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The *Arabidopsis* EIN3 Binding F-Box Proteins EBF1 and EBF2 Have Distinct but Overlapping Roles in Ethylene Signaling [W]

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Ethylene signaling in *Arabidopsis thaliana* converges on the ETHYLENE-INSENSITIVE3 (EIN3)/EIN3-Like (EIL) transcription factors to induce various responses. EIN3 BINDING F-BOX1 (EBF1) and EBF2 were recently shown to function in ethylene perception by regulating EIN3/EIL turnover. In the absence of ethylene, EIN3 and possibly other EIL proteins are targeted for ubiquitination and subsequent degradation by Cullin 1–based E3 complexes containing EBF1 and 2. Ethylene appears to block this ubiquitination, allowing EIN3/EIL levels to rise and mediate ethylene signaling. Through analysis of mutant combinations affecting accumulation of EBF1, EBF2, EIN3, and EIL1, we show that EIN3 and EIL1 are the main targets of EBF1/2. Kinetic analyses of hypocotyl growth inhibition in response to ethylene and growth recovery after removal of the hormone revealed that EBF1 and 2 have temporally distinct but overlapping roles in modulating ethylene perception. Whereas EBF1 plays the main role in air and during the initial phase of signaling, EBF2 plays a more prominent role during the latter stages of the response and the resumption of growth following ethylene removal. Through their coordinated control of EIN3/EIL1 levels, EBF1 and EBF2 fine-tune ethylene responses by repressing signaling in the absence of the hormone, dampening signaling at high hormone concentrations, and promoting a more rapid recovery after ethylene levels dissipate.

INTRODUCTION

The simple gaseous hydrocarbon, ethylene (C_2H_4), controls a myriad of growth and developmental processes in plants, including seedling germination and growth, leaf and petal abscission, fruit ripening, organ senescence, and stress and pathogen responses (reviewed in Schaller and Kieber, 2002). Ethylene responses are mediated through a signal transduction cascade that has been partially defined in *Arabidopsis thaliana* using genetic and molecular approaches (Guo and Ecker, 2004; Chen et al., 2005). The hormone is perceived by five ethylene receptors (ETHYLENE RESPONSE1 [ETR1], ETR2, ETHYLENE-INSENSITIVE4 [EIN4], ETHYLENE RESPONSE SENSOR1 [ERS1], and ERS2), which are in a complex with the Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1). In the absence of ethylene, the receptors stimulate CTR1, which then inhibits downstream events that likely include a mitogen-activated protein kinase (MAPK) cascade. Binding of ethylene to the receptors represses CTR1 action. Once released from inhibition by CTR1, the MAPK cascade eventually activates EIN2 (Alonso et al., 1999), which subsequently induces the accumulation of the transcription factor EIN3 (Chao et al., 1997; Ouaked et al., 2003; Yanagisawa et al., 2003). The pathway between CTR1 and EIN3 is also modulated by the EIN5/XRN4 5′-3′ exoribonuclease, but its direct RNA target(s) is currently unknown (Olmedo et al., 2006; Potushak et al., 2006).

EIN3 is a key positive switch in ethylene perception. For example, mutants in *EIN3* have reduced responses to ethylene, whereas overexpression of EIN3 results in ethylene hypersensitivity or a constitutive ethylene response (Roman et al., 1995; Chao et al., 1997). It works by promoting the transcription of a variety of *ETHYLENE RESPONSE FACTOR* genes that ultimately direct the growth and physiological responses under ethylene control (Solano et al., 1998). During activation, EIN3 binds as a dimer to primary ethylene response DNA elements, which are 28-bp imperfect palindromes found in the promoters of various ethylene-responsive genes (Solano et al., 1998).

In addition to EIN3, *Arabidopsis* encodes five EIN3-Like (EIL) transcription factors, EIL1 to EIL5, that may also contribute to ethylene signaling (Chao et al., 1997; Wang et al., 2002; this work). Mutants in *EIL1* have a weak ethylene insensitivity, and overexpression of *EIL1* results in a constitutive ethylene response, implying that the EIL1 protein functionally overlaps with EIN3 (Chao et al., 1997). In fact, both growth kinetic and phenotypic analyses of *ein3 eil1* double mutants imply that all sustained ethylene responses are generated primarily through the action of EIN3 and EIL1 (Alonso et al., 2003a; Binder et al., 2004a). The cellular roles of the remaining EIL2-5 members of the family remain unclear. The observation that *EIL2* overexpression partially complements the *ein3* mutant phenotype indicates that EIL2 could play a minor role in ethylene perception (Chao et al., 1997).

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It is now apparent that the abundance of EIN3, and possibly the EILs, is a critical regulatory feature governing the strength of ethylene responses. Whereas *EIN3* mRNA levels are unaffected by the hormone, abundance of the EIN3 protein rises dramatically upon ethylene exposure due to a strong stabilization of the protein (Guo and Ecker, 2003; Yanagisawa et al., 2003). This stabilization can be reversed by glucose, a well-known antagonist of ethylene, suggesting that multiple signals converge to regulate EIN3 breakdown (Yanagisawa et al., 2003). The observation of Yanagisawa et al. (2003) that MG132, a potent inhibitor of the 26S proteasome, stabilizes EIN3 even in the absence of ethylene first implicated the ubiquitin (Ub)/26S proteasome system in EIN3 turnover. In this system, a polymer of the Ubs is covalently attached to proteins committed to degradation using a large set of Ub-protein ligases (or E3s) for target selection (for review, see Smalle and Vierstra, 2004). More than 1300 different E3s are predicted to exist in *Arabidopsis* alone, suggesting that each E3 recognizes a limited set of targets through highly specific interactions (Gagne et al., 2002; Smalle and Vierstra, 2004; Gingerich et al., 2005). Once multiple Ubs are bound, the modified target is delivered to the 26S proteasome, which degrades the target but releases the Ub moieties intact for reuse.

How EIN3 is recognized for ubiquitination was recently revealed by us and others through the identification of two E3 components, EIN3 BINDING F-BOX1 (EBF1) and EBF2 proteins, that are essential for proper ethylene perception (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Like other F-box proteins, they form SCF E3 complexes by each assembling with the core subcomplex containing the SKP1, Cullin 1, and RBX1 subunits. EBF1 and 2 also directly bind their target EIN3, using a conserved C-terminal Leu-rich repeat region, and like EIN3, EBF1/2 appear to be nuclear-localized. Importantly, disruption of either *EBF1* or *EBF2* increases EIN3 protein levels and induces a hypersensitivity to exogenous ethylene at both the phenotypic and molecular levels. In fact, a dramatic growth arrest phenotype is observed for confirmed *ebf1 ebf2* double null mutants, which is more severe than any previously published for other ethylene signaling components (Gagne et al., 2004). The double mutants emerge slowly from their seed coat as severely stunted seedlings and fail to progress further developmentally. Other defects include a substantial increase in root hair number, enhanced anthocyanin accumulation, accelerated senescence of the cotyledons, and a failure to develop leaves, stems, and flowers. The *ebf1 ebf2* seedlings have elevated EIN3 levels in the absence of exogenous ethylene, suggesting that ubiquitination of EIN3 (and possibly one or more EILs) by the corresponding $SCF^{EBF1/2}$ complexes is essential for plant growth even in the absence of the hormone (Gagne et al., 2004).

Both phenotypic and molecular analyses suggested that EBF1 and EBF2 work coordinately in ethylene signaling (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). For example, the growth of *ebf1* mutants is repressed in the absence of exogenous ethylene or at low concentrations but is similar to the wild type at saturating levels, whereas the growth of *ebf2* mutants is similar to the wild type at low ethylene concentrations but is markedly more compromised at saturation levels (Guo and Ecker, 2003; Gagne et al., 2004). Here, we demonstrate that EIN3 and EIL1 are the main targets of EBF1 and EBF2. We also delineate the distinct roles of these F-box proteins through the genetic analysis of *ebf1* and *ebf2* mutants in combination with others affecting ethylene perception and through detailed kinetic analysis of hypocotyl growth inhibition of various mutants in response to exogenous ethylene. Collectively, the data reveal that the SCF^{EBF1} and SCF^{EBF2} E3 complexes work in concert to fine-tune the abundance of EIN3/EIL1 in response to ambient hormone levels. The synergistic action of EBF1/2 suggests that the breakdown of other Ub/26S proteasome pathway targets also may be precisely regulated by sets of related E3s working together.

RESULTS

Growth Arrest by *ebf1/2* Mutants Caused by a Defect in Ethylene Signaling

The striking growth arrest of *ebf1 ebf2* double null mutants implied that ethylene perception in *Arabidopsis* is strongly enhanced by a failure to degrade EIN3 and possibly one or more members of the EIL protein family (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). However, given the feed-forward control of ethylene on its own synthesis (Schaller and Kieber, 2002), it remained possible that the growth of the mutant plants was compromised by abnormally high ethylene production in addition to a hypersensitivity to the gas. To test this possibility, we attempted to rescue loss-of-function *ebf1-4 ebf2-4* seedlings (in the Wassilewskija background; Gagne et al., 2004) by growth on either L-α-(2-aminoethoxyvinyl)-glycine (AVG), an ethylene biosynthetic inhibitor, or silver nitrate, a potent inhibitor of perception by the family of ethylene receptors. When used at concentrations that are typically effective (Beyer, 1976; Yang and Hoffman, 1984), neither inhibitor improved the stunted growth of the double homozygous mutant (Figure 1A). We also exposed the *ebf1-4 ebf2-4* seedlings to 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene (Schaller and Kieber, 2002), in an attempt to see if the plants would respond further to the hormone. Under our growth conditions, no additional inhibitory effects of ACC were apparent, suggesting that null *ebf1 ebf2* mutants are saturated for ethylene perception (Figure 1A). Finally, the *ebf1-4 ebf2-4* seedlings were exposed to high concentrations of glucose (1%), which was previously shown to destabilize EIN3 (Yanagisawa et al., 2003) and thus might partially reverse the effects of the mutations if the sugar activated another degradation pathway. Likewise, glucose was without effect in the presence or absence of ACC, AVG, or silver nitrate, with the only change being a promotion of root hair growth under all conditions that included glucose (Figure 1A).

Loss of *CTR1* Exacerbates Defects in *EBF2*

The weak but different responses of the single *ebf1* and *ebf2* mutants to exogenous ethylene suggested that each plays a distinct role in ethylene signaling (Guo and Ecker, 2003; Gagne et al., 2004). To examine these roles further, we combined the monogenic *ebf1-3* and *ebf2-3* mutants (in the Columbia [Col-0] background) with one affecting the negative regulator CTR1. The *ctr1-1* allele blocks action of the CTR1, leading to strong

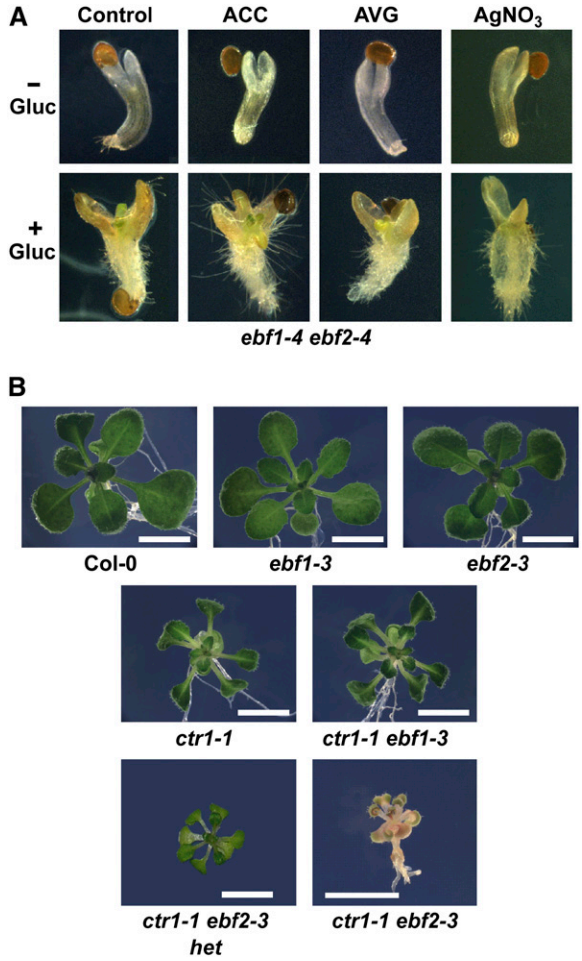


Figure 1. Connection of the *ebf1 ebf2* Mutants to Ethylene Synthesis and Perception.

(A) Development of homozygous *ebf1-4 ebf2-4* seedlings in the Wassilewskija background in response to ACC (0.2 μM), AVG (50 μM), and $AgNO_3$ (100 μM), with or without the inclusion of 2% glucose (Gluc). Seedlings were germinated and grown for 8 d under continuous light.
(B) Synergy of the *ebf1-3* and *ebf2-3* mutations with *ctr1-1*. Plants were grown for 16 d on growth medium under a long-day photoperiod. *ctr1-1 ebf2-3 het* indicates a plant that is homozygous for the *ctr1-1* allele but heterozygous for the *ebf2-3* allele. Bars = 5 mm.

constitutive ethylene signaling even in the absence of the hormone (Kieber et al., 1993). This constitutive response is masked when *ctr1-1* is combined with both *ein3-1* and *eil1-1* (Alonso et al., 2003a), suggesting that the main function of CTR1 is to block accumulation and/or action of EIN3 and relatives, possibly through the control of EBF1/2-mediated turnover.

In the absence of ethylene, light-grown *ctr1-1* seedlings grow slowly and develop epinastic leaves compared with wild-type Col-0 and homozygous *ebf1-3* and *ebf2-3* seedlings, which are phenotypically normal under standard growth conditions (Kieber et al., 1993; Figure 1B). We observed a similar *ctr1-1*–like phenotype for the double mutant of *ctr1-1* with *ebf1-3*, indicating that the corresponding SCF^{EBF1} complex does not play a major role during strong ethylene signaling. By contrast, the growth of *ctr1-1 ebf2-3* seedlings was substantially arrested beyond that observed for *ctr1-1* (Figure 2B). Like *ebf1-3 ebf2-3* mutants, the *ctr1-1 ebf2-3* embryos emerged slowly from the seed coat upon germination and then developed into severely stunted seedlings that failed to produce an inflorescence. However, the *ctr1-1 ebf2-3* seedlings were not as strongly compromised as *ebf1-3 ebf2-3* seedlings; they eventually formed true leaves and a primary root and turned pale green after months of growth. An accentuated *ctr1* phenotype was even observed for plants homozygous for *ctr1-1* but heterozygous for *ebf2-3*, implying that one wild-type copy of *EBF2* is insufficient to restrict the constitutive ethylene signaling induced by CTR1 inactivation (Figure 1B). This strong additive effect of *ebf2-3* with *ctr1-1* indicates that the corresponding SCF^{EBF2} complex in particular plays a major role in modulating ethylene perception once the signaling pathway is engaged.

EIN3 and EIL1 Are the Main Targets of $SCF^{EBF1/2}$

EIN3 is a nuclear-localized DNA binding protein with a set of signature acidic, basic, and Pro-rich domains (Chao et al., 1997; Solano et al., 1998). In addition to EIN3, five structurally related proteins, EIL1-5, are expressed in *Arabidopsis*, which could participate in ethylene perception (Chao et al., 1997; Wang et al., 2002; Figure 2A). The most closely related phylogenetically is EIL1, which like EIN3 contains an amide (Asn/Gln) amino acid–rich domain near its C terminus (Figures 2A and 2B). They share 73% amino acid sequence similarity to each other compared with the next closest relative, EIL2, which is only 43 and 47% similar to EIN3 and EIL1, respectively.

A functional overlap of EIN3 and EIL1 was also revealed by studies showing that EIN3 and EIL1 are the dominant members with respect to ethylene signaling (Chao et al., 1997; Alonso et al., 2003a; Guo and Ecker, 2003; Binder et al., 2004a) and by Potuschak et al. (2003), who found that EIL1, like EIN3, can be recognized by EBF1 and EBF2. Consequently, we tested whether the growth arrest of *ebf1 ebf2* double mutants was caused solely by a stabilization of the EIN3 and EIL1 proteins via a phenotypic analysis of triple and quadruple mutant combinations of *ebf1-3* and *ebf2-3* with strong alleles affecting *EIN3* and *EIL1*. Under the assumption that EIN3 and EIL1 are main targets of $SCF^{EBF1/2}$, their removal should synergistically alleviate the growth repression of *ebf1 ebf2* double mutants. The *ein3-1* mutant used here is a loss-of-function allele previously described by Chao et al. (1997) in the Col-0 ecotype. It contains a premature stop at codon 215, which substantially depresses accumulation of the *EIN3* transcript and prevents translation of most of the protein (Figure 3; Chao et al., 1997). The *eil1-3* and *eil1-4* mutants are new *eil1* alleles that were identified by us in the SIGNAL *Arabidopsis* mutant population generated with the Col-0 ecotype. The T-DNAs were inserted in the only exon for *EIL1* following nucleotides 719 (from the ATG) and 1145 for *eil1-3* and *eil1-4*, respectively (Figure 2C). RT-PCR analysis of homozygous *eil1-3* and *eil1-4* seedlings showed that the insertions block accumulation of the full-length *EIL1* mRNA and are therefore likely to represent null alleles (Figure 2D). Similar to previously described null *eil1* alleles in the *Arabidopsis* Landsberg *erecta* (L*er*) and Col-0 ecotypes (Alonso et al., 2003a), homozygous *eil1-3* and *eil1-4* seedlings display a

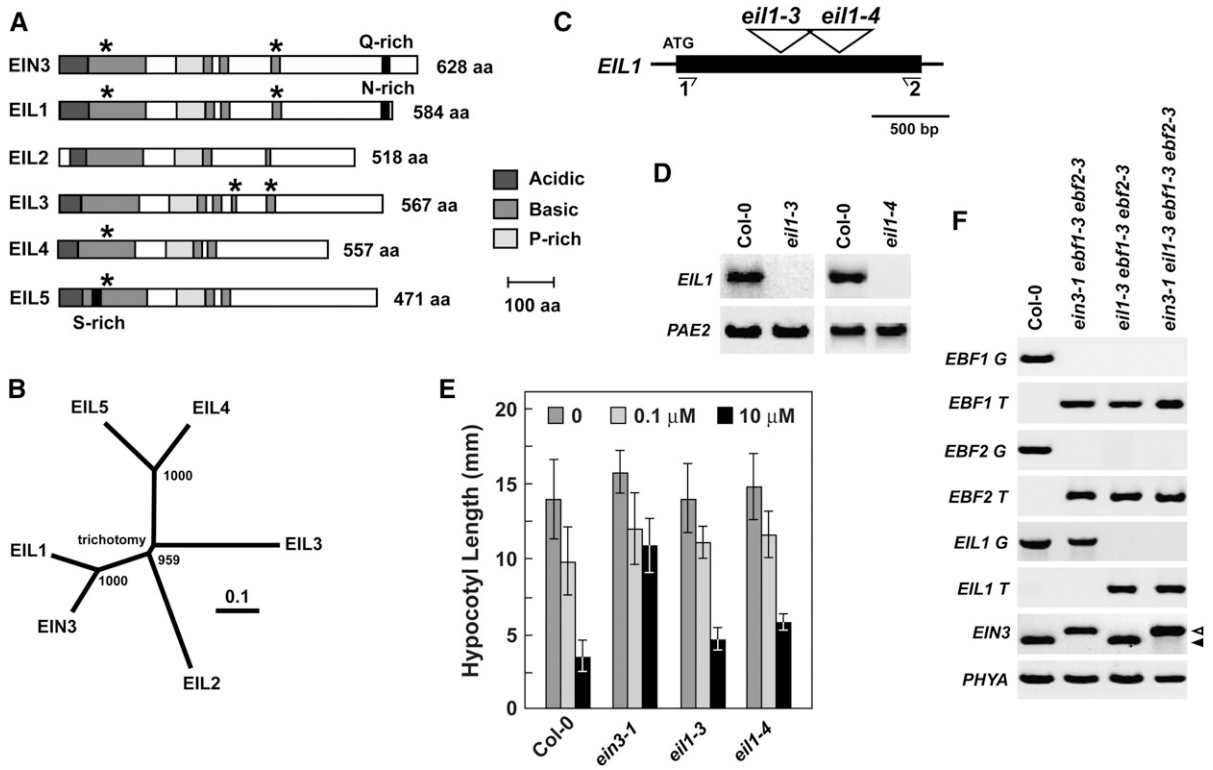


Figure 2. Description of the *Arabidopsis* EIN3/EIL Family and Genetic Analysis of *ein3-1*, *eil1-3*, and *eil1-4* Mutants Alone and in Combination with *ebf1-3* and *ebf2-3*.

(A) Schematic representations of the EIN3 and EIL1-5 proteins. The acidic, basic, Pro-, Ser-, Gln-, or Asn-rich domains are indicated by shaded boxes. The predicted nuclear localization sequences are marked with asterisks. The amino acid (aa) sequence length of each polypeptide is indicated on the right.

(B) An unrooted phylogenetic tree of the EIN3/EIL family. The bar represents a branch length equivalent to 0.1 amino acid changes per residue.

(C) Diagram of the *EIL1* gene. Locations of the T-DNA insertion in the *eil1-3* and *eil1-4* mutants are shown. Arrows indicate the primers used for RT-PCR in **(D)**.

(D) RT-PCR analysis of the *eil1-3* and *eil1-4* mutants. First-strand cDNA was synthesized from total seedling RNA with primer 2 and then amplified by PCR with the primers pairs 1+2. RT-PCR of the *PAC1* transcript is included as a control.

(E) Hypocotyl elongation of the *eil1* mutants in response to ACC. Wild-type Col-0, *ein3-1*, *eil1-3*, and *eil1-4* were grown for 4 d in the dark with or without 0.1 and 10 μM ACC. Each bar represents the average hypocotyl length (±SD) of at least 12 seedlings.

(F) PCR genotyping of the *ein3-1* and *eil1-3* mutations in combination with the *ebf1-3* and *ebf2-3* mutations. G, gene-specific primers; T, a left border T-DNA primer used in combination with a gene-specific primer. For *EIN3*, a pair of gene-specific primers was used for the PCR followed by digestion of the product with *Hae*III. The open and closed arrowheads identify the products from the *ein3-1* and wild-type Col-0 alleles, respectively. PCR amplification of the *PHYA* gene is included as a control.

mild insensitivity to exogenous ethylene. At both 0.1 and 10 μM concentrations of ACC, the elongation of etiolated *eil1-3* and *eil1-4* hypocotyls was less repressed than wild-type Col-0 but still more sensitive than *ein3-1* (Figure 2E).

Owing to the growth arrest and infertility of *ebf1-3 ebf2-3* double mutants (Gagne et al., 2004) and a linkage of *EIL1* with *EBF1* (~3 centimorgans apart on chromosome 2), the various combinations of *ein3*, *eil1*, *ebf1*, and *ebf2* mutants were created by first isolating an *ein3-1 ebf1-3 ebf2-3* triple homozygous plant that was fertile (see below) from F2 progeny of an *ebf1-3 ebf2-3* heterozygote crossed with a homozygous *ein3-1* plant. The triple mutant was then crossed with a homozygous *eil1-3* plant to generate a quadruple heterozygous *ein3-1 eil1-3 ebf1-3 ebf2-3* individual. From self-fertilized F2 progeny of this plant, triple homozygous *eil1-3 ebf1-3 ebf2-3* and quadruple homozygous *ein3-1 eil1-3 ebf1-3 ebf2-3* plants were identified. Given their infertility (see below), we had to individually identify triple homozygous *eil1-3 ebf1-3 ebf2-3* seedlings in a segregating population obtained from a self fertilized plant that was homozygous for *eil1-3* and *ebf2-3* but heterozygous for *ebf1-3*. The genotypes for the *eil1-3*, *ebf1-3*, and *ebf2-3* loci were tracked by PCR of genomic DNA using gene-specific primers that bracket the T-DNAs in combination with a T-DNA left border primer (Figure 2F). The *ein3-1* mutation contains a G-to-A substitution at nucleotide 1598, which removed a *Hae*III restriction site at that position. Consequently, we tracked this mutation by PCR using primers to amplify the region surrounding the mutation and then tested for sensitivity of the amplified product to *Hae*III

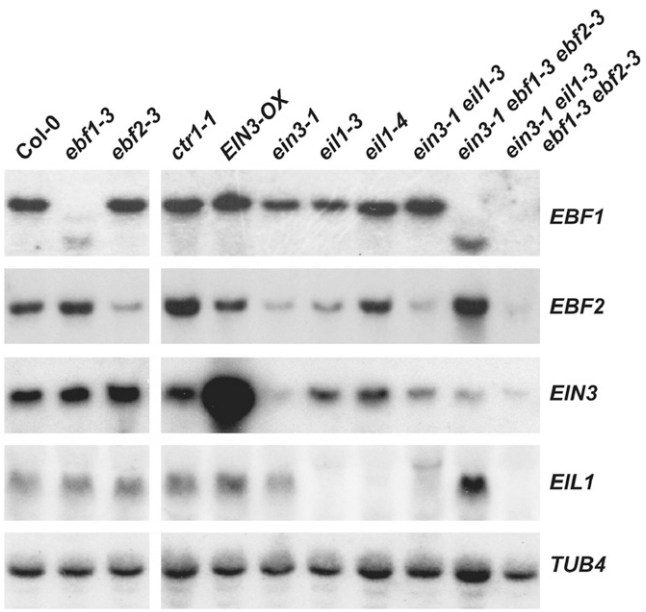


Figure 3. *EBF1*, *EBF2*, *EIN3*, and *EIL1* Transcript Levels in Various Mutant and Overexpression Plants Altered in Ethylene Signaling.

Total RNA was extracted from 2-week-old light-grown plants and subjected to RNA gel blot analysis with the indicated probes. The β-Tubulin-4 (*TUB4*) gene probe was included to verify equal RNA loading.

digestion. As can be seen in Figure 2F, the appropriate mutant alleles were identified by PCR (±*Hae*III digestion for *ein3-1*) using DNA templates isolated from the triple and quadruple homozygous mutants but not using DNA from wild-type Col-0 *Arabidopsis*.

RNA gel blots of 2-week-old light-grown plants also confirmed the effects of the *ein3*, *eil1*, *ebf1*, and *ebf2* mutant combinations on the corresponding transcripts (Figure 3). For example, diminished levels of *EIN3* mRNA were observed in all the mutant combinations bearing the *ein3-1* allele, whereas the *EIL1* transcript was not detected in any line containing either the *eil1-3* or *eil1-4* allele. As described previously (Gagne et al., 2004), the *ebf1-3* mutation induces synthesis of a truncated *EBF1* mRNA. This truncation was detected in the single *ebf1-3* and triple *ein3-1 ebf1-3 ebf2-3* lines as expected but was surprisingly absent in the quadruple mutant (Figure 3). Although it behaves as a null mutation, the *ebf2-3* allele directs synthesis of a near full-length *EBF2* transcript (Gagne et al., 2004). In addition to having reduced levels in the *ebf1-3* lines, the abundance of the *EBF2* mRNA was also substantially lower in the *ein3-1* and *eil1-3* single mutants, the *ein3-1 eil1-3* double mutant, and the quadruple mutant (Figure 3). Reduced levels in the *ein3*/*eil1* backgrounds could reflect previous observations that the transcript abundance of EBF2 is positively regulated by ethylene signaling, which is diminished in these mutant backgrounds (Potuschak et al., 2003, 2006; Gagne et al., 2004). Why the *EBF2* mRNA was also not reduced in the *eil1-4* background was unclear. To avoid this complication, we used the *ebf1-3* allele to construct the triple and quadruple mutants.

As described previously (Gagne et al., 2004), *Arabidopsis ebf1 ebf2* double null mutants displayed a strong growth arrest following germination even in the absence of ethylene, while the *ebf1* and *ebf2* single mutants developed normally (Figures 1B and 4A). Likewise, the *ein3-1* and *eil1-3* single homozygous seedlings and the previously described *ein3-1 eil1-2* double homozygous seedlings (Alonso et al., 2003a) were phenotypically normal in the absence of ethylene in accordance with the nonessential nature of ethylene perception (Figure 4A). Strikingly, when combined with the *ebf1-3 ebf2-3* double mutant, either of the *eil1-3* and *ein3-1* mutations could partially rescue the severely stunted growth of the double mutant (Figure 4). The triple *eil1-3 ebf1-3 ebf2-3* seedlings showed only a modest rescue. Unlike *ebf1-3 ebf2-3* seedlings, the *eil1-3* triple seedlings continued to grow and develop after germination (albeit slowly). Their cotyledons partially expanded and turned pale green following emergence from the seed coat and a stunted root developed that was densely covered with root hairs, all of which were consistent with a partially dampened ethylene response compared with the *ebf1-3 ebf2-3* double mutant. Whereas the *ebf1-3 ebf2-3* plants failed to develop true leaves, one or more leaf pairs eventually emerged after several weeks from the *eil1-3 ebf1-3 ebf2-3* plants (Figure 4A). Unfortunately, the plants never flowered and set seed, thus forcing us to continuously select for *eil1-3* triple mutant individuals from segregating populations.

A much more dramatic rescue of the *ebf1 ebf2* phenotype was apparent upon inactivation of *EIN3*. These triple homozygous seedlings grew rapidly following germination and eventually developed large rosettes of true leaves (Figure 4A). After 3 to 4 weeks, inflorescence stems emerged that ultimately produced fertile flowers and viable seeds (Figure 4B). However, the *ein3-1 ebf1-3 ef2-3* triple mutants were not completely normal; the rosettes produced epinastic leaves with elongated petioles and small leaf blades, and the inflorescence was significantly shorter than wild-type Col-0 (Figures 4A and 4B). This phenotype resembled *ctr1-1* seedlings at both the juvenile and adult stages, suggesting that these plants, like those defective in *CTR1*, were still responding to an apparent ethylene signal (Figures 1B and 4B).

The most complete phenotypic suppression was observed for the quadruple homozygous *ein3-1 eil1-3 ebf1-3 ebf2-3* combination (Figures 4A and 4B). These plants were phenotypically indistinguishable from the wild type at both the juvenile and adult stages when grown under nonstressful conditions in the absence of ethylene. For example, the quadruple mutant plants had flat, fully expanded leaves, tall inflorescences, and morphologically normal flowers that were highly fertile and without a protruding gynoecium. This full development was in stark contrast with plants double homozygous for null *ebf1 ebf2* alleles that fail to generate true leaves, ordered inflorescence stems, and flowers (Gagne et al., 2004; Figure 4A) or to double homozygous *ebf1 ebf2* plants containing a weak allele for *EBF2*, which produce supernumerary epinastic leaves, a dwarfed irregular inflorescence, and mis-shaped flowers with protruding gynoecia and reduced fecundity (*ebf1-1 ebf2-1*; Potuschak et al., 2003). Etiolated *ein3-1 eil1-3 ebf1-3 ebf2-3* seedlings also grew normally in the absence of ethylene and exhibited none of the triple response phenotypes (i.e., exaggerated apical hook, swollen hypocotyl, and reduced root growth; Roman et al., 1995) typical of constitutive ethylene signaling (e.g., *ctr1-1*; Figure 5A).

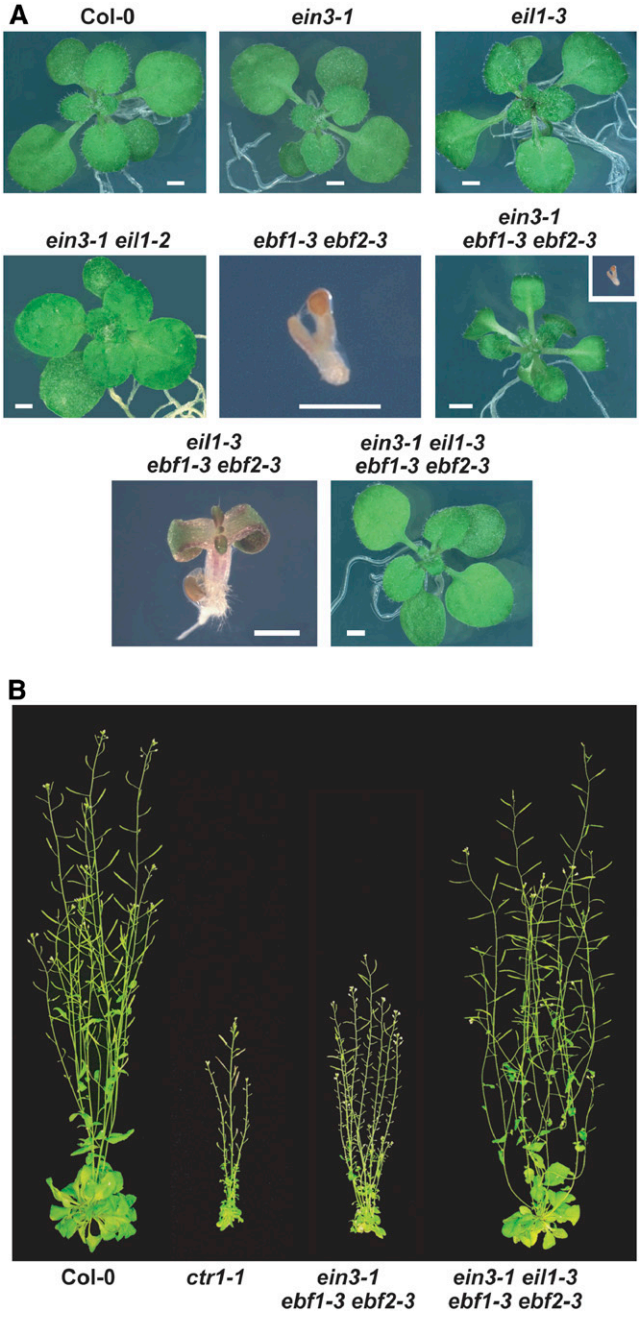


Figure 4. Phenotype of Plants Containing Combinations of the *ein3*, *eil1*, *ebf1*, and *ebf2* Mutations.

(A) Seedlings grown for 16 d under a long-day photoperiod. The inset next to the *ein3-1 ebf1-3 ebf2-3* seedling provides a direct size comparison for an *ebf1-3 ebf2-3* seedling. Bars = 2 mm.
(B) Mature plants grown on soil for 5 weeks under a long-day photoperiod. A *ctr1-1* plant is included for comparison.

To examine whether the various mutant combinations still retained sensitivity to ethylene, we tested the elongation response of etiolated hypocotyls to ACC. (The *ebf1-3 ebf2-3* double mutant and the *eil1-3 ebf1-3 ebf2-3* triple mutant were not tested given their severely stunted growth even without exogenous hormone [see Figures 1A and 4A].) In the absence of ACC, all the mutant combinations appeared wild type and clearly distinct from *ctr1-1* seedlings, which displayed a strong triple response (Figure 5A). In the presence of increasing concentrations of ACC up to 10 μM, a variety of elongation responses were

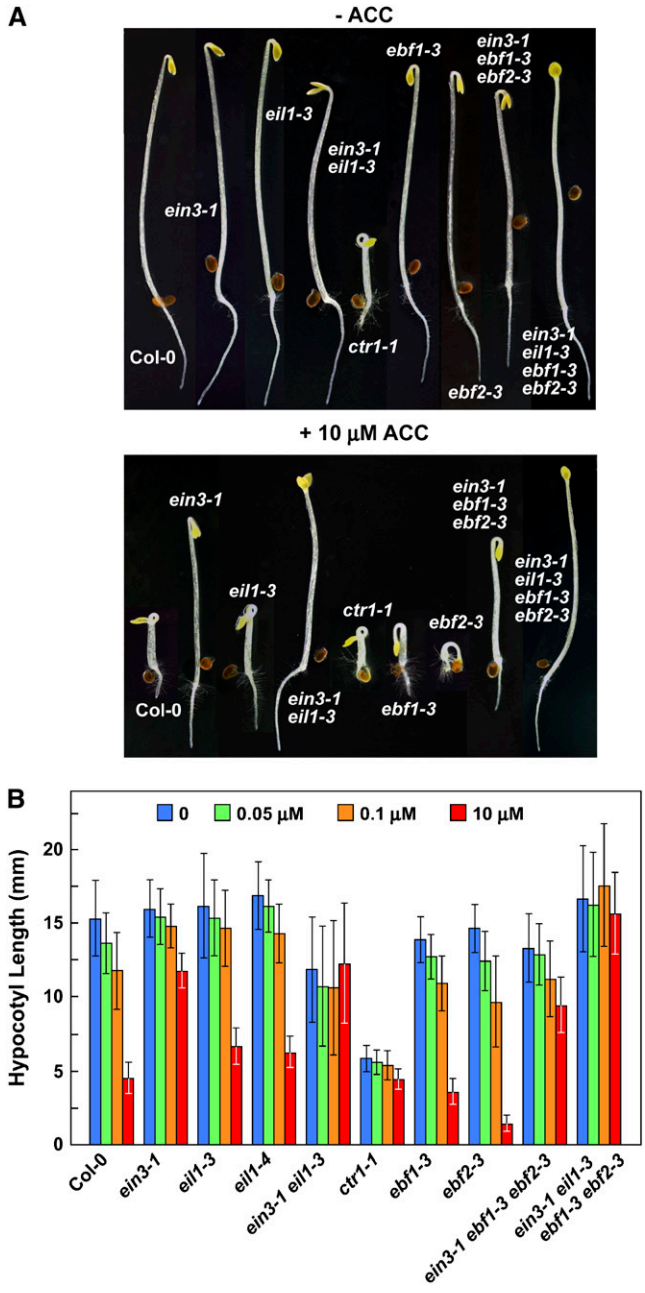


Figure 5. Hypocotyl Elongation of Various Ethylene-Signaling Mutant Combinations in Response to ACC.

(A) Representative plants grown in the dark for 4 d with or without 10 μM ACC.
(B) Graphical quantitation of hypocotyl elongation in response to various concentrations of ACC. Each bar represents the average length (±SD) of at least 25 seedlings.

observed that were all consistent with EBF1 and EBF2 functioning upstream of EIN3 and EIL1 (Figures 5A and 5B). At 10 μM ACC, wild-type Col-0 and *ebf1-3* seedlings now displayed the typical triple response, whereas the *ebf2-3* plants showed a strong exaggeration of this response, which was even more severe than *ctr1-1* plants. Consistent with previous studies (Chao et al., 1997; Figure 2E), *eil1-3* seedlings displayed a mild insensitivity to ACC, while the *ein3-1* seedlings displayed a strong insensitivity with the response additive for the *ein3-1 eil1-3* double mutant. This insensitivity was retained in the *ein3-1 ebf1-3 ebf2-3* triple mutant and the quadruple *ein3-1 eil1-3 ebf1-3 ebf2-3* mutants (Figures 5A and 5B). In fact, the elongation of the quadruple mutant was not detectably inhibited even at a near saturating concentration of ACC. If other targets of EBF1/2 existed that were important to ethylene perception by hypocotyls (e.g., EIL2-5), such a strong insensitivity would be unlikely. Based on the near complete developmental rescue of the quadruple mutant and its strong insensitivity to ethylene, we concluded that EIN3 and EIL1 are the main targets of the $SCF^{EBF1/2}$ complexes and that their stabilization in the *ebf1 ebf2* backgrounds is primarily responsible for the severe growth arrest of these seedlings.

One interesting observation from our ACC sensitivity studies was that seedlings defective in both *EIN3* and *EIL1* often had substantially unfolded apical hooks even when treated with high concentrations of ACC (Figure 5A). In some cases, the cotyledons were presented upwards without an obvious bend, which was in stark contrast with the exaggerated hooks of *ctr1-1* and wild-type seedlings treated similarly. Given the importance of ethylene in forming the apical hook (Lehman et al., 1996; Li et al., 2004; Vriezen et al., 2004), its absence in the *ein3-1 eil1-3* seedlings further supports a role for ethylene and suggests that EIN3 and EIL1 are together required for the asymmetric growth near the hook that forms this bend.

EBF1 and 2 Play Temporally Distinct Roles in Ethylene Signaling

The weaker differential effects of the monogenic *ebf1* and *ebf2* mutants to exogenous ethylene compared with the strong growth arrest of the double mutant suggested previously that the corresponding $SCF^{EBF1/2}$ E3s play distinct but overlapping roles in EIN3 and EIL1 turnover (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). The differential sensitivity of the *ebf1-3* and *ebf2-3* mutants in the *ctr1-1* background further supported this possibility (Figure 1B). To more accurately describe their distinct roles, we applied a time-lapsed imaging system developed by Spalding and colleagues (Parks and Spalding, 1999; Folta and Spalding, 2001) and adapted by Binder et al. (2004a, 2004b) to measure the ethylene growth response of *Arabidopsis* hypocotyls altered in the expression of *EBF1*, *EBF2*, *EIN3*, and *EIL1*. This highly sensitive system has been exploited previously to more precisely classify ethylene perception mutants within the pathway (Binder et al., 2004a, 2004b; Potushak et al., 2006).

Prior studies of young seedlings revealed two phases of ethylene-induced growth inhibition for wild-type Col-0 hypocotyls (Binder et al., 2004a, 2004b; Figure 6). Phase I inhibition begins ~10 min after ethylene is applied; within an additional 15 min, a new, lower, steady state growth rate is reached. This steady state is maintained for another 20 min, after which phase II inhibition is initiated, which further depresses growth within another 20 min to an even lower steady state growth rate. This phase II growth depression is maintained for many hours under continuous hormone exposure. However, if ethylene is removed during phase II, hypocotyl elongation rapidly recovers over ~90 min back to the initial rate in the absence of hormone. Genetic analyses showed that both phases require EIN2 but that only phase II is dependent on EIN3 and EIL1 (Binder et al., 2004a). In the absence of EIN3 and EIL1, hypocotyls enter the phase I growth arrest but fail to enter phase II. Instead, they resume their normal growth rate, even in the presence of saturating ethylene concentrations (see below). Under the conditions used here for 2-d-old seedlings, wild-type and mutant hypocotyls all elongated in air prior to ethylene treatment at rates ranging from 0.28 to 0.47 mm h^{-1} (see Supplemental Table 1 online).

As can be seen in Figure 6A, wild-type Col-0 hypocotyls respond rapidly to exogenous ethylene. Within 10 min after exposure to a saturating dose of 10 μL L^{-1} ethylene, elongation rate dropped dramatically and reached the phase I plateau within 30 min. Soon after, the phase II response became evident, which further depressed elongation. Upon removal of the gas, the elongation rate of the seedlings rapidly increased, such that within ~75 min the rate was nearly restored to that before ethylene exposure. A dampening oscillation was often apparent during recovery that could reflect a negative feedback loop within the ethylene signaling pathway as it attempts to coordinately resume growth within the hypocotyl cylinder (Figure 6A).

When the *ebf1-3* and *ebf2-3* single homozygous mutants were examined similarly, we found that the magnitude of the growth inhibition was similar to the wild type, indicating that neither mutant affected the final strength of the ethylene signal by itself. However, significant changes in the kinetics of the ethylene response and its recovery were observed, indicative of distinct temporal roles. *ebf1-3* seedlings had a similar onset and strength of the phase I growth inhibition to the wild type, but the onset of phase II growth inhibition was markedly faster (Figure 6A). As shown in an expanded version of the kinetics (Figure 6B), *ebf1-3* hypocotyls failed to pause at the intermediate plateau after phase I; instead, they continued to rapidly depress their elongation rate to that eventually reached during a normal phase II response. Following ethylene removal, the elongation rate of *ebf1-3* seedlings recovered with a kinetic similar to the wild type, including the dampened oscillations. By contrast, *ebf2-3* seedlings had a normal initial response to ethylene with a similar onset kinetic, phase I plateau, and final growth rate depression in phase II (Figures 6C and 6D). Unlike wild-type and *ebf1-3* seedlings, their recovery after ethylene removal was substantially delayed, requiring ~75 min longer than the wild type to regain the elongation rate present before ethylene exposure.

The delay in growth recovery for the *ebf2-3* seedlings suggested that EBF2 is particularly important for removing excess EIN3/EIL1 that is stabilized by the ethylene signal. To test this possibility, we examined the growth kinetics of seedlings overexpressing EIN3 (*EIN3-OX*) under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. These seedlings constitutively express a high level of *EIN3* mRNA (Figure 3) and display a heightened ethylene response even in the absence of the

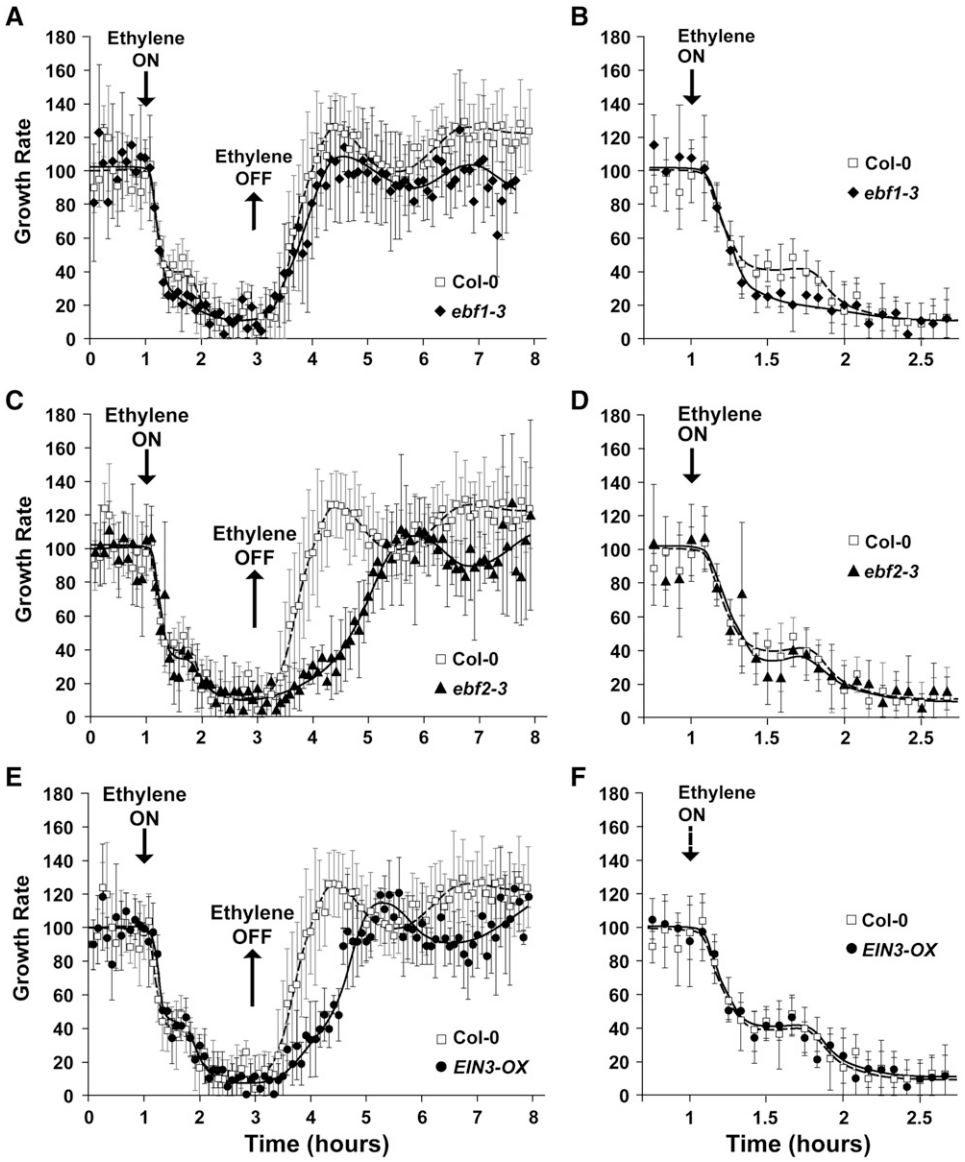


Figure 6. Elongation Kinetics of 2-d-Old Etiolated *Arabidopsis* Hypocotyls in Response to Ethylene for Wild-Type Col-0 Seedlings, Seedlings Homozygous for the *ebf1-3* or *ebf2-3* Mutations, or *EIN3-OX* Seedlings.

Growth rates were recorded for 1 h in air, followed by a 2-h exposure to 10 μL L^{-1} ethylene, and then a 5-h recovery in air. Panels on the left show the full time course. Panels on the right show an enlargement of the response immediately after introducing ethylene. The response of wild-type Col-0 seedlings (open squares) is included in each panel. Data represent the average of at least six seedlings ±SD.

(A) and **(B)** *ebf1-3*.

(C) and **(D)** *ebf2-3*.

(E) and **(F)** *EIN3-OX*.

exogenous hormone (Chao et al., 1997; Guo and Ecker, 2003). As shown in Figures 6E and 6F, the *EIN3-OX* seedlings responded remarkably similar to *ebf2-3* seedlings. The elongation kinetics for both phase I and phase II were indistinguishable with a substantial delay in growth recovery evident upon ethylene removal. For *EIN3-OX* seedlings, it took ~60 min longer than the wild type to reach the pretreatment growth rate after purging the gas.

To further examine the roles of EBF1 and 2, we analyzed previously described transgenic lines that separately overexpress either of the two proteins under the control of the CaMV *35S* promoter (Guo and Ecker, 2003; Potuschak et al., 2006). These lines contain more of the corresponding mRNAs, are less sensitive to ethylene, and have a dampened accumulation of EIN3 after hormone treatment, the latter of which is consistent with a more

effective turnover of EIN3 and EIL1. Retesting the hypocotyl elongation response of *EBF1-OX* and *EBF2-OX* seedlings in the presence of ACC confirmed their decreased ethylene sensitivity. Whereas the elongation of wild-type hypocotyls was dramatically inhibited by increasing concentrations of ACC, both overexpression lines, like the ethylene-insensitive mutant *ein2-1* (Guzman and Ecker, 1990; Alonso et al., 1999), showed little or no response even at saturating concentration (10 μM) (Figure 7A).

From the kinetics of the ethylene response, we found that the *EBF1-OX* and *EBF2-OX* seedlings responded similarly to ethylene in contrast with the different responses of the *ebf1-3* and *ebf2-3* seedlings (Figure 7B). Both overexpression lines displayed a similar onset of growth inhibition and reached the phase I plateau at approximately the same time as the wild type. However, neither appeared to fully enter the stable phase II growth inhibition, which is dependent on EIN3 and EIL1 protein accumulation (Binder et al., 2004a). The slowest growth rate in the presence of ethylene was ~20% faster than wild-type seedlings for both lines. Even before ethylene removal, both the *EBF1-OX* and *EBF2-OX* lines appeared to accelerate growth, and after ethylene removal, they eventually resumed the rapid growth evident before ethylene treatment (Figure 7B).

The mutational analysis and the kinetic data presented here along with prior interaction data showing that both EBF1 and 2 can bind EIN3 and EIL1 (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004) are consistent with the corresponding $SCF^{EBF1/2}$ complexes playing different temporal roles but having similar targets. To further confirm that EIN3 and EIL1 are the main targets, we examined the growth kinetics during prolonged ethylene exposure of the *ein3* and *eil1* mutants either alone, together, or when combined with *ebf1-3* and *ebf2-3*. (*The eil1-3 ebf1-3 ebf2-3* triple mutant was not tested given its already strong growth arrest [see Figure 4A].) Under this prolonged treatment, the elongation rate of wild-type Col-0 seedlings remained strongly inhibited with only a slight recovery after ~5 h. In agreement with Binder et al. (2004a), homozygous *ein3-1 eil1-2* seedlings exposed continuously to ethylene exhibited a normal phase I response but lacked the phase II component, such that the elongation rate of the mutant slowly returned near to that observed before ethylene exposure (Figure 8A). When the contributions of the EIN3 and EIL1 proteins in growth were examined separately, we found that EIN3 plays the dominant role in the phase II response (Figure 8B). Whereas the single *eil1-3* mutant behaved similar to the wild type during both phase I and II growth responses, the single *ein3-1* mutant failed to display a normal phase II response. However, the *ein3-1* seedling did retain a modest ethylene sensitivity (as seen by a depression of the elongation rate) that appeared to strengthen after prolonged exposure (>4 h). This sensitivity could reflect the contribution of EIL1 at later times (Figure 8B).

When the *ein3-1 eil1-3 ebf1-3 ebf2-3* seedlings were tested similarly, we confirmed that EIN3 and EIL1 are the main targets of $SCF^{EBF1/2}$. Whereas the initial response of the quadruple mutant to ethylene was indistinguishable from the wild type, this inhibition was only a transient like that seen for the *ein3-1 eil1-2* double mutant (Figure 8C). Even in the presence of a saturating concentration of ethylene, these seedlings rapidly resumed their growth rate near to that observed before ethylene exposure. The transient growth inhibition within the first hour for the quadruple

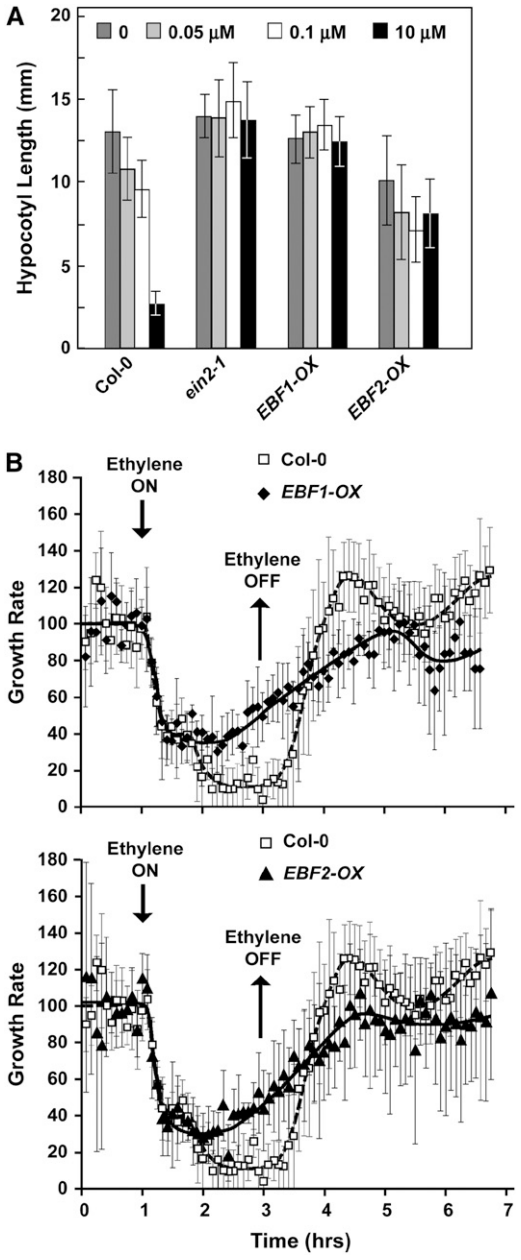


Figure 7. Growth of Etiolated *Arabidopsis* Hypocotyls in Response to ACC and Ethylene for Wild-Type Col-0 Seedlings or Seedlings Overexpressing Either *EBF1* or *EBF2*.

(A) Hypocotyl elongation in response to prolonged ACC treatment. Wild-type Col-0, *ein2-1*, *EBF1-OX*, and *EBF2-OX* seedlings were grown for 4 d in the dark with or without various concentrations of ACC. Each bar represents the average hypocotyl length (±SD) of at least 40 seedlings.
(B) Growth kinetics of 2-d-old etiolated hypocotyls in response to ethylene. Growth rates for Col-0, *EBF1-OX* (top panel), and *EBF2-OX* (bottom panel) were recorded for 1 h in air, followed by a 2-h exposure to 10 μL L^{-1} ethylene, and then a 5-h recovery in air. Data represent the average of at least six seedlings ±SD. The response of wild-type Col-0 seedlings (open squares) is included in each panel.

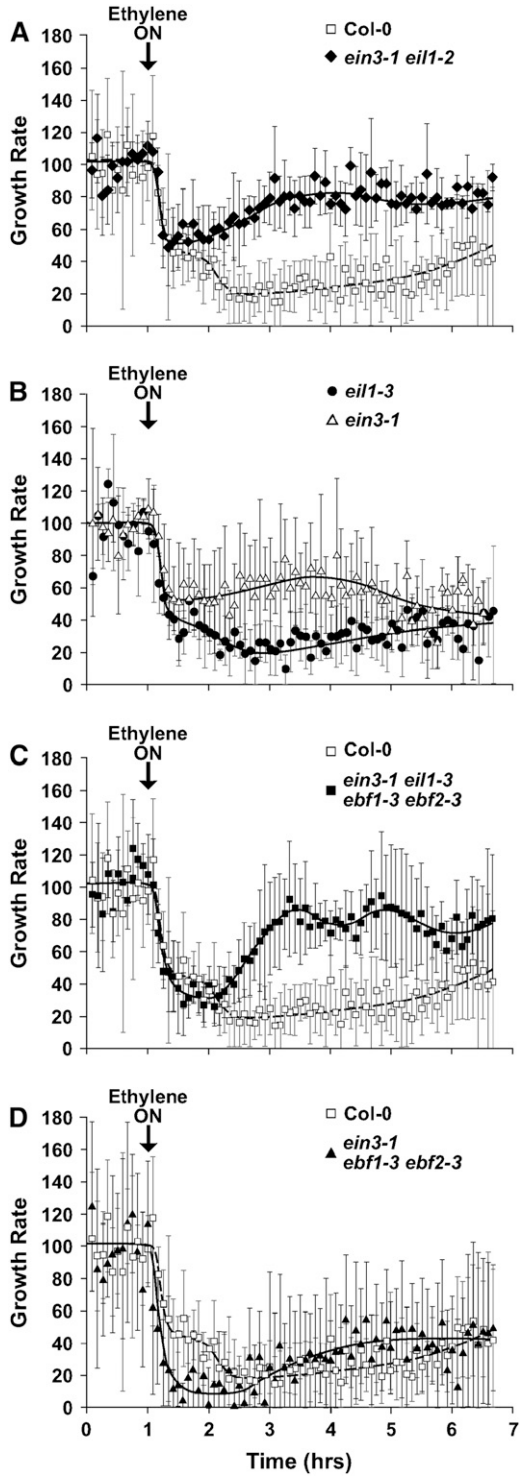


Figure 8. Growth Kinetics of 2-d-Old Etiolated *Arabidopsis* Hypocotyls Containing Various Mutant Combinations Affecting *EIN3*, *EIL1*, *EBF1*, and *EBF2*.

Growth rates were recorded for 1 h in air, followed by a 6-h continuous exposure to 10 μL L^{-1} ethylene. Data represent the average of at least six seedlings ±SD. The response of wild-type Col-0 seedlings is included in **(A)**, **(C)**, and **(D)**.

was noticeably stronger than in that for the *ein3-1 eil1-2* mutant. Whether this reflects a heightened phase I response that is independent of EIN3 and EIL1 is not yet known.

A more complex elongation response was seen for the *ein3-1 ebf1-3 ebf2-3* triple mutant (Figure 8D). The triple mutant began responding to ethylene at the same time as the wild type but once engaged did so at a faster rate. The resulting kinetics did not reveal an obvious phase I plateau with the plants appearing to directly enter the phase II response. At longer times, a slight restoration in growth was detected. While the mechanism for this accelerated response is unclear, one possibility is that EIL1 assumes a more prominent role in the absence of both EIN3 and a mechanism to remove EIN3 and EIL1 (i.e., missing the SCF$^{EBF1/2}$ E3s). Consistent with this possibility, we found that the abundance of the *EIL1* transcript was substantially upregulated in the *ein3-1 ebf1-3 ebf2-3* background (Figure 3).

DISCUSSION

Previous genetic and biochemical data by us and others revealed that the Ub/26S proteasome pathway is a central regulator of ethylene perception in *Arabidopsis* through its control of EIN3 (and possibly the family of related EILs) protein abundance, a transcription factor that directs a key limiting step in the ethylene response pathway (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). This regulation is achieved by SCF E3 complexes containing either of the two F-box–containing substrate recognition factors: EBF1 and EBF2. SCF$^{EBF1/2}$ presumably promote the ubiquitination and subsequent breakdown of EIN3/EILs in the absence of the hormone, but their activity is blocked in the presence of the hormone, thus allowing EIN3/EILs levels to rise and mediate ethylene responses. Here, we showed that EIN3 and EIL1 are the principal targets of SCF$^{EBF1/2}$ and that the two E3s work in a temporally distinct but largely overlapping fashion to control EIN3/EIL1 levels.

Whereas strong *ebf1 ebf2* mutants accumulate high concentrations of EIN3 (and likely EIL1) and display a strong growth arrest even in the absence of ethylene (Gagne et al., 2004), sequential genetic inactivation of the *EIN3* and *EIL1* loci in this background progressively rescues the mutant phenotype with the quadruple *ein3 eil1 ebf1 ebf2* mutant now resembling wild-type plants. Consistent with the nonessential nature of ethylene and the epistasis of *EBF1* and *EBF2* with respect to *EIN3* and *EIL1*, the quadruple mutant grew normally under nonstressed conditions and was almost completely insensitive to prolonged ethylene treatments, at least with respect to etiolated seedling development. Like *ein3-1 eil1-3* seedlings, the quadruple mutant still displays a transient ethylene response during hypocotyls elongation. However, this brief response, which was previously shown to require EIN2 (Binder et al., 2004a), is so short that it has little effect on overall hypocotyl length after prolonged hormone treatments.

(A) *ein3-1 eil1-2* double mutant.
(B) *ein3-1* and *eil1-3* single mutants.
(C) *ein3-1 eil1-3 ebf1-3 ebf2-3* quadruple mutant.
(D) *ein3-1 ebf1-3 ebf2-3* triple mutant.

Our analysis of triple *ein3-1 ebf1-2 ebf2-3* and *eil1-3 ebf1-3 ebf2-3* seedlings confirmed prior studies that showed that EIN3 plays a greater role in ethylene signaling relative to EIL1. Whereas the *ebf1-3 ebf2-3* double mutant was only weakly rescued upon inactivation of *EIL1*, a much stronger rescue occurred upon further inactivation of *EIN3*. Although still dwarfed, the *ein3-1 ebf1-2 ebf2-3* mutant produced a rosette of true leaves and eventually formed a cluster of inflorescences that flowered and generated viable seeds. The plants phenotypically resembled *ctr1* mutants, indicating they were still responding to an apparent ethylene signal in the absence of EBF1 and EBF2. The difference in this response from the wild type likely reflects the action of stabilized EIL1. While there remains speculation that other members of the EIL family (EIL2-5) play important roles in ethylene signaling, both the complete rescue of the *ebf1 ebf2* phenotype in the quadruple mutant at both the etiolated and light-grown stages in the absence of ethylene and the failure of the quadruple etiolated seedlings to retain a durable response to exogenous ethylene implies that their role(s) are minor if acting at all. However, we acknowledge that not all ethylene responses in *Arabidopsis* were evaluated here, leaving open the possibility that EIL2-5 selectively participate in other aspects of ethylene perception (e.g., leaf and floral abscission, and biotic and abiotic stress responses; Schaller and Kieber, 2002). They also could be responsible for the phase I growth response of hypocotyls, which is under EIN2 but not EIN3 or EIL1 control (Figure 8A; Binder et al., 2004b).

The strong phenotypic consequences of simultaneous inactivation of both EBF1 and EBF2 compared with the single mutants clearly indicates that both SCF E3 complexes play essential, overlapping roles in regulating EIN3/EIL1 protein abundance. Distinct functions were first implied from ethylene response studies, which showed only a modest aberrant phenotype for *ebf1* mutants in the absence of ethylene or the presence of low hormone concentrations, while the *ebf2* mutants displayed an ethylene hypersensitivity only at higher or saturating hormone concentrations (Guo and Ecker, 2003; Gagne et al., 2004). Further differences between EBF1 and EBF2 became apparent from expression studies, which showed that the *EBF1* mRNA is abundant even in the absence of ethylene with its level increasing modestly in ethylene, while the *EBF2* mRNA levels are lower initially but strongly upregulated upon hormone exposure (Potuschak et al., 2003; Gagne et al., 2004). The differential contributions of EBF1 and EBF2 are further supported here from analysis of double mutants with *ctr1*. Whereas the strong constitutive ethylene signaling generated by the *ctr1-1* mutation was not accentuated upon combination with the *ebf1-3* mutation, combining the *ctr1-1* and *ebf2-3* mutations strongly compromised seedling growth. In fact, the phenotype of these *ctr1-1 ebf2-3* seedlings resembled that of *ebf1 ebf2* double null mutants (Gagne et al., 2004), the strongest ethylene signaling mutants yet reported. Together, the phenotypes further support the notion that strong ethylene signaling induced by high concentrations of EIN3/EIL1 cannot be overcome by EBF1 but can be by EBF2.

Kinetic analyses of hypocotyl elongation of monogenic *ebf1* and *ebf2* seedlings indicate that one important distinction in EBF1 and EBF2 function is in the temporal control of EIN3/EIL1 levels. Detailed analysis of *ebf1-3* seedlings after ethylene exposure showed that they enter the phase II response, which is dependent on EIN3/EIL1, faster than normal but are indistinguishable from the wild type after ethylene is removed and elongation recovers. Together with the modest upregulation of the *EBF1* mRNA upon ethylene exposure and the inability of the *ebf1-3* mutation to exacerbate the strong constitutive ethylene signaling generated by *ctr1-1*, we propose that a main role of the SCF^{EBF1} complex is to constitutively target EIN3/EIL1 for degradation, thereby repressing ethylene responses at no or low hormone concentrations. Conversely, detailed kinetic analysis of *ebf2-3* seedlings revealed that they are not compromised during the initial stages of ethylene signaling but are substantially delayed in growth recovery following ethylene withdrawal. This pattern was indistinguishable to that of *EIN3*-overexpressing plants, consistent with the notion that the SCF^{EBF2} complex is more important when excess EIN3 (and likely EIL1) is present. Taken together with the data showing that the *ebf2-3* mutation strongly accentuates the phenotype of the *ctr1-1* mutant and that the *EBF2* mRNA is more strongly upregulated by ethylene, we propose that the SCF^{EBF2} complex becomes more important once ethylene signaling is engaged and during recovery after hormone withdrawal. Presumably, through the concerted action of SCF^{EBF1} and SCF^{EBF2}, *Arabidopsis* can repress ethylene signaling in the absence of the hormone, dampen signal at high hormone concentrations, and more rapidly recovery after ethylene levels dissipate.

In addition to playing different temporal roles in ethylene signaling, it is also possible that EBF1 and EBF2 have substrate-specific roles, with one SCF complex recognizing EIN3 and the other recognizing EIL1. The different effects on the elongation kinetics when exposed to ethylene could then reflect the separate temporal functions of the targets and not the degradation machineries. Several observations do not favor such a scenario. First, prior interaction studies showed that EBF1 and EBF2 can both recognize EIN3 and EIL1, indicating that if substrate specificity for the F-box proteins does exist, it is minor (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Second, plants overexpressing *EBF1* or *EBF2* have indistinguishable elongation kinetics after ethylene exposure, with both lines failing to enter the phase II inhibition response that requires EIN3/EIL1 protein accumulation. For example, if EIL1 was required early in the response and EIN3 was required later, a differential effect on elongation should be evident for the *EBF1-OX* and *EBF2-OX* lines that would reflect the target specificity of each SCF^{EBF} complex.

Based on the data reported here and elsewhere, we propose a more complete model for ethylene signaling in *Arabidopsis* (Figure 9). In the absence of ethylene, EIN3/EIL1 is constitutively synthesized and degraded. This breakdown is promoted by CTR1 and is achieved by the SCF E3–directed ubiquitination of EIN3/EIL1 using the EBF1 and 2 F-box proteins as the substrate recognition factors. Both genetic and kinetic analyses are consistent with EBF1 playing the main role in this baseline ubiquitination. The ethylene-induced repression of CTR1 blocks the ubiquitination of EIN3/EIL1 by the $SCF^{EBF1/2}$ E3s. This inhibition allows the rapid accumulation of EIN3/EIL1, which then direct downstream events in ethylene signaling. Their accumulation also activates a negative feedback loop that modestly and strongly upregulates *EBF1* and *EBF2* mRNA levels, respectively. Presumably this upregulation in transcript levels then increases the abundance of the corresponding E3s to prevent excess

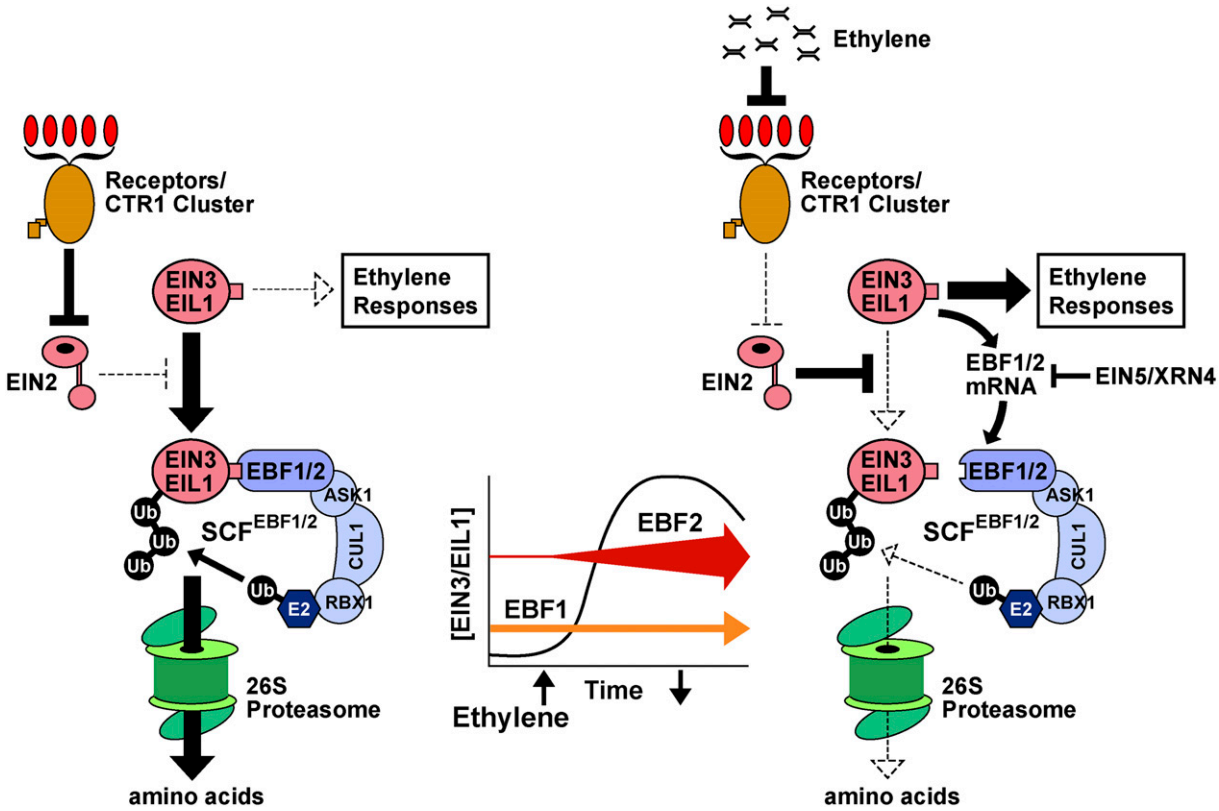


Figure 9. Model for the Action of EBF1 and 2 in the Ethylene Signal Transduction Pathway.

In the absence of ethylene, the receptor family activates the negative regulator CTR1, which leads to inhibition of EIN2. EIN3 and EIL1 levels are kept low by selective ubiquitination of the proteins by SCF^{EBF1} and SCF^{EBF2}, which induces their subsequent breakdown by the 26S proteasome. In the presence of ethylene, the receptors are inhibited, thus reducing the output of CTR1 and its subsequent inhibition of EIN2. EIN2 acts in part to directly or indirectly block the interaction of EIN3 and EIL1 with the SCF E3s containing EBF1 and EBF2. The reduction in ubiquitination allows EIN3 and EIL1 levels to rise to mediate ethylene responses. Over a slower time course, EIN2 activation also leads to an increase in *EBF1* and *EBF2* mRNA and presumably protein levels (shown in inset), which further dampens the accumulation of EIN3 and EIL1. Via an unknown mechanism, the exoribonuclease EIN5/XRN4 dampens the accumulation of the *EBF1* and *EBF2* transcripts. During ethylene signaling, EBF1 plays a special role at no or low hormone levels to maintain low basal levels of EIN3/EIL1. By contrast, EBF2 accumulates during ethylene signaling to prevent excess accumulation of EIN3/EIL1 and to remove EIN3/EIL1 after ethylene levels dissipate.

accumulation of EIN3/EIL1, which could inadvertently prolong ethylene signaling. The strong increase in SCF^{EBF2} levels in particular would also help remove EIN3/EIL1 once ethylene levels dissipate so the plants can more rapidly resume normal growth. Whereas some of this upregulation likely involves increased transcription of the *EBF1* and *EBF2* genes, the recent discovery that the *EIN5*/*XRN4* locus encodes a 5′-3′ exoribonuclease that depresses the steady state levels of *EBF1* and *EBF2* mRNAs suggests that the turnover of these transcripts is also directly or indirectly regulated (Olmedo et al., 2006; Potushak et al., 2006).

Clearly what remains to be determined is how ethylene signaling controls the recognition of EIN3 and EIL1 by the $SCF^{EBF1/2}$ E3s and thus regulates the turnover rates of these two transcription factors. Ethylene could block the assembly and/or action of the $SCF^{EBF1/2}$ complexes and/or prevent them from recognizing EIN3/EIL1 by hormone-induced posttranslational modification of the targets. The observations that EBF1 and EBF2 can bind both EIN3 and EIL1 in a yeast two-hybrid system (which we presume does not respond to ethylene) argues that the interaction of the targets with the E3s does not require prior modification of either (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Consequently, we propose that ethylene induces a modification of either the targets or the F-box proteins that blocks their association. Given the likely participation of a MAPK cascade in ethylene perception (Kieber et al., 1993; Ouaked et al., 2003) and the fact that recognition by SCF E3s is sometimes strongly regulated by target phosphorylation (Deshaies, 1999), a likely mechanism would be that ethylene induces phosphorylation of EIN3/EIL1. These modified forms of EIN3/EIL1 could be both protected from ubiquitination and more active in transcriptional activation. However, the observations that overexpression of either *EBF1* or *EBF2* can negate ethylene signal (Guo and Ecker, 2003; Potuschak et al., 2003; this report) would imply that this protection is either not absolute or is rapidly reversible even in the presence of ethylene.

The realization that *Arabidopsis* encodes for almost 700 F-box proteins has placed dramatic emphasis on the importance of

selective protein turnover by SCF E3s in plant growth and development (Gagne et al., 2002; Smalle and Vierstra, 2004). This large number certainly reflects in part an equally large number of targets that are subject to ubiquitination. For example, the six F-box proteins in the *Arabidopsis* TIR1/AFB family, either working individually or together, may be responsible for recognizing many of the 29 auxin/indole-3-acetic acid proteins that negatively regulate auxin signaling (Dharmasiri et al., 2005). Data presented here for the $SCF^{EBF1/2}$ complexes also support the notion that some E3s work in concert to degrade individual targets. In fact, the observations that glucose can promote EIN3 turnover suggest that a third E3 is involved in EIN3/EIL1 turnover that is regulated by a sugar-sensing pathway (Yanagisawa et al., 2003). However, the glucose-stimulated action of this E3, if it does exist, is insufficient to overcome the strong stabilization of EIN3 and EIL1 in *ebf1 ebf2* seedlings.

Clearly, by using sets of E3s that work in temporally and spatially distinct manners, the abundance of individual targets can be more precisely controlled. While it is clear that EIN3 and EIL1 are key checkpoints in ethylene signaling, a picture is emerging that their action is primarily modulated by the EBF1 and EBF2 F-box proteins and their ability to recognize and presumably ubiquitinate these targets. Coupled with the sophisticated control of *EBF1/2* mRNA levels by both the EIN5/XRN4 exoribonuclease and EIN3/EIL1 themselves (Guo and Ecker, 2003; Potuschak et al., 2003, 2006; Gagne et al., 2004), its appears that providing the appropriate levels of $SCF^{EBF1/2}$ complexes at the appropriate times are critical determinants in defining the correct degree of signaling before and after ethylene perception.

METHODS

Alignments and Phylogenetic Analysis

Amino acid sequences for the five members of the EIL family (EIL1-5) were retrieved by BLAST searches against the *Arabidopsis thaliana* genome database (http://www.arabidopsis.org/blast/). EIN3 and EIL1-5 sequences were aligned, and an unrooted phylogenetic tree with a 1000 bootstrap replicate was generated using ClustalX PC v1.8 (Thompson et al., 1997) and TreeView PC v1.6.0 (Page, 1996). Percentage identities and similarities were calculated using MacBoxshade v2.15 (Institute of Animal Health).

Mutant and Overexpression Lines

Unless indicated, all lines were in the *Arabidopsis* ecotype Col-0 background. The *ebf1-3*, *ebf1-4*, *ebf2-3*, and *ebf2-4* T-DNA insertion mutants were previously described by Gagne et al. (2004). The *ebf1-3* and *ebf2-3* lines were tracked by PCR using the primers 5′-CTGTGGCATTCTTCAAGC-3′ and 5′-TCTTGTCAGGGATCAAGG-3′ (*ebf1-3*) and primers 5′-CCAAAGTATTTGAGATGTCAAGGTCA-3′ and 5′-CGCTTTCTTGGGAAAGTTCTGT-3′ (*ebf2-3*) in conjunction with T-DNA left border–specific primer LBa1 (Alonso et al., 2003b). The homozygous *ein3-1 eil1-2* double mutant and lines overexpressing *EBF1* and *2* under the control of the CaMV *35S* promoter (*EBF1-OX* and *EBF2-OX*) were as described (Alonso et al., 2003a; Guo and Ecker, 2003; Potuschak et al., 2006). The *eil1-2* allele in the *ein3-1 eil1-2* double mutant was originally generated by En-1 transposon insertion in the L*er* background and contains a TCGA insertion at nucleotide 1145 (relative to the start codon) that is typical of an En-1 transposon footprint (Baumann et al., 1998). This mutation was introgressed in the Col-0 background, but sequencing of the closely linked *erecta* locus revealed this line still retains the four-point mutation found in the L*er* background compared with Col-0 (Torii et al., 1996; data not shown).

The *ctr1-1*, *ein3-1*, and *ein2-1* mutants were described previously (Guzman and Ecker, 1990; Kieber et al., 1993; Chao et al., 1997; Alonso et al., 2003b). The *ctr1-1* diepoxybutane mutation was tracked by genomic PCR using the primers 5′-ACTCCTCAGTTTGTCTTGAAGTTTCAGGT-3′ and 5′-ACTATTTAGCTTCCATTGGAAATAGGACC-3′. The products were identified by either DNA sequencing or by digestion with *Tsp*5091 (New England Biolabs). Whereas the 197-bp *CTR1* product from the wild type was not digested with *Tsp*5091, the T-to-A substitution in the *ctr1-1* mutant introduced a *Tsp*5091 site, which generated two fragments of 161 and 36 bp upon *Tsp*5091 cleavage. The *ein3-1* ethyl methanesulfonate allele was tracked by genomic PCR using the primers 5′-GAGCAAGCTAGGAGGAAGAAATGTCTAG-3′ and 5′-TTTAGGCAAACCAAGTTGGATGCCAC-3′. The products were then digested with *Hae*III. Whereas the 438-bp *EIN3* product from the wild type contains a *Hae*III restriction site that upon digestion with *Hae*III generates two fragments of 393 and 45 bp, a G-to-A substitution in the *ein3-1* mutant removed this site. The glufosinate ammonium-resistant *EIN3-OX* line was as described (Yanagisawa et al., 2003). PCR products were detected by ethidium bromide staining following electrophoresis.

The *eil1-3* and *eil1-4* T-DNA insertion alleles (Col-0) were identified in the SIGNAL T-DNA collection (Salk_049679 and Salk_042179) (Alonso et al., 2003b). Both mutants were tracked by genomic PCR using the gene-specific primers 5′-TGAAACGTCTCAAGGAGCAAC-3′ and 5′-ATGTTGCATTTTAGCAACCCC-3′ and the T-DNA–specific left border primer LBa1. Sequencing the *eil1-3* PCR product revealed that the T-DNA is inserted 719 bp downstream of the start codon with the left T-DNA border sequence in 5′ orientation relative to the gene. Amplification of the *PHYA* gene with the primers 5′-GTCAGGCTCTAGGCCG-3′ and 5′-ATTGCAATCAACTATCATCC-3′ was used as a control in PCR genotyping the various mutant backgrounds. The various mutant combinations were generated by appropriate crosses, and the desired genotypes were identified by PCR testing the progeny.

RNA Isolation and Analysis

RT-PCR of *eil1-3* and *eil1-4* seedlings was performed on total RNA isolated by Trizol reagent (Invitrogen). First-strand cDNA was synthesized with primer 2 (5′-TACATCTTGCTGCTGCTGC-3′) and then amplified by PCR with primers 1 (5′-ATGATGATGTTTAACGAGATG-3′) and 2. Amplification of the *PAE2* mRNA was used as a control (Downes et al., 2003). RNA gel blot analysis was performed with total RNA isolated by aurintricarboxylic acid/phenol/chloroform extraction followed by sequential lithium chloride and ethanol/potassium acetate precipitations (Hondred et al., 1999). ^{32}P-labeled RNA probes were synthesized by the riboprobe system using Sp6 or T7 RNA polymerase (Promega) and the *EBF1*, *EBF2*, *EIN3*, and *EIL1* cDNAs as templates. The *EBF1* and *EIL1* probes encompassed the full coding region. The *EBF2* and *EIN3* probes contained 250 and 302 bp of internal coding sequence, respectively. The β-*TUB4* probe was as described (Smalle et al., 2002).

Plant Growth Conditions

Unless otherwise noted, plants were grown on Gamborg's B5 growth medium (Gibco BRL) at 21°C under a long-day photoperiod (16 h light/8 h dark) following a 4-d stratification of the seeds at 4°C. Effects of ACC on hypocotyl growth were measured on 4-d-old etiolated seedlings grown on agar containing half-strength Murashige and Skoog (MS) medium without sugar (Gagne et al., 2004). For inhibitor studies, *ebf1-4 ebf2-4* seeds were germinated on solid half-strength MS medium, pH 5.7, with or without AVG, $AgNO_3$, ACC, and/or glucose.

Hypocotyl Growth Rate Measurements

Kinetic analyses of hypocotyl elongation were generated using etiolated *Arabidopsis* seedlings as described previously (Binder et al., 2004a, 2004b). Mutant and wild-type seeds were surface-sterilized by treatment with 70% ethanol for ~30 s, placed on sterile filter paper to dry, and plated on half-strength MS medium, pH 5.7, containing 0.8% agar and B5 vitamins (100 mg mL^{-1} inositol, 1 mg mL^{-1} nicotinic acid, 1 mg mL^{-1} pyridoxin HCl, and 10 mg mL^{-1} thiamine HCl), and 5 μM AVG (supplied by Tarlochan Dhadialla, Rohm Haas, Philadelphia, PA), with no added sugar.

Seeds were stratified for 2 to 4 d at 4°C and exposed to white light for 2 to 8 h prior to being grown vertically in the dark for 2 d at 22°C. Once seedlings reached a height of 2 to 4 mm, growth rates were measured as described (Binder et al., 2004b). Following a 1-h treatment with air to establish a basal elongation rate, ethylene was introduced at a flow rate of 10 mL min^{-1}, giving a final concentration of 10 μL L^{-1}. Gas flow was maintained at 100 mL min^{-1} using Side-Trak mass flow meters and controller (Sierra Instruments). Hypocotyl growth was measured from digital images that were captured every 5 min for 7 h with either a EDC-1000N CCD camera (Electrim) or an Infinity 2-1M camera (Luminera) and light provided by an infrared light emitting diode. Growth rates were calculated using custom software generated by Edgar Spalding in LabVIEW 5.0 (National Instruments) as previously described (Parks and Spalding, 1999; Folta and Spalding, 2001). All data presented represent the average of at least four seedlings from a minimum of three separate experiments. Data were normalized to the growth rate in air prior to ethylene treatment.

Accession Numbers

Arabidopsis Genome Initiative accession numbers for genes described in this article are as follows: *CTR1*, At5g03730; *EBF1*, At2g25490; *EBF2*, At5g25350; *EIN2*, At5g03280; *EIN3*, At3g20770; *EIL1*, At2g27050; *EIL2*, At5g21120; *EIL3*, At1g73730; *EIL4*, At5g10120; and *EIL5*, At5g65100.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. Average Growth Rate of Etiolated Mutant Seedlings.

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