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EIN3-Dependent Regulation of Plant Ethylene Hormone Signaling by Two *Arabidopsis* F Box Proteins: EBF1 and EBF2

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Summary

The plant hormone ethylene regulates a wide range of developmental processes and the response of plants to stress and pathogens. Genetic studies in *Arabidopsis* led to a partial elucidation of the mechanisms of ethylene action. Ethylene signal transduction initiates with ethylene binding at a family of ethylene receptors and terminates in a transcription cascade involving the EIN3/EIL and ERF families of plant-specific transcription factors. Here, we identify two *Arabidopsis* F box proteins called EBF1 and EBF2 that interact physically with EIN3/EIL transcription factors. EBF1 overexpression results in plants insensitive to ethylene. In contrast, plants carrying the *ebf*1 and *ebf2* mutations display a constitutive ethylene response and accumulate the EIN3 protein in the absence of the hormone. Our work places *EBF1* and *EBF2* within the genetic framework of the ethylene-response pathway and supports a model in which ethylene action depends on EIN3 protein stabilization.

Introduction

Protein degradation via the ubiquitin/26S proteasome pathway is an important posttranscriptional regulatory mechanism in all eukaryotes that allows cells to respond rapidly to signal molecules and changes in environmental conditions (Hershko and Ciechanover, 1998). Ubiquitylation is achieved through an enzymatic cascade involving the sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. Among these enzymes, the E3s play a central role in the selectivity of ubiquitin-mediated protein degradation. To date, several classes of E3s have been reported (Jackson et al., 2000). Major types of E3s are the SCF complexes (Deshaies, 1999), which in *Saccharomyces cerevisiae* are composed of four primary subunits: CDC53 (cullin1), RBX1, SKP1, and an F box protein. The F box proteins contain a degenerated protein domain of approximately 50–60 amino acid residues, identified first in the N-terminal region of cyclin F (Bai et al., 1996), and have in addition protein-protein interaction domains that confer the substrate specificity for ubiquitylation. Thus, the F box proteins are the adaptor subunits that specifically recruit substrates to the core ubiquitylation complex through a physical interaction between the F box domain and the SKP1 subunit.

Recent studies have highlighted the role of several of these F box proteins in various aspects of plant growth and development (Vierstra, 2003). In particular, plant hormone signaling seems to be subjected to SCF-dependent regulation (Frugis and Chua, 2002). Thus, both auxin and jasmonate perception are controlled by SCF-like complexes involving the F box proteins TIR1 (Ruegger et al., 1998) and COI1 (Xie et al., 1998), respectively. Indeed, it has been demonstrated that auxin stimulates the binding of the SCF^{TIR1} to the AUX/IAA proteins leading to their degradation (Gray et al., 2001). AUX/IAA proteins serve as repressors of auxin action by binding to and blocking the ARF transcription factors that activate the auxin-inducible genes (Hellmann and Estelle, 2002). Although the COI1 F box protein is part of a similar SCF complex (Xu et al., 2002), protein substrates involved in jasmonate signaling are presently unknown. More recently, it has been shown that the *Arabidopsis* SLEEPY1 (McGinnis et al., 2003) and the rice GID2 (Sasaki et al., 2003) F box proteins are involved in gibberellin signaling. Similarly to TIR1, these F box proteins are involved in the degradation of negative regulators of the gibberellin response.

Ethylene is a plant hormone involved in a wide range of plant developmental processes, including seed germination, leaf expansion, root hair formation, fruit ripening, timing of vegetative senescence, and response to stress and pathogens (reviewed in Johnson and Ecker, 1998; Wang et al., 2002). The extensive characterization of *Arabidopsis* mutants with an altered ethylene response has led to the identification of ethylene receptors and several downstream components of the ethylene signal transduction pathway (reviewed in Schaller and Kieber, 2002; Wang et al., 2002). The ethylene receptor family is comprised of five members (ETR1, ETR2, EIN4, ERS1, and ERS2) that are similar to two component regulators from bacteria and yeast. Downstream of the ethylene receptors is CTR1, a mitogen-activating protein kinase kinase kinase (MAPKKK), which is a negative regulator in ethylene signaling (Kieber et al., 1993). Therefore, in the absence of ethylene, CTR1 negatively regulates the EIN2 protein (Alonso et al., 1999). EIN2 shares homology with the N-Ramp family of metal transporters. Upon perception of ethylene, CTR1 is inactivated and consequently EIN2 is liberated from repression. Downstream of EIN2 is a transcriptional cascade that is involved in the upregulation of ethylene-responsive genes (Schaller and Kieber, 2002). In this cascade, the EIN2 protein activates the transcriptional activator

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EIN3 and likely several other EIN3-like proteins (EILs). Subsequently these proteins activate other transcription factors, like the ERFs (ethylene response factors) thereby regulating the expression of genes involved in the response to ethylene. How EIN2 activates EIN3 and EILs proteins is presently unknown, but since the level of *EIN3* mRNA is unaffected by treatment of wild-type plants with ethylene (Chao et al., 1997), *EIN3* must be regulated at the posttranscriptional or posttranslational level. Here, we show that two *Arabidopsis* F box proteins EBF1 and EBF2 (for *E*IN3-*B*inding *F* box protein *1* and *2*) physically interact with EIN3 and EIL1 and likely target these proteins for degradation. Genetic epistasis studies demonstrate that *EBF1* and *EBF2* act downstream or independent of *EIN2* and directly control the level of the EIN3 protein.

Results

EBF1 and EBF2 Are Two Nuclear-Localized F Box Proteins Constitutively Expressed in Various *Arabidopsis* Organs

To understand the function of the SCF complex in plant development, we have used a molecular genetic approach to characterize a number of genes encoding F box proteins belonging to the leucine-rich repeat (LRR) family. Here, we report our work concerning two of these proteins.

AtFBL6 (At2g25490), hereafter called EBF1 has been described as an *Arabidopsis* F box protein containing 16 tandem leucine-rich repeats (LRRs) (Xiao and Jang, 2000). EBF1 is structurally related to the budding yeast GRR1 protein that is involved in cell cycle control and glucose signaling (see Hsiung et al., 2001 and references therein). Sequence analysis reveals the presence of a paralogous gene, hereafter called *EBF2* (At5g25350), which is 58.5% identical to EBF1 at the amino acid sequence level. Except for the F box domain, both EBF1 and EBF2 protein sequences consist mainly of the cysteine-containing LRRs (Figure 1A). RNA blot analysis indicates that both *EBF1* and *EBF2* genes are expressed in all plant organs analyzed (Figure 1B) suggesting that they are involved in basic cellular functions.

N-terminal and C-terminal translational fusions to the green fluorescent protein (GFP) were used to investigate the subcellular localization of the EBF1 F box protein in transiently transformed *Nicotiana benthamiana* plants. Whereas the GFP protein alone is detectable in both the cytosol and the nucleus, EBF1 is only detected in the nuclei (Figure 1C), but clearly excluded from the nucleoli. This was observed with both N-terminal (Figure 1C) and C-terminal (data not shown) EBF1 fusion proteins and an N-terminal GFP-EBF2 fusion protein (data not shown). Moreover, in certain transformed cells, we observed a speckled distribution of the EBF1 fusion proteins (Figure 1C). Whether these speckles correspond to ubiquitylation centers is unknown, but the nuclear localization of EBF1 is consistent with the subcellular localization of the plant SCF components, ASK1 and Cullin 1 (Farras et al., 2001; Shen et al., 2002).

The F Box Proteins EBF1 and EBF2 Interact with EIN3 and EIL1 and Components of the SCF Complex

A yeast two-hybrid screen was used to identify proteins that potentially interact with EBF1. The full-length coding sequence of *EBF1* was fused to the Gal4 DNA binding domain to screen a 3-week-old *Arabidopsis* green vegetative tissue cDNA library. From over three million clones screened, 39 clones were identified as potential interactors. One fourth of them represented the ASK1 and ASK2 proteins. These are *Arabidopsis* orthologs of budding yeast SKP1 and represent known subunits of several *Arabidopsis* SCF ubiquitin protein ligases (Gray et al., 1999; Xu et al., 2002). Interestingly, seven individual clones corresponded to the EIN3 (ethylene-insensitive 3, At3g20770) protein (Chao et al., 1997) and one clone to the EIL1 (Ein3-like 1, At2g27050) protein (Chao et al., 1997) (Figure 2A).

We then tested whether EBF2 was able to interact with the EIN3 and EIL1 transcription factors in the yeast two-hybrid assay (Figure 2B). Moreover to investigate the specificity of these interactions, we used two well-characterized *Arabidopsis* F box proteins as controls, TIR1 (Ruegger et al., 1998) and COI1 (Xie et al., 1998) that are involved in auxin and jasmonate signaling, respectively. Like the EBF1 and EBF2 proteins, TIR1 and COI1 are also members of the LRRs family of F box proteins. In our yeast two-hybrid experiments, only EBF1 and EBF2, but not TIR1 and COI1, were able to interact with the EIN3 and EIL1 proteins.

In vitro pull-down assays were subsequently used to examine interactions between the EBF proteins and EIN3 (Figure 2C). For this purpose, we generated GST fusions with EBF1, EBF2, and with COI1, which was used as a control. ^{35}S methionine-radiolabeled EIN3 protein was produced in wheat germ extracts. Thus, we were able to pull down EIN3 with both GST-EBF1 and GST-EBF2, whereas GST alone and GST-COI1 gave negative results.

Finally, to investigate whether EBF1 and EBF2 are part of SCF complexes, we performed pull-down assays as described in Lechner et al. (2002). Both F box proteins, as well as COI1 as a positive control, were expressed in *E. coli* as translational fusion proteins with GST, purified and incubated with total *Arabidopsis* protein extracted from a cell suspension culture. After pull-down, the bound plant proteins were immunoblotted with the ASK1 and AtCUL1 antibodies. Both ASK1 and AtCUL1 copurified with the GST-F Box fusion proteins, but not with the GST protein alone (Figure 2D). The identity of the ASK1 and AtCUL1 proteins was confirmed by competition assays using the peptides used to raise the antibodies (data not shown). From these experiments, we conclude that EBF1 and EBF2 most likely form *Arabidopsis* SCF complexes and that both F box proteins specifically interact with transcription factors involved in ethylene-signaling pathway.

Overexpression of *EBF1* Renders Plants Ethylene Insensitive

Altering the expression of *EBF1/2* might be expected to interfere with the ethylene response if they are compo-

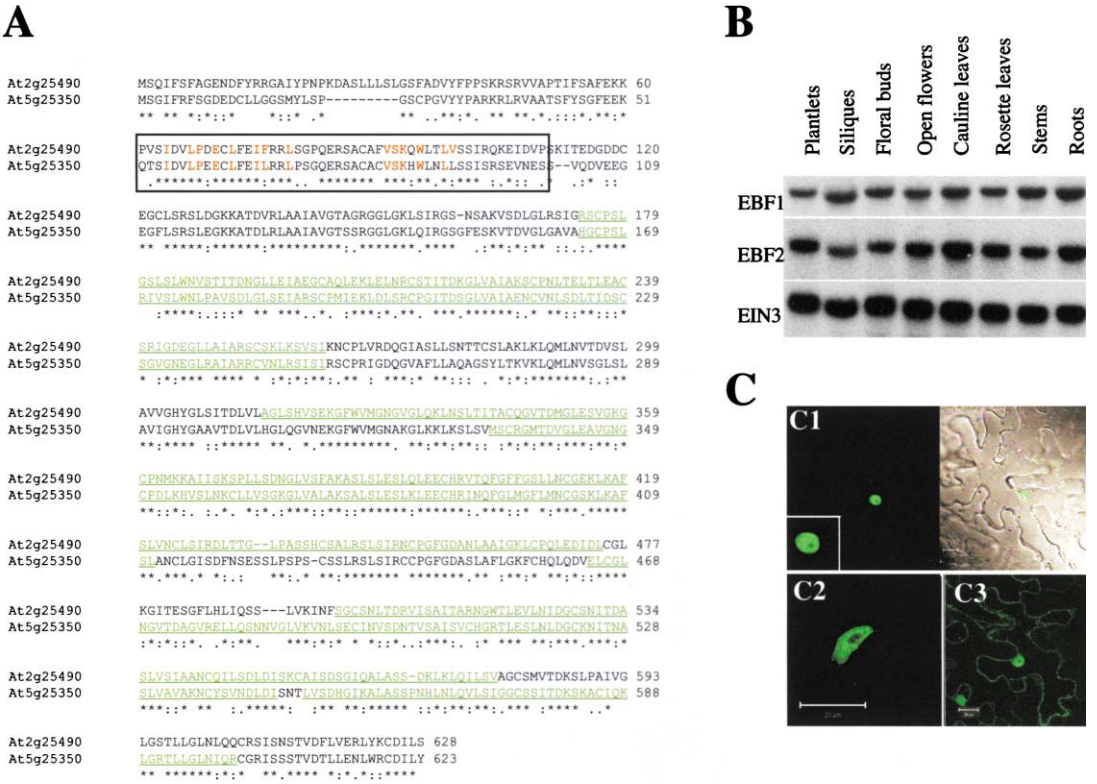

Figure 1. Comparison of EBF1 and EBF2 Amino Acid Sequence, Expression, and Subcellular Localization

(A) The deduced amino acid sequence of EBF1 and EBF2 was aligned using the ClustalW (1.82) program. EBF1 and EBF2 correspond to accession numbers AY072205 and NM_122444, respectively. Numbers show the positions of amino acid residues; asterisks and dots indicate identical and conserved amino acids, respectively. The F box sequences are boxed and the residues corresponding to the F box consensus sequence (Kipreos and Pagano, 2000) are indicated in red. The cysteine-containing LRR sequences (LCC_CC) were identified by motifs scanning in the Prosite profiles database (Falquet et al., 2002) and are indicated in green and underlined.

(B) *EBF1* and *EBF2* mRNA accumulation in various *Arabidopsis* tissues. Total RNA was extracted from 8-week-old plants, siliques, floral buds, open flowers, cauline and rosette leaves, stems, and roots. The RNA blot was hybridized with indicated random-primed ^{32}P-labeled probes.

(C) Subcellular distribution of EBF1 fused to GFP in transiently transformed tobacco cells. *Nicotiana benthamiana* plants were infiltrated with agrobacteria harboring the different plasmids. In (C1), the GFP was fused to the N terminus of EBF1 and shows a clear nuclear localization (left image). The inset shows a magnification of the nucleus whereas the right image shows the corresponding transmitted light reference image viewed by DIC. An identical subcellular localization was observed when the GFP was fused to EBF1 C terminus (data not shown). The (C2) image corresponds to the speckled distribution of N-terminal-fused GFP-EBF1 that was observed in certain cells. (C3) corresponds to nuclear and cytosolic distribution of the GFP protein alone. Scale bar is equal to 20 μm.

nents of the ethylene-signaling pathway. To test this possibility, we generated transgenic *Arabidopsis* lines expressing *EBF1* under the control of the strong CaMV 35S promoter. The resulting transgenic plants belonged to two phenotypic classes, those that were ethylene insensitive and overaccumulated *EBF1* and those that were phenotypically normal and had no detectable transgene expression (Figure 3A). Plants with the highest level of transgene expression had reduced levels of endogenous *EBF1* expression. This was even more striking for *EBF2*, suggesting the existence of a negative-feedback loop for *EBF1/2* expression (Figure 3B).

The ethylene insensitivity of *EBF1* overexpressors is consistent with EBF1 targeting EIN3 for degradation and as such is similar to the phenotype displayed by the loss of function mutant *ein3-1*. While our manuscript was submitted, Yanagisawa et al. (2003) demonstrated that the EIN3 protein was only detectable in plants treated with the ethylene precursor ACC. Thus, we examined whether *EBF1* overexpression had an effect on EIN3 protein level. Indeed transgenic lines that overexpressed *EBF1* showed a reduced level of EIN3 protein compared to Col-0 control plants when grown in presence of 10 μM ACC (Figure 3C). Similarly, Guo et al. (2003, this issue of *Cell*) demonstrated that the EIN3 protein accumulation is impaired in ethylene-treated plants overproducing *EBF1* and *EBF2*.

ebf1 ebf2 Double Mutant Phenocopies *ctr1* in Both Seedlings and Adult Plants

To provide further genetic evidence that *EBF1* and *EBF2* are involved in ethylene signaling, we screened a collection of *Arabidopsis* T-DNA insertion lines (Rios et al., 2002) for mutants in both genes (Figure 3D). Two T-DNA insertions were identified within the coding region of *EBF1*, called *ebf1-1* and *ebf1-2*. The T-DNA insertion was located close to the 3′ end of the coding region in *ebf1-1* and causes a truncation of the distal LRR-repeats. In *ebf1-2*, the T-DNA interrupts most of the

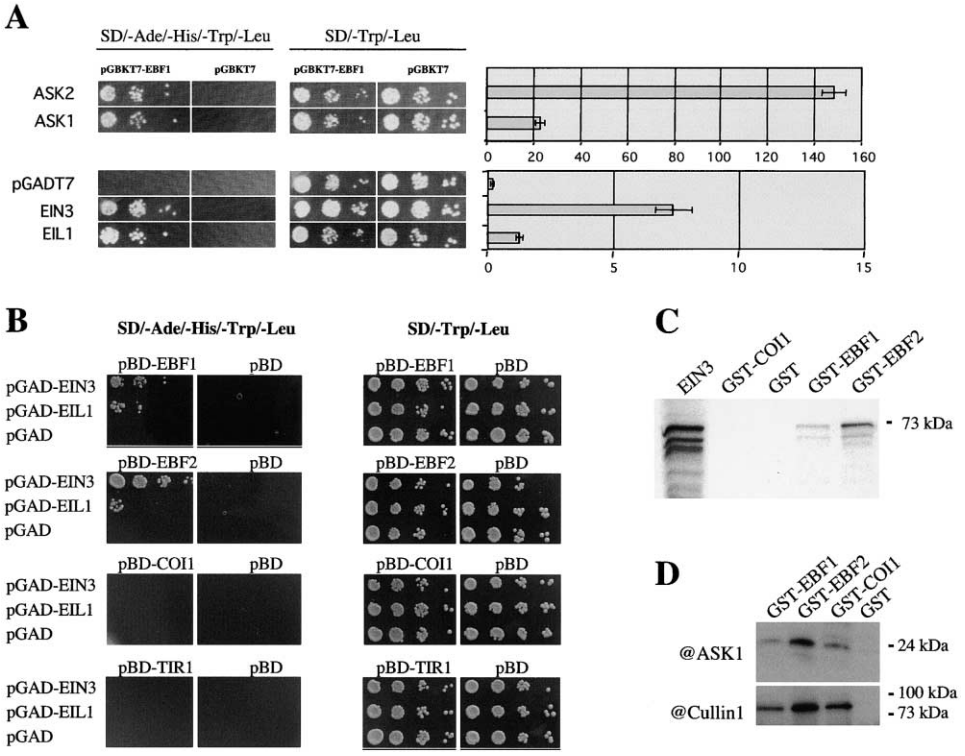

Figure 2. EBF1 and EBF2 Interact with ASK1, ASK2, EIN3, and EIL1

(A) Yeast two-hybrid screening of an *Arabidopsis* green vegetative tissue cDNA library using EBF1 as bait, identified ASK1, ASK2, EIN3, and EIL1 as potential protein interactors. Growth on selective plates lacking adenine, histidine, tryptophan, and leucine (-Ade, -His, -Trp, -Leu) and on control plates lacking only tryptophan and leucine (-Trp, -Leu) is shown. The vectors and expressed proteins are indicated. The plates were photographed after four days. Galactosidase activity is given as the mean ± SD of four independent yeast lines for each combination of constructs.

(B) Yeast two hybrid interactions between EBF1, EBF2, and for comparison COI1 and TIR1, and the EIN3 and EIL1 transcription factors. The vectors and expressed proteins are indicated. The plates were photographed after four days at 30°C on the same medium as in (A).

(C) EIN3 interaction with the F box proteins in vitro. EBF1, EBF2, and COI1 were transcribed and translated in vitro as fusion proteins with GST. These proteins, as well as GST alone, were assayed to pull-down in vitro translated and ^{35}S-labeled EIN3 protein. The same quantities of the GST fusion proteins were used as inputs (data not shown). An aliquot (5 μL) of the EIN3-labeled protein was loaded as a reference.

(D) Pull-down assays using the GST-fused F box proteins or GST alone with total protein extract prepared from an *Arabidopsis* cell suspension culture. The same quantities of purified proteins were used as inputs (data not shown). The bound plant proteins recovered in the pull-down assays were immunoblotted with the ASK1 and AtCUL1 antibodies.

LRRs coding domain. A single line with an insertion in *EBF2* was identified, *ebf2-1*. In this case, the T-DNA was located in the proximal promoter of the gene, resulting in greatly reduced mRNA accumulation (Figure 5A).

Plants homozygous for either *ebf1* or *ebf2* were indistinguishable from wild-type under normal growth conditions. However, when germinated in the dark, all three mutant alleles showed a slightly exaggerated response to ACC (Figures 3E and 3F). Nevertheless, striking developmental alterations resembling the phenotype caused by the well-characterized *c*onstitutive *t*riple *r*esponse*1* (*ctr1*) mutation, were displayed by plants homozygous for both *ebf1-1* and *ebf2-1*. Extensive segregation analysis and PCR testing confirmed that the *ctr1*-like phenotype was dependent on loss of *EBF1* and *EBF2* gene function. We also observed the *ctr1*-like phenotype in the *ebf1-2 ebf2-1* double mutant (data not shown).

When germinated in the dark, the *ebf1-1 ebf2-1* mutant seedlings show a typical constitutive triple response characterized by short hypocotyls, short roots, and exaggerated apical hooks (Figures 4A and 4B). Like *ctr1* (Roman et al., 1995) the *ebf1-1 ebf2-1* mutant is still responsive to ethylene (Figure 4C). Moreover, inhibitors of ethylene perception or biosynthesis including AgNO3 and aminoethoxyvinyl glycine (AVG) could not suppress the constitutive triple response (data not shown), indicating that the phenotypes seen in *ebf1-1 ebf2-1* double mutants are not due to a greatly increased ethylene production or increased sensitivity toward ethylene.

When grown in the light (Figures 4D and 4E), *ebf1-1 ebf2-1* double mutants display slightly longer hypocotyls, curled unexpanded cotyledons, and short and thicker roots with ectopic root hairs (Figure 4F), similar to the *ctr1* mutant (Kieber et al. 1993; Smalle et al., 1997). Moreover, the average area of the leaf epidermal cells was severely reduced in the *ebf1-1 ebf2-1* double mutant (± 1001 μm^2, n = 100) compared to wild-type Col-0 (± 5912 μm^2, n = 100). A ± 5-fold reduction of the mean area of the leaf epidermal cells is also observed for the *ctr1* mutant (± 1158 μm^2, n = 100) as previously reported (Kieber et al., 1993).

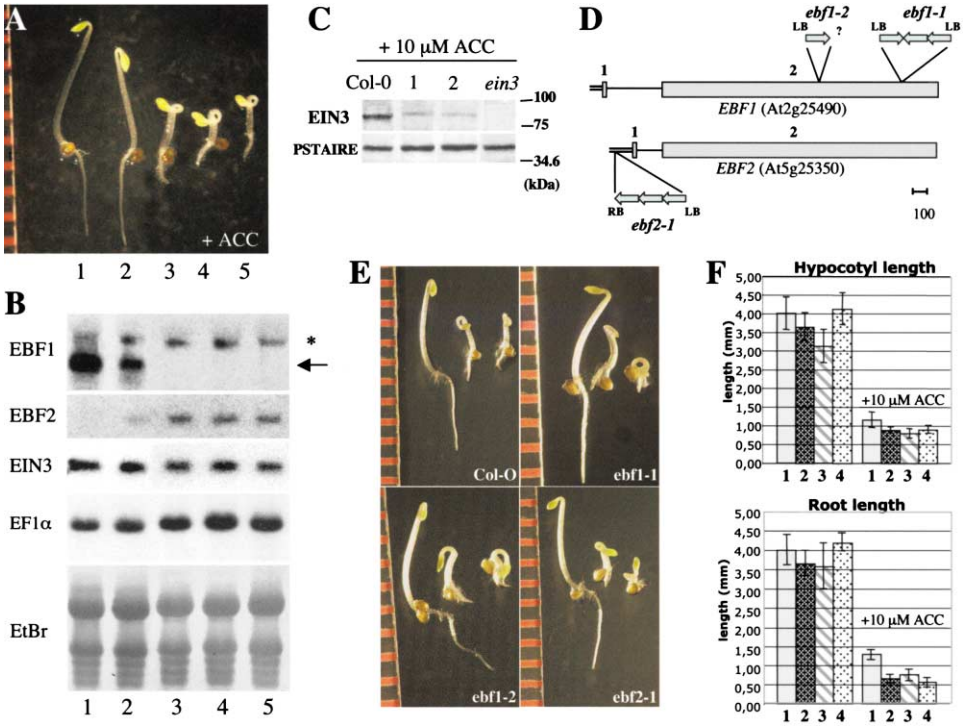

Figure 3. Overexpression of *EBF1* Produces Ethylene Insensitivity and Reduces Expression of the Wild-Type *EBF1/2* Genes, Whereas Single T-DNA Insertion Mutant Lines Are Slightly Hypersensitive to Ethylene

(A) We transformed wild-type *Arabidopsis* plants with a T-DNA vector carrying the *EBF1* open reading frame in the pPILY expression cassette (Ferrando et al., 2000). We tested T3 populations for their response to the ethylene precursor ACC. We found two classes of transformed lines: ethylene-insensitive ones (1, 2) and lines that had a wild-type-like response to ethylene (3, 4). Col-O was used as a control in lane 5.

(B) RNA blot analysis showed that ethylene-insensitive lines (1 and 2) expressed the *EBF1* transgene (arrow) whereas lines that had a wild-type response (3 and 4) lacked transgene expression. Strikingly plants with high levels of transgene expression had reduced expression of endogenous *EBF1* (asterisk), and *EBF2*.

(C) *EBF1* overexpression reduces EIN3 protein accumulation in ACC-treated plants. 5-day-old etiolated seedlings grown on MS medium supplemented with 10 μM ACC were subjected to immunoblot assays for EIN3 protein level. The *ein3* mutant seedlings were used as control to show EIN3 antibody specificity.

(D) Schematic representation of T-DNA insertions in the related F box genes *EBF1* and *EBF2*. LB and RB indicate the orientation of the left and right borders of the T-DNA, respectively. Both genes consist of two exons (gray boxes representing the coding regions). Introns (single lines) and 5′-UTR and promoter sequences (double lines) are indicated.

(E) 3-day-old seedlings germinated in the dark without (left) or with 1 μM (middle) or 10 μM (right) ACC.

(F) Hypocotyl and root length measurements of 3-day-old dark grown Col-0 and *ebf* homozygous mutants in the absence or presence of 10 μM ACC. Each measurement is the average length (mean ± standard error) of >10 hypocotyls or roots.

Similarly to *ctr1*, the *ebf1-1 ebf2-1* double mutant shows also dwarfed growth, supernumerary epinastically curled leaves, early senescence (Figure 4G), and abnormal flowers with the gynoecium protruding from the unopened floral buds (Figure 4H). However, the phenotypes seen in *ebf1 ebf2* plants are more severe than those seen in *ctr1*. This becomes apparent at about two weeks after germination, except for the reduced root growth, which is already obvious at three days after germination. Moreover, unlike the *ctr1* mutation that has a reduced transmission of the *ctr1* allele relative to wild-type and infertile early flowers (Kieber et al., 1993), *ebf1-1 ebf2-1* double mutants have a greatly reduced fertility but can readily be pollinated with wild-type pollen.

Overall our data suggest that EBF1 and EBF2 may play a specific role in the recognition of the EIN3 and (EIL1) transcription factor(s) and facilitate their subsequent SCF-dependent ubiquitylation and degradation. Thus, EIN3 stabilization in the *ebf1 ebf2* double mutant could account for the observed constitutive triple ethylene response phenotype. To test this hypothesis, 12-day-old light grown seedlings were assayed for EIN3 protein abundance. Interestingly, the EIN3 protein accumulated in the *ebf1 ebf2* double mutant in the absence of ACC at a level similar to ACC-treated Col-0 plants (Figure 4I). Moreover, ACC treatment of the *ebf1 ebf2* seedlings did not significantly increase EIN3 protein level (data not shown). The *ebf1 ebf2 ein3* triple mutant (described below) was used to confirm the specificity of the EIN3 antibody.

ERF1 Transcripts Accumulate at High Level in *ebf1 ebf2* Double Mutant and *EBF2* Expression Is Ethylene Responsive in an EIN3-Dependent Manner

Since the *ebf1 ebf2* double mutants resulted in a constitutive "ethylene response phenotype", we investigated whether the mRNA levels of *ERF1*, an immediate target of EIN3, increase in abundance in these plants as well

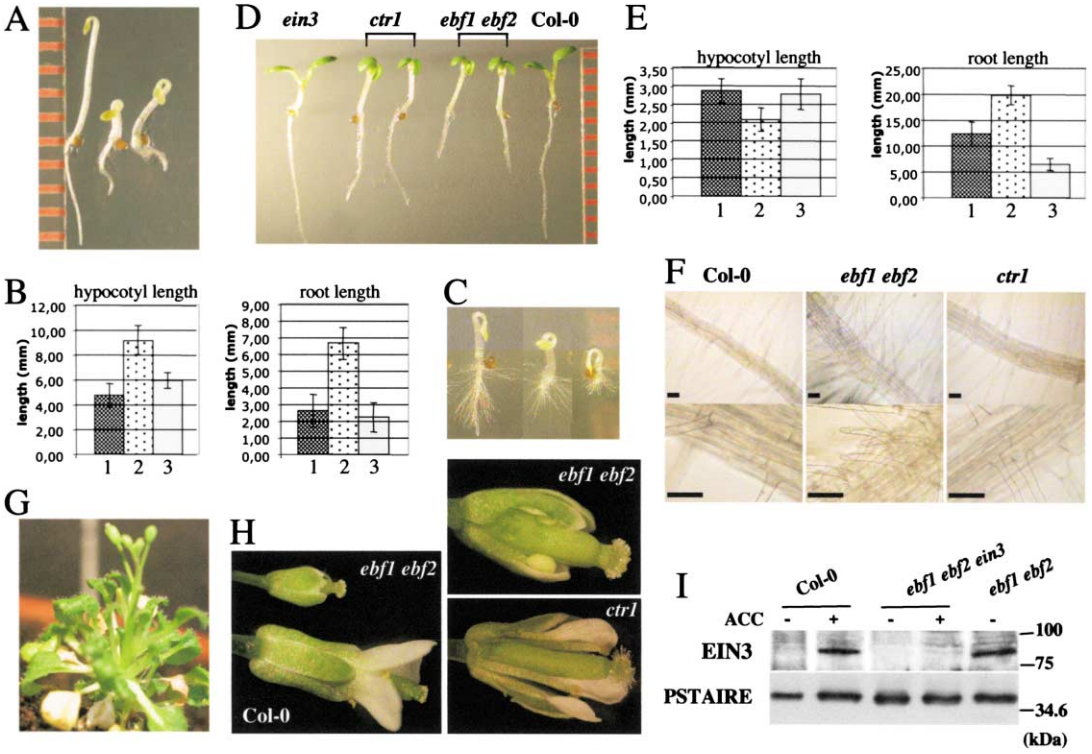

Figure 4. Phenotypic Analysis of *ebf1 ebf2* Double-Mutant Plants

(A) 4-day-old seedlings germinated in the dark; Col-0 (left), *ctr1* (middle) and *ebf1-1 ebf2-1* (right). *ctr1* and *ebf1-1 ebf2-1* display the triple response in absence of ethylene.

(B) Hypocotyl and root length measurements of 4-day-old dark grown *ctr1* (1), Col-0 (2) and *ebf1-1 ebf2-1* (3) seedlings. Each measurement is the average length (mean ± standard error) of >10 hypocotyls or roots.

(C) *ebf1-1 ebf2-1* double-mutant plants are still responding to ethylene. Dark grown 4-day-old *ebf1 ebf2* plants germinated on MS (left), MS + 1 μM ACC (middle), and MS + 10 μM ACC (right). The plants were grown on vertical plates resulting in increased growth of root hair.

(D) 10-day-old light grown *ein3*, *ctr1*, *ebf1-1 ebf2-1* mutants, and Col-0 seedlings.

(E) Hypocotyl and root length measurements of 10-day-old light grown *ctr1* (1), *Col-0* (2), and *ebf1-1 ebf2-1* (3) seedlings. Each measurement is the average length (mean ± standard error) of >10 hypocotyls or roots.

(F) *ebf1-1 ebf2-1* double mutants exhibit a thicker root with more root hairs than the control plant. They also have a high frequency of ectopic rot hairs (root hairs in adjacent vertical cell files), similar to *ctr1*. Magnifications on root hairs are shown in the lower images. Scale bar is equal to 100 μm.

(G) Detailed view of 8-week-old *ebf1-1 ebf2-1* double-mutant plant grown on soil showing dwarfed growth, epinastic curvature of the leaves, supernumerary leaves, and early senescence.

(H) *ebf1-1 ebf2-1* double mutants flower morphology. They have dwarfed flowers that do not open, except for some late flowers in senescing plants. The gynoecium is protruding from the unopened flower in a way that is reminiscent of the *ctr1* mutation. The *ebf1 ebf2* double mutant and the *ctr1*-mutant flowers (on right images) have been opened to reveal the internal structures.

(I) The EIN3 protein accumulates in the *ebf1 ebf2* double mutant in the absence of ACC treatment. 12-day-old light Col-0 or *ebf1 ebf2 ein3* triple-mutant seedling were treated or not during 1 hr with 50 μM ACC. The *ebf1 ebf2* double mutant was not ACC-treated. Total protein extracts were subjected to immunoblot assays.

as in other known ethylene-response mutants. Indeed the steady-state level of *ERF1* mRNA was elevated in the *ebf1-1 ebf2-1* double mutant (Figure 5A) and was even more abundant than that found in *ctr1* mutant plants, previously shown to have constitutive expression of *ERF1* (Solano et al., 1998). The increase of *ERF1* transcript levels is consistent with the high accumulation of EIN3 protein in the *ebf1 ebf2* mutant (Figure 4I).

Interestingly, we also found that *EBF2* mRNA level, and to a lesser extent *EBF1* mRNA levels, were altered in the ethylene-signaling mutants (Figure 5A). *EBF2* transcript levels were elevated in the *ctr1* mutant but less abundant in the *ein2*, *ein3*, and *ein6* mutants. Similarly, the accumulation of *EBF1* transcript was reduced in the *ein3* mutant. These observations are in apparent disagreement with the ethylene-insensitive phenotype of *EBF1* overexpressing plants as one might have expected that *EBF* transcript levels should positively correlate with ethylene insensitivity. Thus, we suspected the existence of a negative-feedback loop in which accumulated EIN3 protein induces its future breakdown by activating *EBF2* transcription either directly or through components of the transcription cascade downstream of EIN3.

To investigate this issue, 2-week-old wild-type Col-0 or *ein3* in vitro grown plants were treated with 10 μM ACC (Figure 5B). Previous work has shown that EIN3 mRNA levels are not responsive to ethylene treatment (Chao et al. 1997). However, both the abundance of *ERF1* and *EBF2* mRNA significantly increases in ACC-

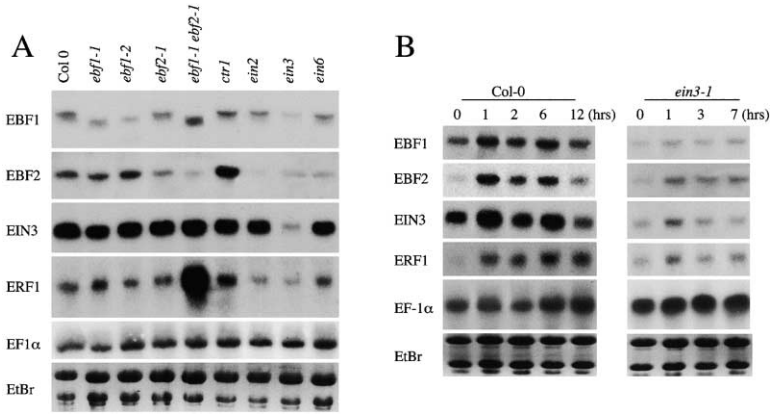

Figure 5. *EBF1*, *EBF2*, *EIN3*, and *ERF1* Transcript Levels in Different Mutants of Ethylene Signaling and Following Ethylene Treatment

(A) RNA was extracted from rosette leaves of 4-week-old plants and subjected to RNA gel blot analysis with the indicated probes. Note that *ebf1-1* and *ebf1-2* plants express a truncated *EBF1* transcript due to the T-DNA insertion in the gene whereas *ebf2-1* expresses an apparent full-length *EBF2* transcript albeit at a greatly reduced level of expression.

(B) RNA was extracted from 2-week-old Col-0 and *ein3* plantlets at different time points after 10 μM ACC treatment and subjected to RNA blot analysis and hybridized with the indicated probes.

treated Col-0 plants, but not in ACC-treated *ein3* mutant plants. From these experiments, we conclude that *EBF2* transcription is regulated by ethylene in an EIN3-dependent fashion. Such a negative-feedback loop might allow the plant to rapidly respond to changing ethylene concentrations and to have strict temporal control over EIN3 protein abundance/activity.

The *ebf1 ebf2* Double-Mutant Phenotype Requires Functional *EIN3*

We next investigated the relationship between EIN3 and EBF1 and EBF2 using a genetic approach and tested whether the constitutive ethylene response of *ebf1 ebf2* mutants might simply reflect increased levels of EIN3. Thus, we generated the triple-mutant combination *ein3-1 ebf1-1 ebf2-1*. As expected, no EIN3 protein was detectable in the *ein3-1 ebf1-1 ebf2-1* plants, even when ACC-treated (Figure 4I). These plants had an ethylene-insensitive phenotype similar to the one seen in *ein3* mutant plants showing that *ein3* is epistatic to *ebf1 ebf2* (Figure 6A). Unlike *ebf1 ebf2* mutant plants, *ein3 ebf1 ebf2* mutant plants exhibited normal leaf and flower morphology and were fully fertile (Figures 6B and 6C). However, *ein3-1 ebf1-1 ebf2-1* triple-mutant plants were slightly smaller than *ein3-1* plants (Figure 6B).

From these observations, we conclude that the constitutive triple ethylene response phenotype of the *ebf1-1 ebf2-1* double mutant is indeed dependent on the presence of functional EIN3 protein. Moreover, the weak differences between the *ein3* and *ein3-1 ebf1-1 ebf2-1* mutants suggest that in addition to EIN3, mutations of the *EBF1* and *EBF2* may also affect the function of EIL1 or other still unknown proteins involved in the ethylene signaling. This is not unexpected as it was recently shown that EIL1 acts in parallel with EIN3 (Alonso et al., 2003) and therefore the stabilization of EIL1 in *ein3-1 ebf1-1 ebf2-1* plants could account for the observed slight reduction in ethylene insensitivity and for the reduced plant size.

The *EBF1* and *EBF2* Gene Products Act Downstream or Independent of the *EIN2* Gene Product

The *EIN2* gene encodes a transmembrane protein with partial homology to mammalian metal transporters and has been shown to act upstream of EIN3 (Alonso et al., 1999). We generated *ein2 ebf1 ebf2* triple mutants. We found that, as young seedlings, *ein2 ebf1 ebf2* triple-mutant plants had a intermediate phenotype with greatly reduced hypocotyl and root length compared to *ein2* and wild-type seedlings when germinated in the dark in the absence of ethylene. (Figure 6D). However the reduction in hypocotyl length and root length was not as severe as seen in *ebf1 ebf2* plants and in contrast to *ebf1 ebf2* plants, *ein2 ebf1 ebf2* triple mutants lacked an exaggerated apical hook. As these plants matured, they showed the characteristic *ctr1*-like morphology of *ebf1 ebf2* double-mutant plants (Figures 6E–6G) with epinastic cotyledons, epinastic leaves, reduced leaf size, and altered flower morphology. Because of the *ebf1 ebf2*-like phenotype for most of the lifespan of the *ein2 ebf1 ebf2* triple mutant, we tentatively place EBF1 and EBF2 downstream of EIN2, although we cannot rule out that they act independently of EIN2.

Discussion

Based on the biochemical and genetic interactions between EBF1/2 and EIN3 and the previous knowledge, we propose a model in which EIN3 is expressed constitutively, but is unable to accumulate because it is subjected to permanent proteolysis mediated by the two F box proteins, EBF1 and EBF2. Only after perception of ethylene, EIN3 becomes stabilized and acts on its target promoters.

This model is consistent with several experimental observations. (1) EIN3 exhibits a constitutive mRNA accumulation that is not affected by treatment with ethylene and remains constant in ethylene-response mutants (Chao et al., 1997), including in the *ebf-1 ebf2* double mutant. (2) EIN3 is rapidly degraded trough a proteasome-mediated way, but is stabilized upon ethylene treatment (see the accompanying paper from Guo et al., 2003 and also Yanagisawa et al., 2003). (3) Massive overexpression of EIN3 leads to constitutive ethylene response (Chao et al., 1997), whereas overexpression of EBF1 results in ethylene insensitivity and reduced EIN3 protein level, indicating an inverse correlation between EIN3 and the F box protein levels. (4) Both EBF1 and EBF2 proteins, as well as other SCF components (Farras et al., 2001; Shen et al., 2002) are localized in the nucleus, where EIN3 is supposed to act. (5) The *ebf1*

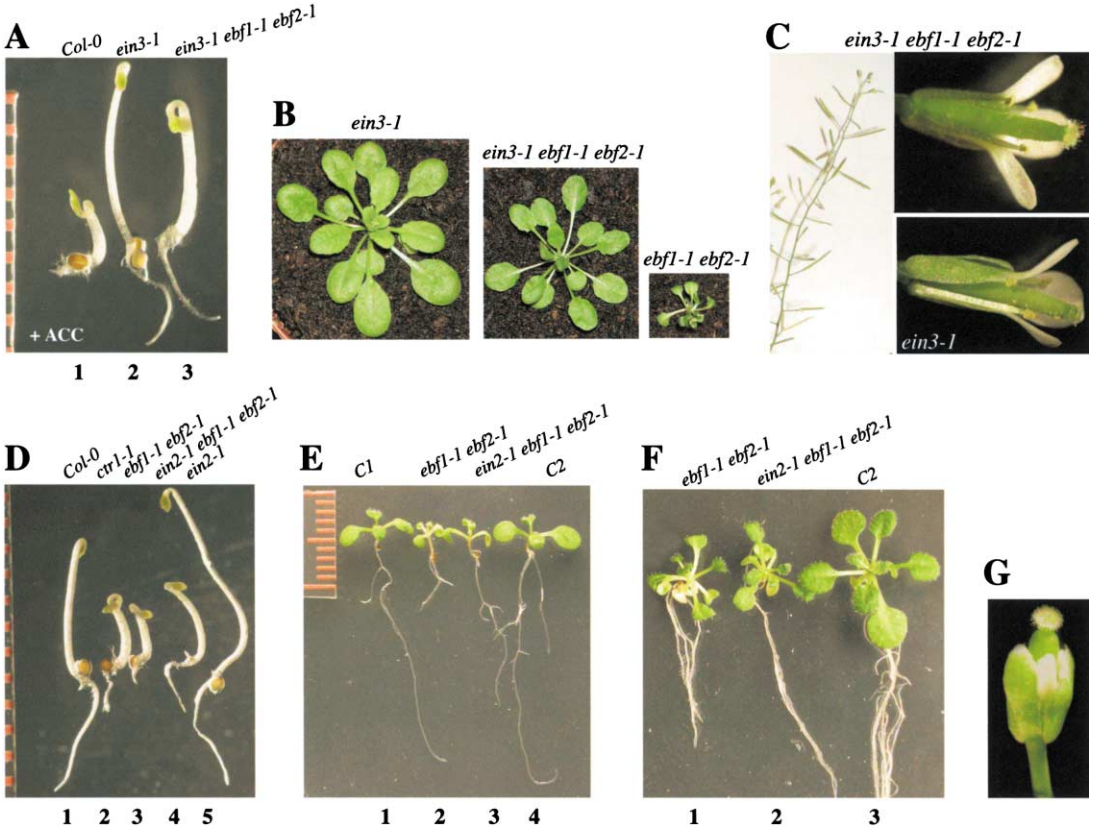

Figure 6. Effect of *ein3* and *ein2* Mutations on the *ebf1 ebf2* Mutant Phenotype

(A) When germinated in the dark in the presence of ACC, *ein3-1 ebf1-1 ebf2-1* triple mutant plants display an ethylene-insensitive phenotype, similar to the *ein3-1* control.

(B) *ein3-1*, *ebf1-1 ebf2-1* mutant plants lack the striking phenotype of the *ebf1-1 ebf2-1* double mutant.

(C) *ein3-1 ebf1-1 ebf2* triple-mutant plants, unlike *ebf1-1 ebf2-1* double-mutant plants, are fully fertile. There are no aberrations in flower morphology or silique morphology visible when compared to the *ein3-1* plant.

(D) *ein2-1 ebf1-1 ebf2-1* triple-mutant plants display an intermediate phenotype when germinated in the dark in the absence of ethylene. They have a strongly reduced hypocotyl and root lengths when compared to the *ein2-1* or Col-O control, but are lacking an exaggerated apical hook and have increased hypocotyl and root length when compared to *ebf1-1 ebf2-1* or *ctr1-1* seedlings.

(E) 10 days after germination in the light *ein2-1 ebf1-1 ebf2-1* seedlings have the characteristic morphology of *ebf1-1 ebf2-1* seedlings with epinastic cotyledons and leaves. However, *ein2-1 ebf1-1 ebf2-2* plants are less severely affected than *ebf1-1 ebf2-1* plants and primary root growth is almost unimpaired when compared to control plants that are heterozygous for the *ebf1-1* and *ebf2-1* insertions and wild-type (C1) or *ein2-1* (C2) with respect to the *EIN2* gene.

(F) 3 weeks after germination in the light *ein2-1 ebf1-1 ebf2-1* plants display leaf epinasty and reduced leaf area similar to the *ebf1-1 ebf2-1* double mutant. However senescence and root growth are less impaired than in the *ebf1-1 ebf2-1* plant. C2 is as in (E).

(G) *ein2-1 ebf1-1 ebf2-1* triple-mutant plants show defects in floral morphology similar to the *ebf1-1 ebf2-1* mutant plants.

ebf2 mutant plants displayed a constitutive ethylene response similar to, but more severe than, *ctr1*. The *ebf1 ebf2* double-mutant plants clearly showed a high accumulation of the EIN3 protein and displayed a strong transcriptional upregulation of the direct EIN3 target *ERF1* gene in the absence of hormone induction. (6) Finally, the *ebf1-1 ebf2-1 ein3-1* triple mutant fails to display a *ctr1*-like phenotype. This later result is noteworthy because it indicates that under normal growth conditions, the EIN3 protein, and not EIL1 (or other EILs), is mainly responsible for the triple response mutant phenotype. This is in agreement with *ein3* loss-of-function mutant being hormone insensitive (Chao et al., 1997) and an *eil1* mutant being only weakly ethylene insensitive (Alonso et al., 2003).

According to our model, EIN3 degradation needs to be switched off to allow EIN3 accumulation after ethylene stimulation. Our genetic results indicated that EIN2 act upstream or parallel to EBF1/2, however its function in the turnover of the EIN3 protein is still highly speculative. To explain how ethylene regulates EIN3 stability several mechanisms can be envisaged (Figure 7). Either the $SCF^{EBF1/EBF2}$ complexes are negatively regulated after ethylene perception to allow EIN3 accumulation. For example EBF1/2 F box proteins might selectively be degraded or excluded from the nucleus in an EIN2-dependent way. Or, EIN3 is directly protected from the $SCF^{EBF1/EBF2}$ ubiquitin protein-ligases. Many targets for SCF-dependent proteolysis are modified by phosphorylation (Deshaies, 1999) or even by N-glycosylation (Yoshida et al., 2002). However, these modifications are prerequisites for their recognition by the F box proteins. In ethylene signaling, this rule does not seem to apply since we observed a direct interaction between EIN3 and the F box proteins

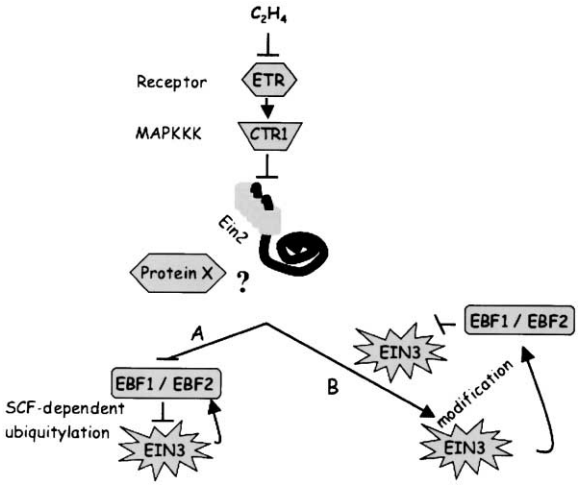

Figure 7. Models Showing How EBF1 and EBF2 Function during Ethylene Signaling

Two models are proposed to explain how EIN3 accumulates after ethylene perception. Both models assume that the $SCF^{EBF1/EBF2}$ complexes permanently degrade EIN3, in the absence of ethylene. Upon ethylene perception, the MAPKKK CTR1 is inactivated and consequently EIN2 is activated. In model (A) EIN2, or another yet unidentified component of the signaling pathway (called protein X), acts as a negative regulator of EIN3-dependent ubiquitylation by the $SCF^{EBF1/EBF2}$; whereas in model (B), it acts as a positive regulator of EIN3 stability by protecting EIN3 from EBF1/EBF2-mediated turnover. Thus, in the presence of ethylene, the EIN3 protein will be able to accumulate and trigger ethylene-responsive gene expression. EIN3 then induces its own degradation by inducing EBF2 transcription, resulting in a negative-feedback loop.

in vitro and in yeast two-hybrid assays. Therefore, it is possible that a posttranslational modification of EIN3 either does not occur at all or that it has acquired other roles than tagging the protein for proteolysis. For example, it might serve to protect EIN3 from proteolysis. Future research will be needed to resolve this issue and it is more than likely that other components of the ethylene-response pathway still await discovery.

In addition to position EBF1/2 in the ethylene-response pathway by biochemical and genetic means, we found that *EBF1* and *EBF2* transcription is regulated in an ethylene-responsive way. The transcription of both genes is misregulated in known mutants of the ethylene-response pathway and *EBF2* transcription is rapidly induced after application of ethylene. Moreover, the ethylene-inducible upregulation of *EBF2*, as well as the steady-state transcription levels of *EBF1*, is greatly reduced in *ein3* mutant plants. Based on this observation, we propose a negative-feedback loop (Figure 7), in which the stabilization of EIN3 results in the strong transcriptional induction of *EBF2*, which subsequently promotes the degradation of EIN3, thus enabling the plant to have very strict temporal control over EIN3 activity.

It is noteworthy that in other known plant hormone signaling pathways in which the SCF is involved, the protein targets are thought to act as transcription repressors, such as the AUX/IAA proteins in auxin signaling (Tiwari et al., 2001, Gray et al., 2001), the DELLA domain-containing proteins in gibberellin response (Dill et al., 2001) and a still unknown repressor protein in jasmonate signaling. In these pathways, the repressors are degraded after hormone signaling enabling the transcription of downstream targets. Protein degradation in ethylene signaling seems to be different since it appears that EIN3, a transcriptional activator, is permanently degraded via the SCF and becomes stabilized only after hormone signaling. However, the constitutive degradation of a transcription activator is not unique to plant, since in mammals, the HIF1α transcription factor, which activates the transcription of genes encoding angiogenic and growth factors, is also constitutively expressed and degraded under normoxic conditions. Only when oxygen levels are low, the protein is able to accumulate (Ivan et al. 2001). Nevertheless the HIF1α protein is recognized by a different class of ubiquitin protein-ligase (the ECS-type for ElonginC-Cul2-SOCS box) and specifically requires the hydroxylation of a proline residue for its degradation. In the future, it will be interesting to compare and identify the advantages in signaling pathways of this mode of regulation, versus the degradation of transcriptional repressors.

Experimental Procedures

Plant Material, Transformation, and Treatments

The *Arabidopsis* plants were of the Columbia (Col-0) ecotype. The *ebf1-1, ebf1-2*, and *ebf2-1* knockout lines were identified by PCR-screening the Köln-*Arabidopsis* T-DNA mutant collection (Rios et al., 2002) using gene specific primers and T-DNA specific primer FISH1 and FISH2. The T-DNA insertion sites were determined by DNA sequencing. In the *ebf1-1* and *ebf1-2* mutants, the T-DNA were inserted in the EBF1 coding sequence at position 1642 and 1048, respectively. In the *ebf1-1* mutant, the last 81 amino acids are replaced by a 20 amino acid peptide (VQDIFNCKWLHVREIYMDQQ). In *ebf1-2*, the mutation gives rise to a truncated protein in which the 267 last amino acids are replaced by a 7 amino acid sequence (TPGYILQ). In the *ebf2-1* mutant, the T-DNA is inserted 221 nucleotides upstream of the EBF2 coding sequence, which based on the sequences of available full-length cDNA clones, belongs to the proximal promoter region.

Transgenic *Arabidopsis* plants were obtained by *Agrobacterium*-mediated transformation using the floral dip method (Bechtold et al. 1993). Plants were grown on soil with 12 hr day and 12 hr night. In vitro plants were grown onto half strength Murashige and Skoog medium M0255 (Duchefa) supplemented with 1% sucrose (conditions used: 16 hr day and 8 hr night at 20°C).

ein3-1, *ctr1-1*, *ein2-1*, and *ein6* have been described (Chao et al., 1997, Kieber et al., 1993, Alonso et al., 1999) and were obtained from the Nottingham *Arabidopsis* Stock center. With the exception of *ein6,* which is in the Landsberg background, they are in the Columbia background. The sequence of the PCR markers used for segregation analysis is available on request.

ein3-1 ebf1-1 ebf2 plants were generated by crossing *ein3-1* to a plant that was double heterozygous for *ebf1-1* and *ebf2*. In the F1 generation, triple heterozygous plants were identified by PCR testing; in the F2 generation, plants that were homozygous for the *ein3-1* mutation were selected on the basis of their ACC-insensitive phenotype. Thirty-two plants were transferred to soil, the *ein3-1* homozygously was confirmed by PCR testing and PCR product sequencing, and the segregation of the T-DNA insertions in *EBF1* and *2* was determined by PCR testing. Two plants homozygous for both T-DNA insertions were found, perfectly matching the expected segregation. The ethylene-response phenotype was confirmed and statistically analyzed in the F3 generation.

Likewise, *ein2-1 ebf1-1 ebf2* plants were generated by pollinating an *ebf1 ebf2* double-mutant plant. In the F2 generation, plants that were phenotypically *ebf1 ebf2* double mutants were selected and tested for the presence of the *ein2-1* mutation. Fifty-three plants were screened and four were found to be homozygous for the *ein2-1* mutation. The close genetic linkage of EBF2 and EIN2 on chromo-

some 5 can explain the reduced number of double mutants. Offspring of plants homozygous for *ein2-1* and heterozygous for both *ebf* insertions was used to identify and analyze *ein2-1 ebf1 ebf2* triple homozygous seedlings.

Cell Size Measurements

The leaf epidermal cell size measurements of 22 days postgerminated seedlings were performed as described in Lechner et al. (2002).

Fluorescence Imaging of the F Box Proteins

The GFP-EBF1 and the EBF1-GFP fusions were generated by PCR modifying the EBF1 EST clone APZL08dO8R and introduction into the pSKGFP3 (P.G., unpublished data) vector. The resulting fusion genes were subsequently introduced into the T-DNA vector pER8 (Zuo et al., 2002).

The GFP fusion proteins were transiently expressed in agro-infiltrated leaves of *Nicotiana benthamiana* as described by Voinnet et al. (1998) in the presence of 5 μM estradiol. Three days after infection, the infiltrated leaves were peeled and observed. Confocal images were obtained by a Zeiss LSM510 laser-scanning confocal microscope with argon laser excitation at 488 nm and through 505–550 emission filter-set and using a C-APOCHROMAT (63 × 1, 2W Korr) water objective lens. The images are presented as single sections. Transmitted light reference images were taken using differential interference contrast (Nomarski) optics and argon laser illumination at 488 nm.

EBF1 Overexpression Constructs

The open reading frame of EBF1 was PCR modified to clone it into the pPILY plant expression cassette (Ferrando et al., 2000). This expression cassette containing EBF1 was subsequently introduced into the pCAMBIA1380 vector (CAMBIA, Australia). Col-O was used for transformation.

RNA Gel Blotting

RNA gels were performed with 20 μg of total RNA per lane. The integrity and the amount of RNA applied to each lane were verified by ethidium bromide staining and hybridization with a probe encoding the translation elongation factor 1-α (EF-1α) (cDNA clone 232A19T7, gene At1g07920).

Yeast Two-Hybrid Screening and Assays

The yeast host strain AH109 (Clontech) was first transformed with pGBKT7-EBF1 as a bait, followed by transformation with a 3-week-old *Arabidopsis* (Co) green vegetative tissue cDNA library in pGAD10 vector (Clontech). About 3×10^6 yeast transformants were screened on the selective medium SD/-His/-Trp/-Leu with 1.5 mM 3-amino-1,2,4,-triazole (3-AT) and the positive clones were than subsequently plated on SD/-Ade/-His/-Trp/-Leu medium. Around forty-five clones were able to grow on this medium and gave a positive β-galactosidase activity. Plasmids were extracted, transformed into *E. coli*, and cDNA inserts were analyzed by restriction enzyme digestions and sequenced.

To further confirm and validate our clones, we retransformed the yeast AH109 strain containing either the pGBKT7-EBF1 or the pGBKT7 empty vector with the candidate plasmids and plated different dilutions of the transformants either on SD/-Ade/-His/-Trp/-Leu or on SD/-Trp/-Leu media. Thirty-nine transformants were able to grow on SD/-Ade/-His/-Trp/-Leu , with both the candidate plasmids and pGBKT7-EBF1 and not with the empty vector, and were considered as positive.

To test the β-galactosidase activities, the candidate plasmids were retransformed in the yeast Y187 strain (transformed with pGBKT7-EBF1) since this strain has a stronger promoter to drive *lacZ* expression than AH109.

Moreover, the cDNAs coding EBF1, EBF2, COI1, and TIR1 were introduced into the pGBT9 vector (Clontech), and tested against the obtained EIN3 and EIL1 plasmids. A full-length clone of EIN3 was furthermore put in a pGADT7 clone without any change in the two-hybrid interaction. The yeast two-hybrid analysis was performed using the GAL4-based matchmaker two-hybrid system (Clontech) according to the manufacturer's protocol.

Immunoblot Assays

The EIN3 polyclonal antibody (Yanagisawa et al. 2003) was affinity purified with the His-tagged recombinant EIN3 protein expressed from *E. coli*. Samples of 15 μg proteins were separated by SDS gels and blotted onto Immobilon-P membrane (Millipore, Bedford, MA). The blots were probed first with the EIN3 antibody and subsequently stripped and immunoblotted with the Cdc2 (PSTAIRE) polyclonal antibody, used as loading control.

In Vitro Protein-Protein Interaction Studies

The EBF1, EBF2, and COI1 coding sequences were cloned into the gateway vector pDEST™15 (Invitrogen) by recombination for expression in *E.coli* strain BL21(DE3)pLysE. In these constructs, GST is placed in frame at the N terminus of the fusion protein. After 4 hr of 0.5 mM IPTG induction, the fusion proteins were purified in the native form, under nondenaturing conditions on bulk glutathione-Sepharose 4B (Pharmacia).

For the GST pull-down assays, the EIN3 protein was translated in vitro, using the TNTT7-coupled wheat germ extract system (Promega) and radio-labeled with ^{35}S methionine. The GST pull-down assays were realized with 4 μg of purified GST or GST-F box fusion proteins fixed to glutathione-Sepharose 4B resin (Amersham) and incubated with 2.5 mg of total protein extracts prepared from an *Arabidopsis* cell suspension culture, as described in Lechner et al. (2002).

To detect the AtCul1 protein, we used the purified rabbit polyclonal anti-Cul1 antibody (Shen et al., 2002) diluted 1: 800. To detect the ASK1 protein, we used a purified rabbit polyclonal antibody raised against a peptide of the *Arabidopsis* ASK1 protein (P.G., unpublished data) diluted 1: 3000. The immunoreactive proteins were detected using peroxidase-conjugated goat antirabbit antibodies (Dianova) and the ECL Western blot analysis system from Amersham Biosciences.

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