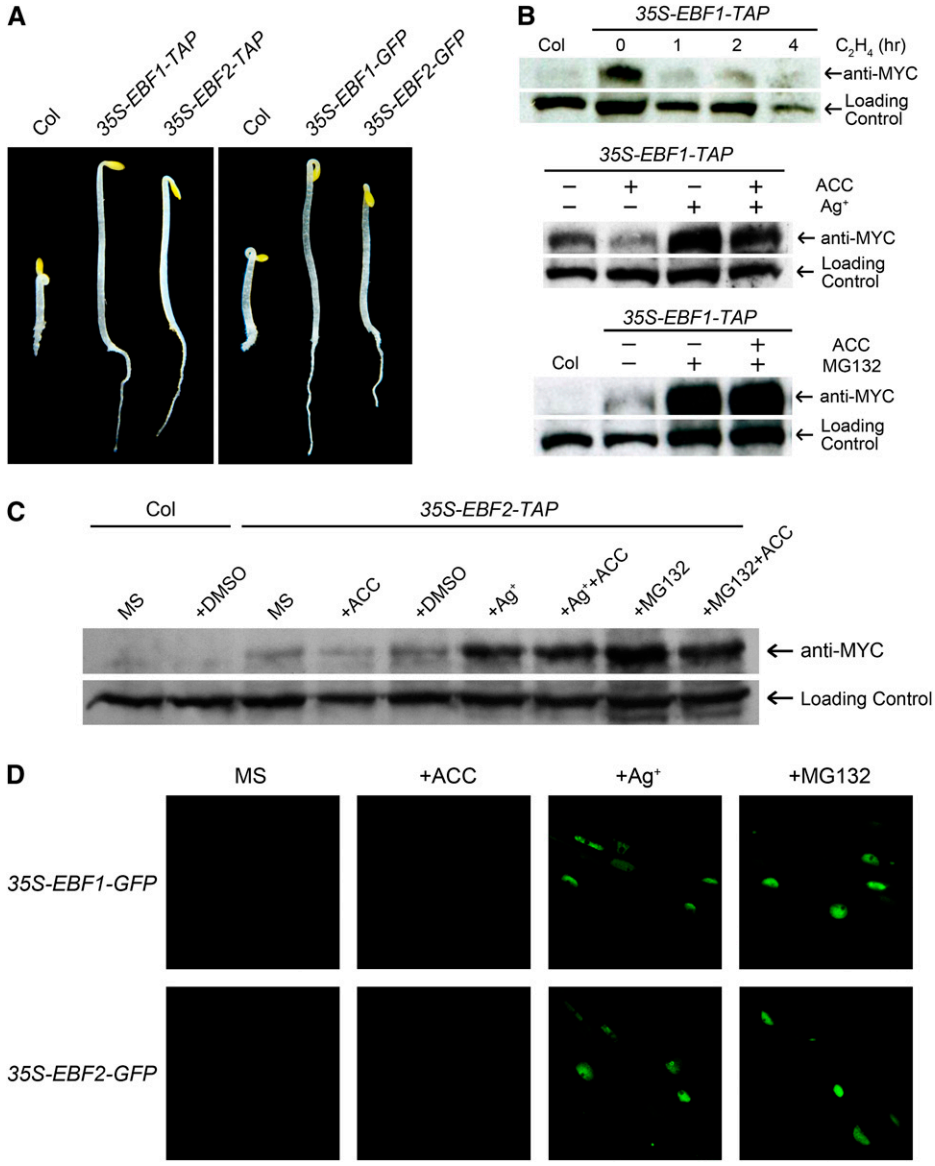

Figure 6. The Levels of EBF1/EBF2 Proteins Are Upregulated by Ag^+ and MG132.

(A) Reduced ethylene sensitivity of *35S-EBF1-TAP*, *35S-EBF2-TAP*, *35S-EBF1-GFP*, and *35S-EBF2-GFP* etiolated seedlings grown on MS medium supplemented with 10 μM ACC for 3 d.

(B) Immunoblot assay of EBF1-TAP protein (detected by anti-MYC antibody) in 6-d-old light-grown *35S-EBF1-TAP* seedlings.

(C) Immunoblot assay of EBF2-TAP protein (detected by anti-MYC antibody) in 6-d-old light-grown seedlings.

(D) Treatments with Ag^+ and MG132 but not ACC promoted the accumulation of EBF1-GFP and EBF2-GFP in the nuclei of the transgenic seedlings. Seedlings grown on MS medium were treated with ethylene (20 ppm) for the indicated time, or ACC (100 μM), $AgNO_3$ (20 μM for GFP detection and 100 μM for immunoblot assays), and MG132 (50 μM) for 4 h before being subjected to immunoblot assays or GFP detection.

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directly phosphorylates and thus stabilizes EIN3 (Yoo et al., 2008). To ascertain the role of EIN2 in the ethylene signaling that leads to the stabilization of EIN3/EIL1, we constructed transgenic plants that overexpressed EIN3 in the *ein2* mutant background. Two alternative approaches were taken toward this end. First, we crossed *ein2* with a previously generated EIN3-overexpressing transgenic plant (*EIN3ox*) (Chao et al., 1997), and the double homozygous lines were obtained. Second, we isolated several alleles of *EIN3ox ein2* from a genetic screen that was originally designed to identify mutations that suppressed *EIN3ox* phenotypes. Of the six isolated *seo* (for suppressor of EIN3 overexpression) mutants, five were found to be new *ein2* mutant alleles (see Supplemental Figure 3A online). Compared with the parent *ein2* and *EIN3ox* plants, etiolated *EIN3ox ein2*

seedlings exhibited an intermediate phenotype in terms of hypocotyl and root lengths (Figure 7A). We detected a reduced EIN3 protein level in *EIN3ox ein2-5* progeny compared with that of *EIN3ox* parent plants (Figure 7B), providing an explanation for the intermediate phenotype.

Notably, the *EIN3ox ein2* and five *seo* seedlings were completely unresponsive to ethylene application, similar to *ein2* (Figure 7A; see Supplemental Figure 3B online). In addition, treatment with ACC failed to enhance the accumulation of EIN3 protein in *EIN3ox ein2-5* (Figure 7B). We also found that the expression levels of *ERF1* and *EBF2* were not further increased by ethylene in the *EIN3ox ein2* plant (Figure 7C). By contrast, ethylene application enhanced EIN3 protein accumulation and *ERF1* and *EBF2* gene expression in the *EIN3ox* plants (Figures 7B

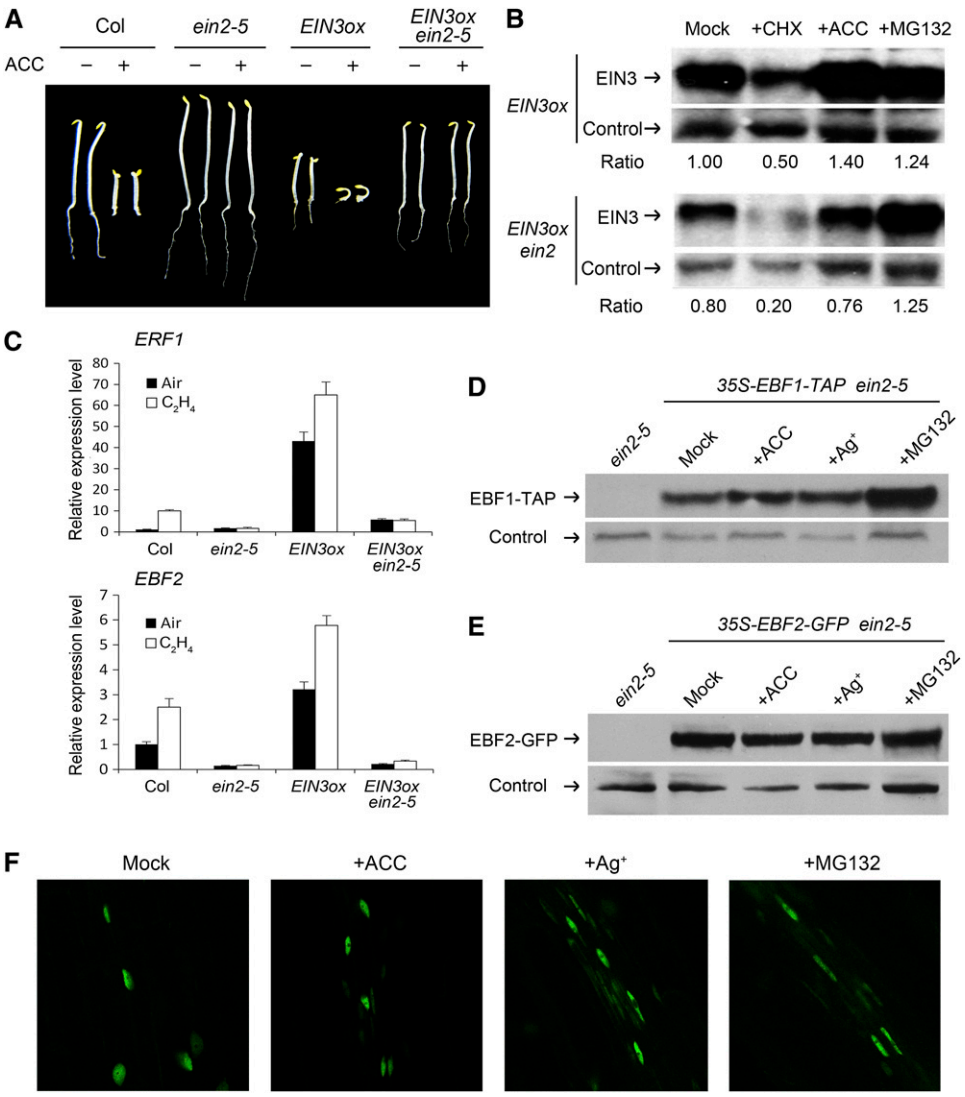

Figure 7. EIN2 Is Required for Ethylene-Induced EIN3 Stabilization and EBF1/EBF2 Degradation.

(A) The triple-response phenotype of 3-d-old etiolated seedlings grown on MS medium supplemented with or without 10 μM ACC.
(B) Immunoblot assay of EIN3 protein in *EIN3ox* and *EIN3ox ein2* seedlings treated with mock (0.5% DMSO), ACC (100 μM), CHX (100 μM) or MG132 (50 μM) for 4 h. The relative EIN3 protein levels were calculated after normalization with loading controls and listed. Experiments were repeated two times with similar results.
(C) Analysis of ethylene-regulated gene expression (*ERF1* and *EBF2*) by quantitative real-time RT-PCR for 6-d-old light-grown seedlings treated with or without ethylene (20 ppm) for 4 h. Data presented are mean values of three biological repeats with standard deviation.
(D) Immunoblot assay of EBF1-TAP protein (detected by anti-MYC antibody) in 6-d-old light-grown *35S:EBF1-TAP ein2* seedlings.
(E) Immunoblot assay of EBF2-GFP protein (detected by anti-GFP antibody) in 6-d-old light-grown *35S:EBF2-GFP ein2* seedlings.
(F) The GFP fluorescence was detected in the roots of 3-d-old *35S:EBF2-GFP ein2* etiolated seedlings treated with 100 μM ACC, 20 μM $AgNO_3$, or 50 μM MG132 for 4 h.
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and 7C). Collectively, these data suggest an essential role for EIN2 in mediating ethylene-induced EIN3 accumulation.

We next investigated whether EIN2 perturbs EIN3 protein degradation. We treated both *EIN3ox ein2-5* and *EIN3ox* seedlings with the protein synthesis inhibitor cycloheximide (CHX). We found that the rate of EIN3 proteolysis in *EIN3ox ein2* seedlings was approximately 2 times faster than in *EIN3ox* seedlings upon CHX treatment (Figure 7B). Moreover, treatment with MG132 (but not ACC) resulted in a pronounced accumulation of EIN3 in *EIN3ox ein2* seedlings (Figure 7B). These results indicate that EIN2 is required for EIN3 stabilization by repressing its proteasomal degradation.

Because destruction of EBF1/EBF2 leads to EIN3/EIL1 stabilization (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Figure 1F), we then determined whether EIN2 promotes the destruction of EBF1/EBF2 and thus stabilizes EIN3. We found that EBF1-TAP protein dramatically accumulated in the *ein2 EBF1-TAPox* seedlings, and this accumulation was not affected by treatment with ACC or silver ion (Figure 7D). Likewise, we also detected remarkable accumulation of EBF2-GFP protein using anti-GFP antibody in the *ein2 EBF2-GFPox* seedlings, which was not affected by treatment with ACC, silver ion, or MG132 (Figure 7E). Consistent with this, we could readily detect GFP fluorescence in the nuclei of the *ein2 EBF2-GFPox* seedlings, regardless of chemical treatments (Figure 7F). Therefore, we conclude that EIN2 is required for ethylene-induced proteasomal degradation of EBF1/EBF2 proteins.

Ethylene-Induced EBF1/EBF2 Degradation Is Independent of EIN3/EIL1

The ethylene signaling pathway is subject to multiple feedback mechanisms. For instance, the expression of *EBF2* is transcriptionally induced by EIN3 to dampen the ethylene signaling (Binder et al., 2007; Konishi and Yanagisawa, 2008). To investigate whether ethylene-induced EBF1/EBF2 proteolysis results from a feedback regulation that is dependent on EIN3/EIL1, we generated the *EBF1-GFPox/ein3 eil1* and *EBF2-GFPox/ein3 eil1* transgenic lines. While the basal levels of EBF1-GFP and EBF2-GFP proteins (indicated by the GFP fluorescence) were not detectable, treatments with Ag^+ and MG132 led to considerable accumulation of both nuclear proteins in the *ein3 eil1* background, reminiscent of the scenario in the wild-type background (Figure 8). Therefore, ethylene-induced EBF1/EBF2 proteolysis is independent of EIN3/EIL1-mediated transcription, ruling out the possibility of feedback regulation in this proteolysis process. We thus conclude that ethylene-induced EBF1/EBF2 proteolysis is a primary signaling event occurring upstream of EIN3/EIL1 but downstream of EIN2.

MKK9 Is Not Involved in the Ethylene Signal Transduction

The results described above supported the hypothesis that the ethylene signal is transduced through a pathway involving EIN2 and EBF1/EBF2 to stabilize EIN3/EIL1. This scenario seems to disagree with the requirement of MKK9-MPK3/MPK6 for ethylene signaling and EIN3 stabilization that virtually bypasses the function of EIN2 (Yoo et al., 2008). To clarify such discrepancy,

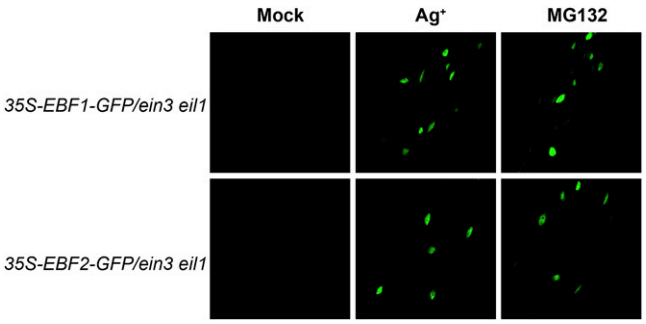

Figure 8. Ethylene-Induced EBF1 and EBF2 Degradation Is Independent of EIN3/EIL1.

Ag^+ and MG132 treatments promote the nuclear accumulation of EBF1-GFP and EBF2-GFP in the *ein3 eil1* mutant background. The GFP fluorescence was detected in the roots of 3-d-old etiolated seedlings treated with mock (0.5% DMSO), 20 μM $AgNO_3$, or 50 μM MG132 for 4 h.

we reexamined the functional requirement of MKK9 for ethylene signaling. *mkk9-5*, a T-DNA insertion line disrupting *MKK9* (see Supplemental Figure 4 online), was treated with ethylene and the seedling growth phenotype was quantified. We found that *mkk9-5* plants displayed ethylene responses nearly identical to those of the wild type (Figures 9A and 9B). We also found that the *mkk9-5 ctr1-1* double mutant showed constitutive ethylene responses similar to *ctr1* in etiolated seedlings, light-grown seedlings, and adult plants (Figures 9C to 9F). Moreover, immunoblot assays showed that the protein levels of EIN3 were similarly enhanced in wild-type and *mkk9* mutant plants after ethylene treatment, whereas EIN3 protein constitutively accumulated in *mkk9 ctr1*, just as in *ctr1* (Figure 9G). Consistent with these phenotypes, the expression levels of ethylene response genes (e.g., *ERF1*) were comparable between wild-type and *mkk9* plants, as well as between *ctr1* and *mkk9 ctr1* plants (Figure 9H). On the other hand, dexamethasone (a synthetic glucocorticoid)-induced expression of a constitutively activated form of MKK9 (*MKK9^{DD}*) resulted in an *eto*-like (ethylene overproduction) phenotype, which could be reversed by Ag^+ or aminoethoxyvinylglycine (AVG; a ethylene synthesis inhibitor) treatment (Xu et al., 2008). Consistent with this finding, treatments with Ag^+ or AVG remarkably reduced MKK9^{DD}-induced EIN3 accumulation (see Supplemental Figure 5A online). Furthermore, we found that the *MKK9^{DD}*-induced *eto* phenotype was suppressed similarly by *etr1-1*, *ein2-5*, or *ein3 eil1* mutations (see Supplemental Figure 5B online), further supporting a role of MKK9 in the ethylene biosynthetic pathway. Collectively, our findings support that MKK9 participates in inducing ethylene production rather than transducing ethylene signaling.

DISCUSSION

The EIN3/EIL family of transcription factors is a class of plant-specific DNA binding proteins. Previous studies revealed that plant responses to ethylene are mediated by SCF$^{EBF1/EBF2}$-dependent proteolysis of EIN3 (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). In this study, we reported that,

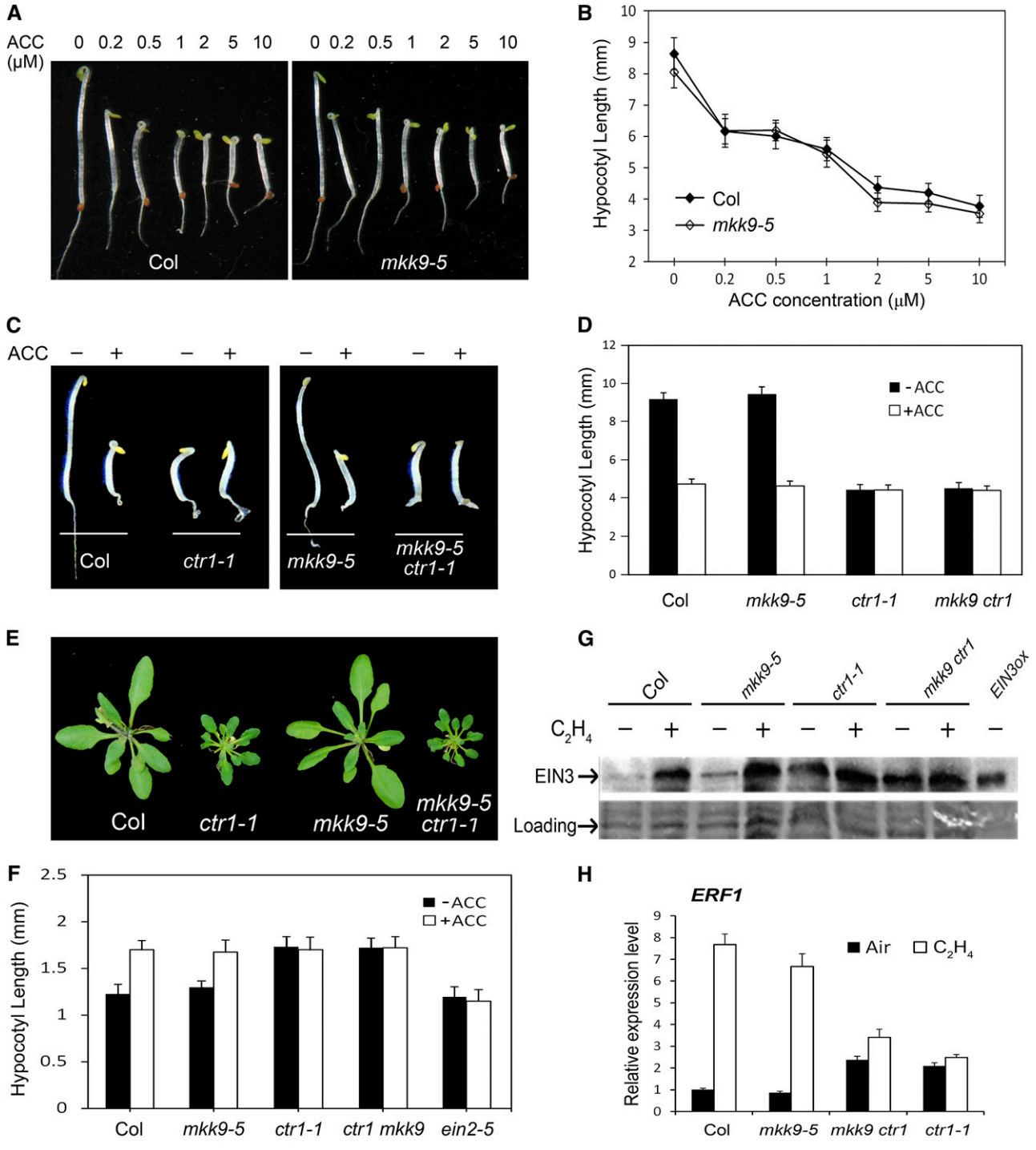

Figure 9. MKK9 Is Not Involved in the Ethylene Signaling Pathway.

(A) The triple response phenotype of 3-d-old Col-0 and *mkk9-5* etiolated seedlings grown on MS medium supplemented with indicated concentrations of ACC, showing similar ethylene sensitivity.

(B) Graphical quantification of hypocotyl lengths in **(A)**.

(C) The triple response phenotype of indicated genotypes grown on MS medium supplemented with or without 10 μM ACC for 3 d, showing a similar phenotype between *mkk9 ctr1* and *ctr1*.

(D) Graphical quantification of hypocotyl lengths in **(C)**.

(E) The rosette phenotype of 35-d-old plants, showing no difference between *mkk9 ctr1* and *ctr1*.

like EIN3, EIL1 protein is also targeted by $SCF^{EBF1/EBF2}$-dependent proteolysis and ethylene stabilizes it and that EIN3 and EIL1 cooperatively but differentially regulate a wide array of ethylene responses. Our studies also revealed that EIN2 and EBF1/EBF2 are indispensible signaling transducers for ethylene to induce the accumulation of EIN3/EIL1 proteins in the nucleus. Moreover, we discovered that ethylene promotes the proteasomal degradation of EBF1/EBF2 proteins, which requires EIN2 but not EIN3/EIL1. These studies illustrate that EIN3 and EIL1 are two master transcription factors that coordinate the regulation of myriad ethylene responses. Our results also indicate that ethylene signaling acts through EIN2 to promote the accumulation of EIN3/EIL1, which disagrees with a recent report that ethylene seems to bypass EIN2 to stabilize EIN3 through a MKK9 cascade. To resolve this disagreement, we reexamined the role of MKK9 in ethylene response and concluded that MKK9 is involved in ethylene biosynthesis rather than ethylene signaling. Together, our studies provide new insights into the signaling mechanism by which ethylene regulates master transcription factors to influence plant growth and development and help clarify controversial questions in the ethylene biology field.

EIL1 Is Another Target Protein of EBF1/EBF2 and Plays a Distinct Role in Ethylene Signaling

Genetic studies suggest that EIL1, another member of the EIN3/EIL family, also plays a role in mediating ethylene responses, including the triple response, pathogen defense, and gene expression (Alonso et al., 2003a). However, the activation mechanism of EIL1 by ethylene has not been explored. Our study presented compelling evidence to indicate that EIL1 is a nuclear protein also degraded by the $SCF^{EBF1/EBF2}$ complex and that ethylene acts to inhibit its proteolysis. Normal function of the receptors and EIN2 is required for ethylene-induced EIL1 accumulation, whereas CTR1 represses its stabilization. When EBF1 and EBF2 are functionally disrupted, the transgenically expressed EIL1-GFP protein constitutively accumulates in the nucleus and thus activates ethylene-inducible gene expression. Therefore, EIL1 undergoes fairly similar regulation as EIN3, and the ethylene signal seems to activate both transcription factors by repressing their proteasomal degradation.

EIN3 and EIL1 are thought to function redundantly in the ethylene signaling pathway, with EIL1 being a minor player (Alonso et al., 2003a). Our studies revealed that EIL1 is functionally distinct from EIN3 in mediating ethylene signaling. For instance, mutation of *EIN3* effectively suppresses the growth-arrested phenotype of the *ebf1 ebf2* mutant, while *eil1* does not, suggesting that EIN3 has a predominant role in regulating seedling growth and development. Conversely, EIL1 is necessary for regulating the inflorescence height of adult plants, as *eil1* shows a stronger suppression of the *ebf1* semidwarf phenotype than does *ein3*. In the meantime, EIL1 and EIN3 have overlapping effects in a wide array of ethylene responses. The *ebf1 ebf2 ein3* (*tm*) plants show a reduction in plant stature, apical dominance, silique fertility, and rosette size. Inactivating one copy of *EIL1* greatly suppresses the defects of the *tm* plants, highlighting the importance of EIL1 in regulating various ethylene responses. Loss of two *EIL1* copies fully rescues the abnormalities of the *tm* mutant, indicating that EIN3 and EIL1 are the major transcription factors cooperatively mediating the ethylene response pathway.

Despite the similarity between EIN3 and EIL1 in subcellular localization, interaction partners, and regulatory mode, constitutive overexpression of *EIN3* and *EIL1* driven by the same 35S promoter leads to dissimilar phenotypes, as EIN3 overexpressors usually display stronger ethylene response phenotypes, implying that EIN3 protein is more effective than EIL1 in activating ethylene responses. Because the EIN3/EIL family proteins share a highly conserved N-terminal half responsible for DNA binding and a more diverse C-terminal half that is believed to mediate protein–protein interactions (Guo and Ecker, 2003), it is likely that the divergent C-terminal fragments determine the functions and activities of different family members. In support of this possibility, a recent study revealed that EIL3, a member with limited C-terminal sequence similarity to EIN3/EIL1, is instead a regulator of sulfate acquisition and glucosinolate degradation under sulfur-free conditions (Maruyama-Nakashita et al., 2006), suggesting that distant members of EIN3/EIL family might function in ethylene-unrelated pathways.

Ethylene-Induced EBF1/EBF2 Degradation Is a Primary Signaling Event Downstream of CTR1 and EIN2 but Upstream of EIN3/EIL1

Two alternative models had been previously proposed to explain how EIN3 is regulated by ethylene: one is that the ethylene signal directly modulates EIN3 activity, and the other is that ethylene inhibits the function of $SCF^{EBF1/EBF2}$ (Guo and Ecker, 2003; Kepinski and Leyser, 2003; Potuschak et al., 2003; Gagne et al., 2004). In this study, we provided several lines of genetic and biochemical evidence to demonstrate that EBF1/EBF2 are required for the transmission of ethylene signaling to regulate EIN3 and EIL1 stabilization, supporting the second model whereby ethylene inhibits the function of EBF1/EBF2. First, while the *ein3* and *eil1* mutants are partially ethylene sensitive, the *ein3 ebf1*

Figure 9. (continued).

(F) Quantitative analysis of hypocotyl lengths. Five-day-old light-grown seedlings germinated on water-agar medium supplemented without or with 10 μM ACC.

(G) Immunoblot assay of EIN3 protein in 4-d-old etiolated seedlings of the indicated genotypes treated with or without ethylene gas (20 ppm) for 4 h.

(H) Analysis of ethylene-regulated gene *ERF1* expression by quantitative real-time RT-PCR in 6-d-old light-grown seedlings treated with or without ethylene (20 ppm) for 4 h. Error bars indicate SD ($n = 3$).

Each bar in **(B)**, **(D)**, and **(F)** represents the average length (±SD) of at least 20 seedlings. Experiments were repeated three times with similar results. [See online article for color version of this figure.]

ebf2 and *eil1 ebf1 ebf2* triple mutants are unresponsive to ethylene application, implying that the endogenous EIL1 and EIN3 in the respective triple mutants are not further activated by ethylene. Second, transgenic plants overexpressing EIL1-GFP in the *ein3 ebf1 ebf2* mutant or inducibly expressing EIN3 in the *ein3 eil1 ebf1 ebf2* mutant are not responsive to ethylene, in contrast with the ethylene hypersensitivity phenotype of *EIL1-GFPox*, *EIL1ox*, or *EIN3ox* in the wild-type background. Third, *ein3 ebf1 ebf2* fully suppresses *ein2* and *ctr1* in etiolated seedlings and adult plants, while being fully suppressed by *eil1*, providing additional genetic confirmation of EBF1/EBF2 in the ethylene signaling pathway. Finally, the levels of EBF1/EBF2 proteins are downregulated by ethylene through the 26S proteasome pathway, and this effect is reversed by silver ion or *EIN2* mutation but is independent of *EIN3*/*EIL1* mutations. Collectively, these data evidently indicate that EBF1 and EBF2 act as integral signaling transducers downstream of CTR1 and EIN2 but upstream of EIN3/EIL1, and ethylene stimulates EIN3/EIL1 accumulation by promoting EBF1/EBF2 proteasomal degradation.

Further studies will focus on the understanding of how ethylene induces the proteasomal degradation of EBF1/EBF2. EIN2 seems to be particularly important for inducing EBF1/EBF2 proteolysis, as both EBF proteins are very stable in the *ein2* mutant. A recent study reported that EIN2 interacts with several components of the CSN, such as CSN3, CSN6A, and CSN6B (Christians et al., 2008). Mutation in *EER5*, encoding a putative CSN subunit protein that also interacts with the C terminus of EIN2, leads to hypersensitivity to ethylene (Christians et al., 2008), suggesting that CSN might be a negative regulator of ethylene signaling. It has been shown that the CSN complex is responsive for removing Nedd8 or Rub moieties from the Cul1 subunit of the SCF complex in both animals and plants (Lyapina et al., 2001; Cope et al., 2002). Suppression of CSN functions results in significant reduction of the levels of multiple F-box proteins due to enhanced autoubiquitination of these F-box proteins (Cope and Deshaies, 2006; Stuttmann et al., 2009). It is thus possible that EBF1 and EBF2 might undergo autoubiquitination processes that lead to their self-destruction and that EIN2 mediates ethylene signaling to repress the action of CSN, which facilitates the stabilization of EBF1/EBF2 by inhibiting their autoubiquitination. Further investigations on whether EBF1/EBF2 proteins are autoubiquinated and how CSN modulates the ubiquitination and degradation of EBF1/EBF2 and EIN3/EIL1 will help fill in the gap of signaling events between EIN2 and EIN3/EIL1.

Stabilization of EIN3/EIL1 by Ethylene Requires EIN2 but Not MKK9

A recent study proposed that ethylene bypasses EIN2 to activate the MKK9-MPK3/MPK6 cascade that phosphorylates EIN3 and stabilizes it (Yoo et al., 2008). Our data seem to disagree with this proposal. First of all, we demonstrated that EIN2 is absolutely required for ethylene-induced EIN3/EIL1 stabilization (Guo and Ecker, 2003; Figure 1A). Whereas *EIN3ox* shows hypersensitivity to ethylene, *EIN3ox ein2* does not respond to ethylene based on the analyses of the triple response phenotype, the levels of EIN3 protein, and the expression levels of ethylene-inducible genes (e.g., *ERF1*). Further studies revealed that EIN2 is also required for ethylene-induced EBF1/EBF2 degradation, as both EBF1 and EBF2 proteins constitutively accumulate in the nuclei of *ein2*, while ACC and Ag^+ treatments have no effect on their accumulation. Together, these data indicate that EIN2 acts to repress the accumulation of EBF1/EBF2 that target EIN3/EIL1 for degradation, and the ethylene signal requires EIN2 to stabilize EIN3/EIL1.

On the other hand, we also provided evidence to argue against the fact that MKK9 is involved in the ethylene signal transduction. Based on comparison of ethylene response phenotypes, EIN3 protein accumulation, and downstream gene expression,

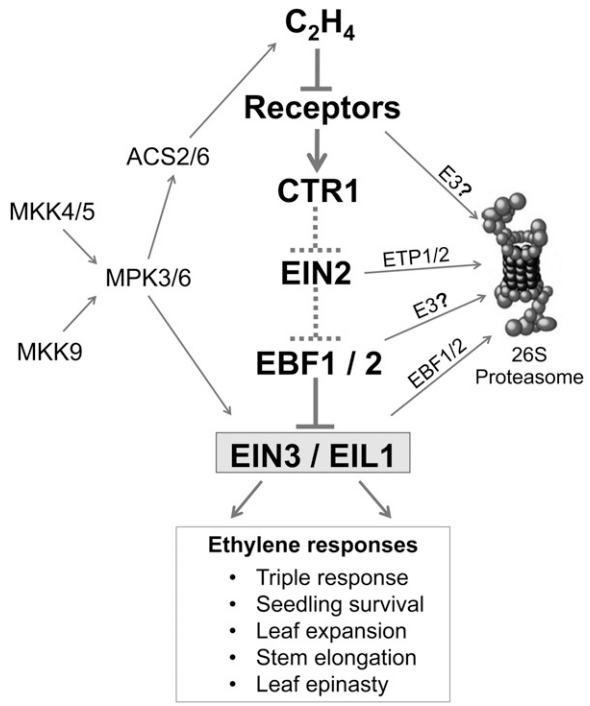

Figure 10. A Proposed Model Illustrating the Ethylene Signaling Pathway That Requires EIN2 and EBF1/EBF2 to Regulate the Functions of EIN3 and EIL1.

The ethylene signal is perceived by a linear pathway involving the receptors, CTR1, EIN2, and EBF1/EBF2, to ultimately activate EIN3 and EIL1 transcription factors. Ethylene acts to promote the proteasomal breakdown of EBF1/EBF2, which form the SCF complex to target EIN3/EIL1 for proteolysis. Both EIN2 and EBF1/EBF2 are indispensible for ethylene-induced EIN3/EIL1 accumulation. EIN3 and EIL1 act cooperatively but differentially in the ethylene response pathway. The MKK4/MKK5- and MKK9-activated MPK3/MPK6 kinases are believed to enhance ethylene production by upregulating ACS abundance, although MKK9-MPK3/MPK6 were reported to directly phosphorylate EIN3 and modulate its stability (Yoo et al., 2008). Several ethylene signaling components (ETR2, EIN2, EBF1/EBF2, and EIN3/EIL1) are subject to ubiquitin/proteasome-mediated degradation, with the responsible E3 ligases for the receptors and EBF1/EBF2 remaining unidentified. Arrows and bars represent positive and negative regulations, respectively. The solid lines indicate direct regulation, whereas the dotted lines indicate either indirect regulation or regulation in an unknown manner.

we found little difference between the wild type and *mkk9-5* or between *ctr1* and *mkk9-5 ctr1*. Recently, two independent studies documented that MPK3 and MPK6 function in ethylene biosynthesis by positively regulating the gene expression and protein stability of ACS2 and ACS6 (Liu and Zhang, 2004; Joo et al., 2008; Xu et al., 2008). They also found that the ethylene overproduction phenotype induced by *MKK9^{DD}* (that could constitutively activate MPK3/MPK6) is reversed by AVG and silver ion (Xu et al., 2008). Consistent with these findings, our results also indicated that MKK9^{DD}-induced seedling phenotype or EIN3 accumulation is suppressed when ethylene perception or signaling is impaired. Taking these results together, we propose a model in which MKK9-MPK3/MPK6 participate in ethylene responses by modulating ethylene production, instead of transmitting the ethylene signal via the canonical ethylene signaling pathway (Kendrick and Chang, 2008; Stepanova and Alonso, 2009). Moreover, EIN2 and EBF1/EBF2 are essential signaling transducers required for the perception of ethylene to stabilize EIN3 and EIL1, which work cooperatively but differentially in the ethylene response pathways (Figure 10). The previously reported phosphorylation and stabilization of EIN3 elicited by MPK3/MPK6 might mediate the effects on EIN3 activity of other signaling pathways (e.g., environmental stress), rather than of ethylene signaling.

It seems that protein degradation plays an essential role in nearly every step of the ethylene signaling pathway (Figure 10). It has been shown that ETR2, EIN2 (by two F-box proteins ETP1 and ETP2), and EIN3 (by two F-box proteins EBF1 and EBF2) are all regulated by the ubiquitin/proteasome pathway (Chae et al., 2003; Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Wang et al., 2004; Qiao et al., 2009). We now report that EBF1 and EBF2 are also subject to proteasome-mediated proteolysis, although the responsible E3 ligases have yet to be identified. Ethylene plays a crucial role in regulating plant development and stress responses, whereby impairment of the ethylene response pathway diminishes tolerance or resistance to environmental stresses, but excess activation of ethylene signaling leads to growth inhibition or even plant death. It is thus necessary and advantageous for plants to evolve elaborate strategies to dynamically equilibrate the ethylene signaling level within a controllable range. Proteasomal degradation of several ethylene signaling components would provide multifaceted regulatory layers to reset and fine-tune the ethylene signal output in plant response and adaptation to the environment.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was the parent strain for all mutants and transgenic lines used in this study. *ein3-1* (Chao et al., 1997), *eil1-1* (Alonso et al., 2003a), *ein2-5* (Alonso et al., 1999), *ein3-1 eil1-1* (Alonso et al., 2003a), *ebf1-1*, *ebf2-1*, *ein3-1 ebf2-1*, *ein3-1 ebf1-1* (Guo and Ecker, 2003), *etr1-1* (Hua and Meyerowitz, 1998), *eto1* (Guzman and Ecker, 1990), and *ctr1-1* (Kieber et al., 1993) mutants, and *EIN3ox* (*35S:EIN3*), *EIL1ox* (*35S:EIL1*) (Chao et al., 1997), and *MKK9^{DD}* (Xu et al., 2008) transgenic plants with Col-0 background were described previously. *eil1-3* (Salk_042113), used for getting the *ein3-1 eil1-3 ebf1-1 ebf2-1* quadruple mutant, and *mkk9-5* (SAIL_60_H06) were identified from the SIGnAL T-DNA collection (Alonso et al., 2003b).

Double, triple, and quadruple mutants were generated by genetic crosses, and homozygous lines were identified by comparison with the parental phenotype and through PCR-based genotyping (primers are listed in Supplemental Table 1 online).

Surface-sterilized seeds were plated on MS medium (4.3 g/L MS salts, 1% sucrose, pH 5.7 to 5.8, and 8 g/L agar) and imbibed for 4 d at 4°C. For the triple response, the plates were wrapped in foil and kept in a 22°C incubator before the phenotypes of seedlings were analyzed. For adult plant phenotypic analysis, light-grown seedlings from plates were transferred to soil and grown to maturity at 22°C under a 16-h-light/8-h-dark cycle.

Chemical Solutions

All chemicals were from Sigma-Aldrich, unless specified otherwise, and prepared as stock solutions. DMSO was used to dissolve MG132 (10 mM), CHX (100 mM), and β-estradiol (10 or 20 mM). ACC (10 mM) and $AgNO_3$ (100 mM) were dissolved in water. Final concentrations were 50 μM MG132, 100 μM CHX, 100 μM $AgNO_3$, and 100 μM ACC for immunoblot experiments, 20 μM $AgNO_3$ for GFP fluorescence detection, and 10 μM ACC for germination.

Ethylene treatment of *Arabidopsis* seedlings grown on plates was performed in containers by flowing through hydrocarbon-free air supplemented with 20 ppm ethylene or hydrocarbon-free air alone (Kieber et al., 1993).

Antibody Preparation and Immunoblot Assays

The coding region corresponding to residues 448 to 583 of EIL1 protein was PCR amplified from reverse transcription product using primers 5′-AAGTTTCATATGGGTGGAATG-3′ and 5′-TATTCGGATCCATATTGATACATC-3′, digested with *Nde*I and *Bam*HI, and inserted into pET-16b plasmid. His-EIL1 protein was purified from *Escherichia coli* by Ni-NTA agarose and used to raise polyclonal antibodies in rabbits. Immunoblot assays were performed as described previously (Lin et al., 1995). In brief, 4-d-old dark-grown seedlings were treated with 20 ppm ethylene or hydrocarbon-free air for 1, 4, or 12 h, or 3-month-old *Arabidopsis* suspension cell cultures derived from 3-d-old Col-0 etiolated seedlings were treated with MG132, ACC, or MG132 plus ACC for 4 h before protein samples were extracted for immunoblot analysis. The blot was probed with anti-EIL1 antibody (1:1000 dilution), and a nonspecific band was used as a loading control.

For EIN3-3FLAG immunoblots, 6-d-old light-grown *pER8-EIN3-3FLAG* transgenic seedlings were transferred to MS liquid medium with different concentrations of β-estradiol for 8 h and then treated with ACC for an additional 4 h. EIN3-3FLAG fusion proteins were visualized by immunoblots using anti-FLAG antibody (Sigma-Aldrich).

For EIN3 immunoblots, 3-d-old etiolated seedlings were treated with ethylene gas for the indicated times or treated with different combinations of ACC, MG132, or CHX for 4 h. Total protein extracts were subjected to immunoblot with anti-EIN3 antibody (Guo and Ecker, 2003), and a nonspecific band was used as a loading control. The relative EIN3 protein levels were calculated after normalization with loading controls using the Tanon Gel Image System.

For EBF1/2-TAP immunoblots, 3-d-old dark-grown *35S-EBF1-TAP* or 6-d-old light-grown *35S-EBF1/2-TAP* or *35S-EBF1-TAP ein2* seedlings were treated with indicated chemicals for 4 h, total protein extracts were subjected to immunoblots with anti-MYC antibody (Upstate), and a nonspecific band was used as a loading control.

For EBF2-GFP immunoblots, 6-d-old light-grown *35S-EBF2-GFP ein2* seedlings were treated with the indicated chemicals for 4 h, total protein extracts were subjected to immunoblots with anti-GFP antibody (Roche), and a nonspecific band was used as loading control.

RT-PCR and Real-Time Quantitative RT-PCR Analysis of Gene Expression

Total RNA was prepared using the TRIzol reagent (Invitrogen). Two micrograms of total RNA treated with DNase I (Promega) was added in a 20-μL reverse transcription reaction using the M-MLV reverse transcription system (Promega), and an appropriate amount of cDNA products was then used for a 15-μL PCR amplification reaction using the gene-specific primers listed in Supplemental Table 1 online (the genotyping primers of *eil1-1* are also used for RT-PCR analysis). Real-time PCR was performed using SYBR Green Mix (Takara) and the specific primers listed in Supplemental Table 1 online. The expression level was normalized to that of the *UBIQUITIN5* (*UBQ5*) control. Data presented are mean values of three biological repeats with standard deviation.

Plasmid Construction and Generation of Transgenic *Arabidopsis* Plants

The *35S-EIL1-GFP*, *35S-EBF1-GFP*, and *35S-EBF2-GFP* constructs were created by cloning the *EIL1*, *EBF1*, and *EBF2* coding regions into *Kpn*I and *Bam*HI, *Kpn*I and *Sal*I, and *Kpn*I and *Sal*I sites of the binary vector pCHF3-GFP (Yin et al., 2002), respectively. The *35S-EBF1-TAP* and *35S-EBF2-TAP* constructs were created by cloning the *EBF1* and *EBF2* coding regions into pC-TAPa vectors (Rubio et al., 2005) by the Gateway method (Invitrogen). Primer sequences used for the PCR are summarized in Supplemental Table 2 online. All constructs were verified by DNA sequencing analysis.

The resulting constructs were introduced into *Agrobacterium tumefaciens* strain C58, and subsequently the *35S-EIL1-GFP* construct was transformed into *Arabidopsis* wild-type (Col-0) and *ein3-1 ebf1-1 ebf2-1* plants; *35S-EBF1-GFP* and *35S-EBF2-GFP* constructs were transformed into wild-type (Col-0), *ein2-5*, and *ein3 eil1* plants; *35S-EBF1-TAP* and *35S-EBF2-TAP* constructs were transformed into wild-type (Col-0) and *ein2-5*; and the pER8-EIN3-3FLAG construct (Chen et al., 2009) was transformed into the *ein3 eil1 ebf1 ebf2* quadruple mutant by vacuum infiltration (Bechtold and Pelletier, 1998).

Confocal Laser Microscopy

A Leica TCS SP2 inverted confocal laser microscope with ×40 objectives was used to detect GFP fluorescence. The excitation wavelength was 488 nm, and a band-pass filter of 510 to 525 nm was used for emission. Roots from etiolated transgenic plants expressing GFP fusion proteins were mounted on standard microscope slides in the presence of water, ACC (100 μM), Ag^+ (20 μM), or MG132 (50 μM), and GFP fluorescence was detected using the confocal microscope.

Suppressor Screening of *EIN3ox* and Genetic Mapping

EIN3ox seeds were mutagenized with 0.25% ethyl methanesulfonate following routine procedures. About 50% of mutagenized seeds (referred as to the M1 population) grew in soil and generated M2 seeds at 22°C under a 16-h-light /8-h-dark photoperiod. M2 seeds were routinely plated on MS medium containing 10 μM ACC to screen for mutants suppressing the phenotype of *EIN3ox* in the dark. Seedlings with hypocotyls and roots longer than *EIN3ox* were transplanted to soil to set seeds.

F2 seeds derived from crosses between *seo* (suppressor of *EIN3ox*) mutants and wild-type Landsberg *erecta* were grown on MS medium containing 10 μM ACC in darkness for 3 d, and seedlings with longer hypocotyls and roots were selected for genetic mapping. The *seo* mutations were roughly mapped using simple sequence length polymorphism markers. Genomic DNA was isolated from *seo* mutants and then PCR-based amplification of *EIN2* was used for sequencing. The sequence results were then compared with the wild-type sequences.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *EIN3* (At3g20770), *EIL1* (At2g27050), *CTR1* (At5g03730), *EBF1* (At2g25490), *EBF2* (At5g25350), *EIN2* (At5g03280), *ETR1* (At1g66340), *MKK9* (At1g73500), *ERF1* (At3g23240), *ACO2* (At1g62380), *UBQ5* (At3g62250), and *TUBULIN8* (At5g23860).

Supplemental Data

The following materials can be found in the online version of this article.

Supplemental Figure 1. Ethylene Positively Regulates the Protein Levels of EIL1.

Supplemental Figure 2. The *eil1* Mutation Fully Suppresses the Morphological Phenotypes of the *ein3 ebf1 ebf2* Triple Mutant.

Supplemental Figure 3. Five *seo* Mutants Were Identified as New Alleles of *ein2 EIN3ox* That Showed Complete Ethylene Insensitivity.

Supplemental Figure 4. Identification of the T-DNA Mutant of *MKK9*.

Supplemental Figure 5. Inhibition of Ethylene Perception or Signal Transduction Suppresses $MKK9^{DD}$-Induced EIN3 Accumulation and Ethylene Response.

Supplemental Table 1. Primers for Genotyping and RT-PCR.

Supplemental Table 2. Primers for Construction of Plant Transformation Vectors.

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