ETHYLENE-INSENSITIVE5 encodes a 5′→3′ exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2

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Ethylene is a gaseous plant growth regulator that controls a multitude of developmental and stress responses. Recently, the levels of *Arabidopsis* EIN3 protein, a key transcription factor mediating ethylene-regulated gene expression, have been demonstrated to increase in response to the presence of ethylene gas. Furthermore, in the absence of ethylene, EIN3 is quickly degraded through a ubiquitin/proteasome pathway mediated by two F-box proteins, EBF1 and EBF2. Here we report the identification of *ETHYLENE-INSENSITIVE5* as the 5′→3′ exoribonuclease *XRN4*. Specifically, we demonstrate that EIN5 is a component of the ethylene signal transduction cascade acting downstream of CTR1 that is required for ethylene-mediated gene expression changes. Furthermore, we find that the ethylene insensitivity of *ein5* mutant plants is a consequence of the over-accumulation of *EBF1* and *EBF2* mRNAs resulting in the under-accumulation of EIN3 even in the presence of ethylene gas. Together, our results suggest that the role of EIN5 in ethylene perception is to antagonize the negative feedback regulation on EIN3 by promoting *EBF1* and *EBF2* mRNA decay, which consequently allows the accumulation of EIN3 protein to trigger the ethylene response.

Arabidopsis | growth regulation | signal transduction

The plant hormone ethylene regulates a variety of developmental and stress responses in plants, including seed germination, cell elongation, cell fate, sex determination, fruit ripening, flower senescence, leaf abscission, defense against pathogens, and responses to mechanical trauma (1, 2). The most widely documented ethylene response is the so-called "triple response" of dark-grown (etiolated) seedlings. In the reference plant *Arabidopsis thaliana* the triple response is characterized by inhibition of hypocotyl and root elongation, a thickened hypocotyl, and an exaggerated apical hook (3). The triple-response phenotype has been used extensively to isolate components of the ethylene signal transduction pathway (4). Several mutants that display an aberrant triple response have been isolated in *Arabidopsis*. One class of mutants (i.e., *etr1*, *etr2*, *ein2*, *ein3*, *ein4*, *ein5*, *ein6*, and *ein7*) shows a reduction or complete loss of ethylene responsiveness in the presence of exogenous ethylene or its biochemical precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). A second class of mutants (*eto1*, *eto2*, *eto3*, and *ctr1*) constitutively exhibits a triple response, either because of the overproduction of ethylene (Eto^- mutants) or as a consequence of constant activation of the ethylene signaling pathway (Ctr^-). The third class of mutants, which includes *ebf1*, *ebf2*, *eer1*, and *rte1*, exhibits an enhanced sensitivity to exogenous ethylene, suggesting they are negative regulators of the ethylene signaling pathway (5–10). A genetic and molecular analysis of these mutants has unraveled a largely linear ethylene signal transduction pathway leading from initial hormone perception to transcriptional regulation (4, 11). Briefly, ethylene gas is perceived by a family of five endoplasmic reticulum-localized, membrane-bound receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) that have been demonstrated to be negative regulators of ethylene signaling (12, 13). To be functional, the ethylene receptors require a copper cofactor whose loading likely depends on RAN1, a homolog of the human Menkes Wilson P-type ATPase copper transporters (14, 15). Recent data suggest that RTE1, an evolutionarily conserved protein with unknown biochemical activity, plays an important role in the function of the ethylene receptor ETR1 (6). Interestingly, the tomato homolog of RTE1, GR, has been demonstrated to be required for ethylene responsiveness in fruit tissues (16). The first signaling component downstream of the ethylene receptors is CTR1, which is also a negative regulator of ethylene signaling (17). EIN2, an integral membrane protein of unknown function, is genetically downstream of CTR1 and is a major positive regulator of the pathway because loss-of-function mutations result in complete ethylene insensitivity (18). Functioning downstream of EIN2 is a small family of DNA-binding transcription factors that includes EIN3 and various EIN3-like proteins (5, 19). Loss-of-function mutations in *EIN3* cause partial ethylene insensitivity, whereas loss-of-function mutations in *EIL1* or *EIL2* result in weak ethylene insensitivity (19, 20). Moreover, ectopic expression of *EIN3* or *EIL1* is sufficient to induce a complete ethylene response phenotype, suggesting that these proteins act as master regulators of the ethylene response (19, 21). Interestingly, recent studies have suggested that EIN3 protein levels rapidly increase in response to ethylene and that this response requires several ethylene signaling pathway components, including the ethylene receptors (ETR1 and EIN4), CTR1, EIN2, EIN5, and EIN6 (5). Furthermore, in the absence of or upon depletion of ethylene gas, EIN3 is quickly degraded through a ubiquitin/proteasome pathway mediated by two F-box proteins, EBF1 and EBF2 (5, 9, 10, 22).

In this study we describe the identification of *ETHYLENE-INSENSITIVE5* as the previously described 5′→3′ exoribonucle-

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. BT026022), and gene expression and tiling array data have been deposited in the Gene Expression Omnibus database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5174).

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ase XRN4. We show that the *ein5* mutation is able to partially suppress the constitutive ethylene response phenotype of *ctr1* in both seedling and adult plants, thereby suggesting that EIN5 is a component of the ethylene signal transduction cascade acting downstream of CTR1. The involvement of EIN5 in the ethylene signaling cascade is further suggested by the finding that *ein5* mutant plants are defective for ethylene-mediated gene expression. In addition, the mRNAs for the EIN3-regulating F-box proteins EBF1 and EBF2 accumulate in *ein5* mutant plants in the presence and absence of ethylene gas, likely resulting in the under-accumulation of EIN3 and ultimately ethylene insensitivity. Mutation of either *EBF1* or *EBF2* in *ein5* mutant plants suppresses the ethylene insensitivity phenotypes of these plants. Taken together, these results suggest that the role of EIN5 in ethylene perception is to antagonize the negative feedback regulation on EIN3 by promoting *EBF1* and *EBF2* mRNA decay, which consequently allows the accumulation of EIN3 protein to trigger the ethylene response.

Results

***ein5* Mutants Specifically Affect the Ethylene Response.** The *EIN5* locus is one of five novel ethylene-insensitive (Ein$^-$) complementation groups identified as a result of a large-scale screen for ethylene response mutants in *Arabidopsis* (23). Mutations at the *EIN5* locus confer insensitivity to high levels of exogenous and endogenous ethylene, which is characterized by the elongated hypocotyl and root compared with those of wild-type seedlings (Fig. 1*A*). Mutation of the *CTR1* gene confers a constitutive triple-response phenotype on plants (17). CTR1 is a Raf-like protein kinase that negatively regulates downstream ethylene signaling events upon response to the ethylene receptors (17). Thus, the absence of CTR1 results in constitutive triggering of the ethylene response pathway. We wished to determine whether EIN5 function is epistatic to the CTR1 kinase. To do this, we made crosses between *ein5-1* and *ctr1-1* mutant plants. We found that in *ein5-1 ctr1-1* double-mutant plants *ein5* was able to partially suppress the constitutive ethylene phenotype of *ctr1* in both seedling and adult stages (Fig. 1 *B* and *C–F*, respectively), thus demonstrating that EIN5 affects a step in the ethylene signaling pathway downstream of CTR1.

***EIN5* Identified as the 5′→3′ Exoribonuclease XRN4.** We cloned the *EIN5* gene using a map-based approach (Fig. 2*A*). *ein5-1* and *ein5-7* as well as new alleles were mapped to the lower half of chromosome 1, near marker nga128 (Fig. 2*A*). Using the 128-bp deletion-containing allele *ein5-11* as a tool, we were able to precisely identify *EIN5* as coding for the previously described 5′→3′ exoribonuclease XRN4 (Fig. 2*B*). Confirmation of *EIN5*'s identity as *XRN4* was provided by examination of multiple *ein5* allele sequences. Seven of the 10 sequenced *ein5* alleles caused frame-shift mutations that predicted a premature termination of the protein (Fig. 2*C* and Table 1). In addition, the mutation in *ein5-4* caused a complex rearrangement of the gene, whereas *ain1-2* and *ain1-7* have G-to-A transitions that affect, respectively, the donor and acceptor sites of exon 3 (Fig. 2*A*). It has previously been reported that XRN4/EIN5 shares homology with two 5′→3′ exoribonucleases from *Saccharomyces cerevisiae*, Xrn1p and Rat1p (24). In addition, many other eukaryotic organisms also have homologs of XRN4/EIN5 (Fig. 2*D*; see also Fig. 7, which is published as supporting information on the PNAS web site). Interestingly, mutation of *S. cerevisiae XRN1* results in pleiotropic defects including meiotic arrest, reduced spore viability, and slow growth (25). Correspondingly, we were interested to learn whether *XRN4/EIN5* could complement the pleiotropic growth defects associated with an *xrn1* mutant yeast strain. To do this, we introduced *EIN5* into a *S. cerevisiae xrn1* mutant strain using a yeast expression plasmid. We observed that a yeast expression plasmid carrying either *EIN5* or the *S. cerevisiae Xrn1* gene could complement an *xrn1* mutant (Fig. 8*B*, which is published as supporting information on the PNAS web site). Similarly, as previously described, we observed that that introduction of *XRN2* rescues the *rat-1ts* mutation but *EIN5* does not (26) (Fig. 8*A*).

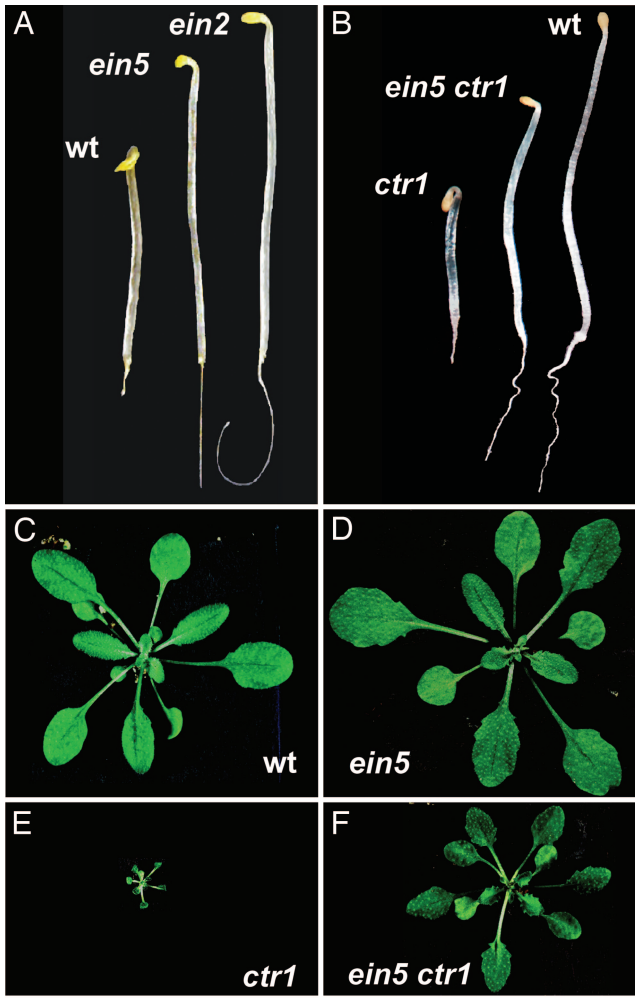

Fig. 1. Ethylene insensitivity of *ein5* and partial suppression of the constitutive phenotype of *ctr1*. (*A*) Comparison of 3-day-old etiolated wild-type, *ein5*, and *ein2* seedlings grown in 10 ppm ethylene in hydrocarbon-free air. Wild type displays the ethylene-mediated triple-response phenotype, which is absent in *ein2* and significantly reduced in *ein5*. (*B–F*) *ein5* partially suppresses the constitutive ethylene phenotype of *ctr1* in both seedling and adult stage. (*B*) Comparison of 3-day-old etiolated *ctr1*, *ein5/ctr1*, and wild-type seedlings grown in hydrocarbon-free air. *ctr1-1* displays a constitutive triple response in the absence of exogenous ethylene. (*C–F*) Three- to 4-week-old adult wild-type (wt), *ein5*, *ctr1*, and *ein5/ctr1* plants.

Altered Ethylene-Mediated Gene Expression in *ein5* Mutants. The observed Ein$^-$ phenotype of *ein5* mutant plants suggests that XRN4/EIN5 plays an important role in the ethylene signal transduction pathway. Therefore, mutation of *XRN4/EIN5* might be expected to result in altered ethylene-mediated gene expression. To examine whether XRN4/EIN5 is required for proper ethylene-mediated gene expression, we performed Northern blot analysis using total RNA from 3-day-old etiolated seedlings of wild-type Col-0, *ein5-1*, *ein5-6*, *ein5-11*, and *ein3-1* grown in air or 10 ppm ethylene. We found that wild-type Col-0 seedlings treated with ethylene (Fig. 3*A*, Col-0+) exhibited a dramatic increase in expression of the ethylene-responsive genes *AtGST2*, *AtEBP* (coding for the ethylene-responsive protein

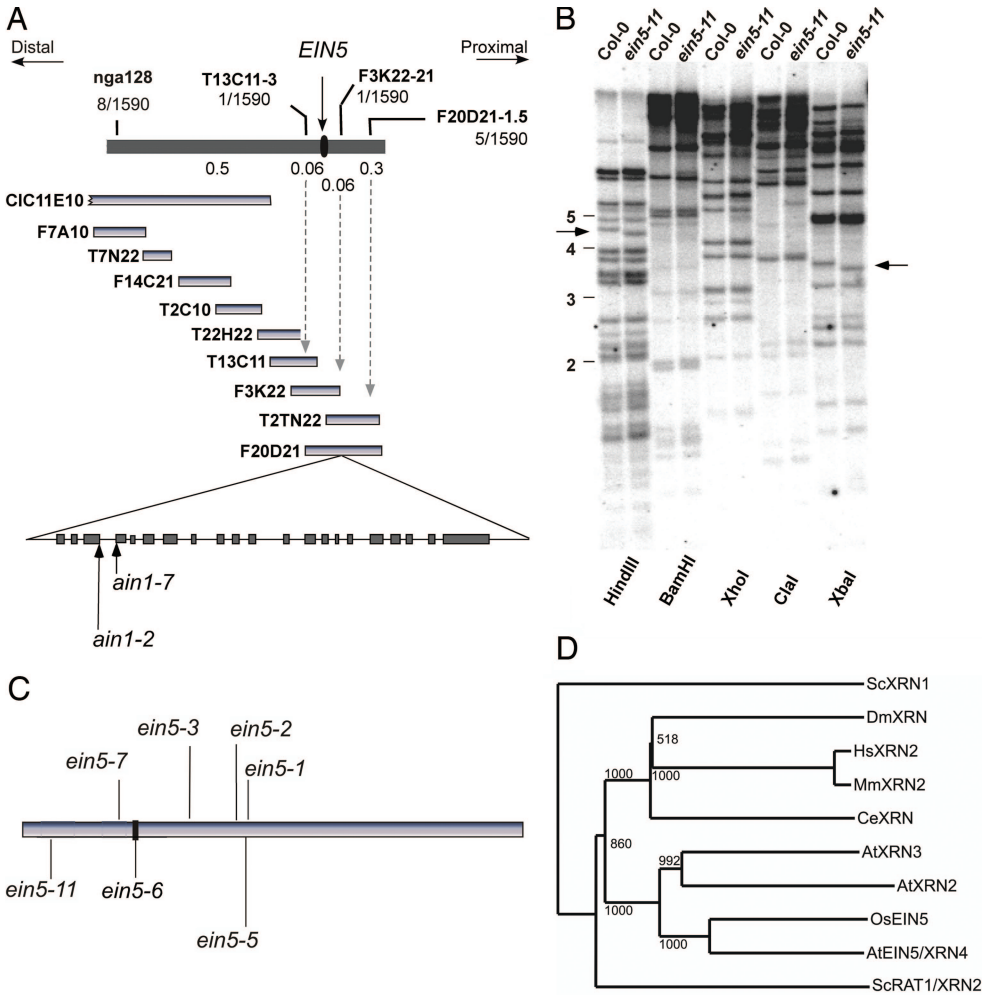

Fig. 2. Map-based cloning of *ETHYLENE-INSENSITIVE5*. (*A*) Partial genetic map of *Arabidopsis* chromosome 1. *EIN5* was mapped to SSLP marker nga128 and to restriction fragment-length polymorphisms identified within BACs T13C11, F3K22, and F20D21. The position of markers is indicated above the chromosome line, and under each one is displayed the number of recombinants between the marker and *ein5* per number of chromosomes examined. A physical map of the region was constructed by using one yeast artificial chromosome, clone CIC11E10, and nine BAC clones. The genetic distance to *EIN5* is shown below the chromosome line, and a broken arrow points to the location in the BAC containing the marker. At the bottom, the intron–exon structure of *EIN5* and the location of mutations in *ain1-2* and *ain1-7* alleles are schematically shown. Both of these alleles have G→A transitions that affect the donor and acceptor sites of exon 3. (*B*) Identification of polymorphisms between the genomes of *ein5-11* and wild-type plants. The allele *ein5-11*, which contains a 128-bp deletion, was used to locate *EIN5*. BAC F20D21 was used to identify polymorphisms between the genomes of *ein5-11* and wild type. Southern blot analysis of restriction enzyme digests of Col-0 and *ein5-11* DNA was performed with ^{32}P-labeled DNA from BAC F20D21. The arrows point to the observed polymorphisms. (*C*) Schematic representation of mutations in *ein5* alleles. The nucleotide positions for each mutation are as follows: *ein5-1*, 1-bp deletion at position 4292; *ein5-2*, 4-bp deletion at 3393; *ein5-3*, 5-bp deletion at 2417; *ein5-5*, 23-bp deletion at 4217; *ein5-6*, 22-bp deletion at 1724; *ein5-7*, 1-bp deletion at 1749; *ein5-11*, 128-bp deletion at 163. Position is given with reference to the ATG marking the start of the ORF in the unspliced sequence. (*D*) Phylogenetic analysis of XRN 5′→3′ exonucleases. The tree includes representative XRN genes from *Drosophila* (Dm, *Drosophila melanogaster)*, humans (Hs, *Homo sapiens*), mice (Mm, *Mus musculus*), worms (Ce, *Caenorhabditis elegans*), rice (Os, *Oriza sativa*), yeast (Sc, *S. cerevisiae*), and *Arabidopsis* (At, *A. thaliana*). The phylogenetic tree was generated by using the neighbor-joining method with a bootstrap value of 1,000 replicates and analyzed by using NJplot.

element), *AtACO2*, basic chitinase (*BCH1*), and defensin (*PDF1.2*) compared with Col-0 seedlings grown in hydrocarbon-free air (Fig. 3*A*, compare Col-0+ to Col-0−). Conversely, this same set of genes showed a reduced ethylene induction of expression in *ein5* mutant seedlings (Fig. 3*A*) as compared with wild-type plants, although not as dramatic as the reduction observed in *ein3* mutant seedlings (Fig. 3*A*). All three *ein5* alleles tested (*ein5-1*, *ein5-6*, and *ein5-11*) exhibited a clearly reduced expression of defensin, basic-chitinase (*BCHI*), and GST (*ATGST2*) as compared with wild-type plants (Fig. 3*A*). Taken together, these findings provide further evidence for the involvement of EIN5 in the ethylene signaling pathway.

To confirm and expand on these observations we used Affymetrix (Santa Clara, CA) *Arabidopsis* gene expression arrays, which interrogate the expression of >22,000 genes (27). We hybridized mRNA samples from 3-day-old etiolated seedlings of wild-type Col-0, *ein5-1*, and *ein2-5* grown in air or treated for 4 h with 10 ppm ethylene gas to compare transcription profiles. We found that the expression of 244 genes was induced and 384 genes were repressed at least 2-fold after 4 h of ethylene exposure in wild-type etiolated seedlings (Table 2). Among these transcripts there are several previously known ethylene responsive genes such as *ERF1* (21) and many genes involved in cell wall metabolism, confirming the success of the ethylene treatment for this experiment (data not shown). Fig. 3*B* shows graphically the 50 transcripts induced and repressed at the highest level (100 total) in wild-type Col-0 seedlings upon ethylene treatment and a comparison between expression levels of these same 100 genes

Table 1. Molecular nature of *EIN5* alleles

Allele	Mutagen	Position of mutation†	Ecotype	Source
ein5-1	X-ray	A 1-bp deletion at 4292; exon 15	Col-0	23
ein5-2	X-ray	A 4-bp deletion at 3393; exon 12	Col-0	23
ein5-3	Fast neutron	A 5-bp deletion at 2417–2421; exon 8	Col-0	This work
ein5-4	Fast neutron	Rearrangement	Col-0	This work
ein5-5	T-DNA	A 23-bp deletion at 4217; exon 15	Col-0	J. M. Alonso, R. Solano, and J.R.E., unpublished observations
ein5-6	T-DNA	A 22-bp deletion plus 5 bp at 1724–1745; exon 5	Col-0	J. M. Alonso, R. Solano, and J.R.E., unpublished observations
ein5-7 (*ein7*)	X-ray	A 1-bp deletion at 1749; exon 5	Col-0	23
ein5-11	T-DNA	A 128-bp deletion at 163–291	Col-6	J. M. Alonso, R. Solano, and J.R.E., unpublished observations
ain1-2‡	EMS	G→A at the donor site of the third exon/intron junction at 1074	C24	Gift from D. Van der Straeten (University of Ghent, Ghent, Belgium)
ain1-7‡	EMS	G→A at the acceptor site of the third exon/intron junction at 1149	Col	Gift from A. Bleecker (University of Winsconsin, Madison, WI)

†Relative to the start ATG of the unspliced gene.
‡*ain,* ACC-insensitive mutants.

in *ein2-5* and *ein5-1* seedlings upon hormone treatment (see also Tables 3 and 4, which are published as supporting information on the PNAS web site). We found that the loss of ethylene-mediated gene expression was more significant in *ein2-5* mutant plants compared with *ein5-1* mutant plants (Fig. 3*B*). In *ein2-5* mutant plants there was almost no induction or repression of the 100 genes that are ethylene-responsive in wild-type Col-0 plants. Interestingly, in *ein5-1* mutant plants these genes exhibited an intermediate level of expression and repression between wild-type Col-0 and *ein2-5*, as expected because of the observed intermediate ethylene insensitivity of *ein5* mutant plants. Taken together, these results suggest that, rather than affecting a subset of the genes that respond to ethylene, *ein5* affects the expression levels of most ethylene-responsive genes.

EIN5 Regulates the EIN3-Targeting F-Box Proteins EBF1/2 mRNAs. Next, we wanted to identify potential target RNAs regulated by XRN4/EIN5 that could possibly elucidate its function in the ethylene signaling pathway. To do this, we used a set of custom Affymetrix genome tiling arrays that span the entire *Arabidopsis* genome at high resolution (28). We prepared RNA samples from 10-day-old light-grown seedlings of wild type (Col-0) and *ein5-1* to compare transcript profiles. From the expression profiling we found that *EBF1* and *EBF2* were highly overexpressed in *ein5-1* mutant plants compared with wild-type Col-0 plants (Fig. 4 *A* and *B*). This result was confirmed by examination of the data previously obtained using the gene expression arrays. In addition, using whole-genome tiling arrays allowed us to examine the expression of many other genes not present on the gene expression arrays (see also Fig. 9, which is published as supporting information on the PNAS web site, and data not shown).

To validate these results and to further characterize the regulation of *EBF1* and *EBF2* mRNA levels by EIN5, we performed Northern blot analysis using total RNA from 3-day-old etiolated seedlings of wild-type Col-0 and *ein5-6* grown in air or 10 ppm ethylene for various amounts of time. We found that *ein5-6* mutant seedlings accumulated significantly more *EBF1* mRNA than did wild-type Col-0 seedlings both before and during ethylene treatment (Fig. 4*C*). Furthermore, the levels of *EBF1* mRNA in the *ein5-6* mutant seedlings accumulated to even higher levels compared with wild-type Col-0 plants after 4 and 12 h of growth in ethylene (Fig. 4*C*). Similar to the previous results, we also found that *ein5-6* mutant seedlings accumulated significantly more *EBF2* mRNA than did wild-type Col-0 seedlings both before and during ethylene treatment (Fig. 4*D*). Interestingly, we observed that, upon treatment with ethylene, wild-type Col-0 plants accumulated increased levels of *EBF2* mRNA. However, the increase of *EBF2* mRNA was still significantly higher in the *ein5-6* mutant seedlings compared with wild-type Col-0 seedlings after treatment with ethylene (Fig. 4*D*).

Next we wanted to examine whether *EBF1* and *EBF2* mRNA levels were also significantly higher in light-grown adult *ein5* mutant plants compared with wild-type Col-0 plants. We performed Northern blot analysis using total RNA from rosette leaves of 3-week-old wild-type Col-0, *ein5-1*, *ein5-6*, and *ein5-7* plants. As was the case in 3-day-old etiolated seedlings, the *ein5* mutant plants accumulated significantly higher levels of *EBF1* and *EBF2* mRNAs than did wild-type Col-0 plants (Fig. 4 *E* and *F*). Taken together, these results demonstrate that the XRN4/EIN5 exoribonuclease regulates *EBF1* and *EBF2* at the post-transcriptional level and suggest that regulation of these EIN3-targeting F-box proteins is the role of XRN4/EIN5 in the ethylene signal transduction pathway.

Mutation of *EBF1* and *EBF2* Can Suppress the Ein$^-$ Phenotype of *ein5* Mutant Plants. We wanted to determine whether regulation of *EBF1* and *EBF2* mRNA levels was relevant to the role of EIN5 in ethylene signal transduction. We generated double mutants between *ein5* and *ebf1* or *ebf2*. As shown in Fig. 5 *A* and *B*, *ebf2-1* completely suppressed the Ein$^-$ phenotype of *ein5-1*, whereas *ebf1-1* partially suppressed the Ein$^-$ phenotype of *ein5-1*. Additionally, three independent attempts were made to obtain *ein5-1 ebf1-1 ebf2-1* triple-mutant plants. We were never able to obtain a homozygous triple mutant plant, but we found that *ein5-1 ebf1-1 ebf2-1*$^{+/-}$ and *ein5-1 ebf1-1*$^{+/-}$ *ebf2-1* were highly hypersensitive to ethylene (data not shown). Therefore, the inability to obtain triple-mutant plants was likely caused by embryo lethality of this combination of mutations. Taken together, these results suggest that the Ein$^-$ phenotype of *ein5* mutant plants is caused by the over-accumulation of *EBF1* and *EBF2* mRNAs, likely resulting in a decrease in EIN3 levels.

To test directly whether EIN3 levels are depleted in *ein5* mutant plants compared with wild-type Col-0, Western blot analysis with an EIN3-specific antibody was done by using total protein from 3-day-old etiolated seedlings of wild-type Col-0 *ein5-1*, *ein5-1 ebf1-1*, and *ein5-1 ebf2-1* grown in air or 10 ppm ethylene. As expected, upon ethylene treatment EIN3 accumulated in wild-type Col-0 plants, and this ethylene-induced accumulation of EIN3 was ablated in *ein5-1* mutant plants (Fig. 5*C*)

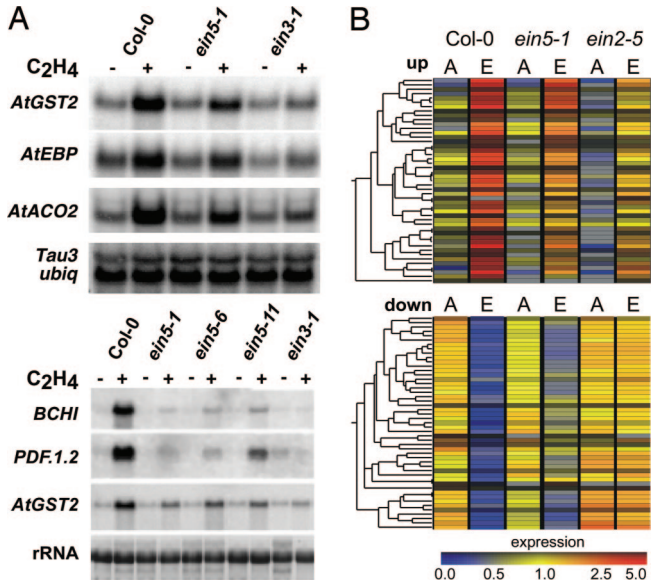

Fig. 3. Defects in ethylene-mediated gene expression of *ein5* mutant plants. (*A*) RNA gel blot analysis of total RNA from 3-day-old etiolated seedlings of wild-type Col-0, *ein5-1*, *ein5-6*, *ein5-11*, and *ein3-1* grown in air or 10 ppm ethylene (C2H4). (*Upper*) Ten micrograms of total RNA from 3-day-old etiolated seedlings of wild-type Col-0, *ein5-1*, and *ein3-1* grown in air (−) or 10 ppm ethylene (+) were sequentially hybridized with probes to GST (*AtGST2* and At4g02520), ethylene-responsive binding protein (*AtEBP* and At3g16770), and ACC oxidase (*AtACO2* and At1g62380). Finally, the blot was rehybridized with probes to ubiquitin and Tau3 as a loading control. (*Lower*) Twenty micrograms of total RNA from 3-day-old etiolated seedlings of wild-type Col-0, *ein5-1*, *ein5-6*, *ein5-11*, and *ein3-1* grown in air (−) or 10 ppm ethylene (+) were sequentially probed with probes to basic chitinase (*BCH1* and At3g12500), defensin (*PDF1.2* and At5g44420), and *AtGST2*. Finally, the blot was rehybridized with a probe to 25S rRNA, and this blot is shown as a loading control. (*B*) Hierarchical cluster analysis of ATH1 microarray data obtained from etiolated seedlings exposed to ethylene (E) or hydrocarbon-free air (A). (*Upper*) Clustering of 50 genes up-regulated >3.0 times. (*Lower*) Clustering of 50 genes down-regulated >3.0 times. (Genes are listed in Tables 3 and 4.) Each cell corresponds to the level of expression of each gene in a given condition. Red indicates an increased level of expression, and blue indicates a decreased level of expression. Expression level is relative to the average of normalized expression of two GeneChips per condition. Genes are clustered together based on similarities of expression level. The bar is the scale for normalized expression.

(5). Interestingly, ethylene-induced accumulation of EIN3 was completely restored in *ein5-1 ebf2-1* double-mutant plants but not in *ein5-1 ebf1-1* double mutant plants, providing a likely explanation for the difference in ability of the *ebf1* and *ebf2* to suppress the Ein$^-$ phenotype of *ein5-1* mutant plants (Fig. 5). Taken together, these results suggest that the over-accumulation of *EBF1* and *EBF2* mRNAs in *ein5* mutant plants results in an inability to accumulate EIN3 in response to ethylene, thereby causing ethylene insensitivity.

Table 2. Genes exhibiting an altered expression in *ein5-1* and *ein2-5* with respect to Col-0

Allele	No. of genes increased*	No. of genes decreased*	% altered with respect to Col-0 (air)†
ein5-1	127	123	1.56
ein2-5	193	119	1.95

*At least 2-fold difference.
†Sixteen thousand genes expressed.

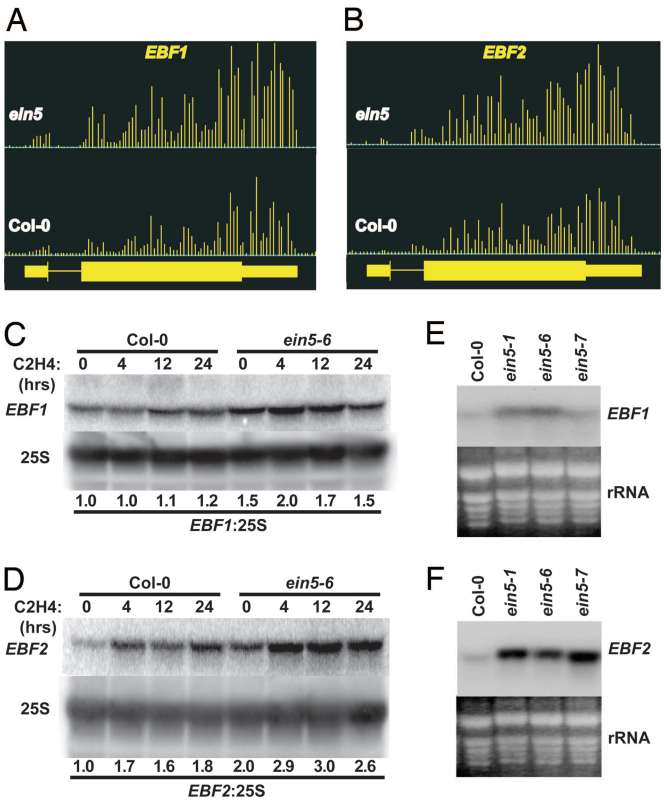

Fig. 4. EIN5 regulates the level of mRNA for EIN3-targeting F-box proteins EBF1 and EBF2. (*A*) Levels of *EBF1* mRNA were determined by Affymetrix *Arabidopsis* tiling array expression analysis. The top track displays the level of *EBF1* mRNA from *ein5-1* mutant plants. The middle track displays the level of *EBF1* mRNA from wild-type (Col-0) plants. The bottom track is the annotated gene model for the *EBF1* locus. (*B*) Levels of *EBF2* mRNA determined by Affymetrix *Arabidopsis* tiling array expression analysis. The top track displays the level of *EBF2* mRNA from *ein5-1* mutant plants. The middle track displays the level of *EBF2* mRNA from wild-type (Col-0) plants. The bottom track is the annotated gene model for the *EBF2* locus. (*C*) RNA gel blot analysis of 10 μg of total RNA from 3-day-old etiolated seedlings of wild-type Col-0 and *ein5-6* grown in air or treated for various times with 10 ppm ethylene (C_2H_4) gas with a 3′ *EBF1* probe. The times of ethylene treatment (or lack thereof) are indicated above the lanes. After stripping, the blot was rehybridized with a 25S rRNA probe, and this blot is shown as a loading control. Normalized values of *EBF1* mRNA to the rRNA control (with Col-0 without ethylene treatment set at 1.0) are indicated at the bottom of the rRNA blot. (*D*) RNA gel blot analysis of 10 μg of total RNA from 3-day-old etiolated seedlings of wild-type Col-0 and *ein5-6* grown in air or treated for varying times with 10 ppm ethylene (C2H4) gas with a 3′ *EBF2* probe. The times of ethylene treatment (or lack thereof) are indicated above the lanes. After stripping, the blot was rehybridized with a 25S rRNA probe, and this blot is shown as a loading control. Normalized values of *EBF2* mRNA to the rRNA control (with Col-0 without ethylene treatment set at 1.0) are indicated at the bottom of the rRNA blot. (*E*) RNA gel blot analysis of 10 μg of total RNA from rosette leaves of 3-week-old wild-type Col-0, *ein5-1*, *ein5-6*, and *ein5-7* plants with a 3′ *EBF1* probe. The rRNA is shown as a loading control. (*F*) RNA gel blot analysis of 10 μg of total RNA from rosette leaves of 3-week-old wild-type Col-0, *ein5-1*, *ein5-6*, and *ein5-7* plants with a 3′ *EBF2* probe. The rRNA is shown as a loading control.

Discussion

Here we show that EIN5 is a component of the ethylene signaling pathway that acts downstream of CTR1, which is necessary for proper ethylene-mediated gene expression. Interestingly, microarray analysis carried out on wild-type Col-0, *ein2*, and *ein5* etiolated seedlings (exposed to air or to ethylene) (Fig. 3) revealed that a set of genes that are highly induced by ethylene treatment in the wild-type plant exhibits an intermediate expression level in *ein5* as compared with *ein2*, where there is only

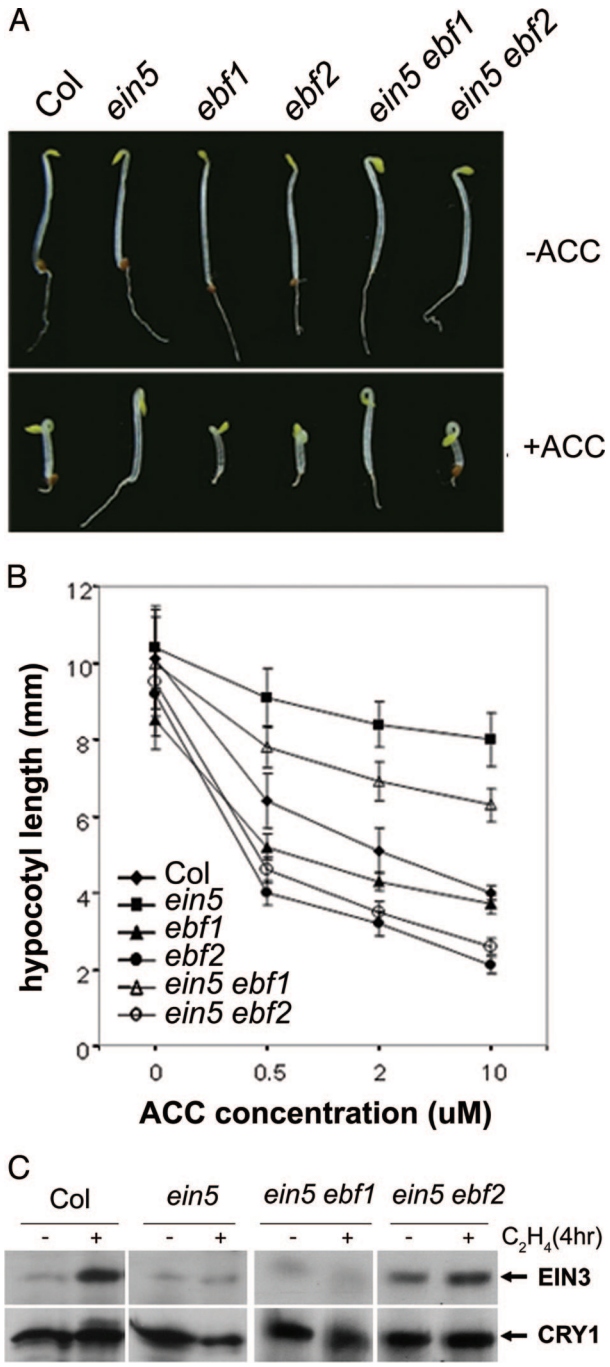

Fig. 5. *ein5* defect is suppressed by *ebf1* and *ebf2* mutations. (*A*) Triple-response phenotype of 3-day-old etiolated seedlings grown on MS medium supplemented with (+ACC) or without (−ACC) 10 μM ACC. (*B*) Dosage response of wild-type Col-0 and various mutants. Etiolated seedlings were grown on MS medium supplemented with different concentrations of ACC for 3 days. The length of hypocotyls from 10 seedlings was measured, and the mean values and standard deviations were plotted. (*C*) Immunoblot assays of EIN3 protein in *ein5* and *ein5 ebf* double mutants. Etiolated seedlings grown in air for 3 days were treated with or without ethylene (C_2H_4) gas (10 ppm) for 4 h before tissues were harvested for immunoblot assays. CRY1 (*Arabidopsis* cryptochrome 1) protein was used as a loading control.

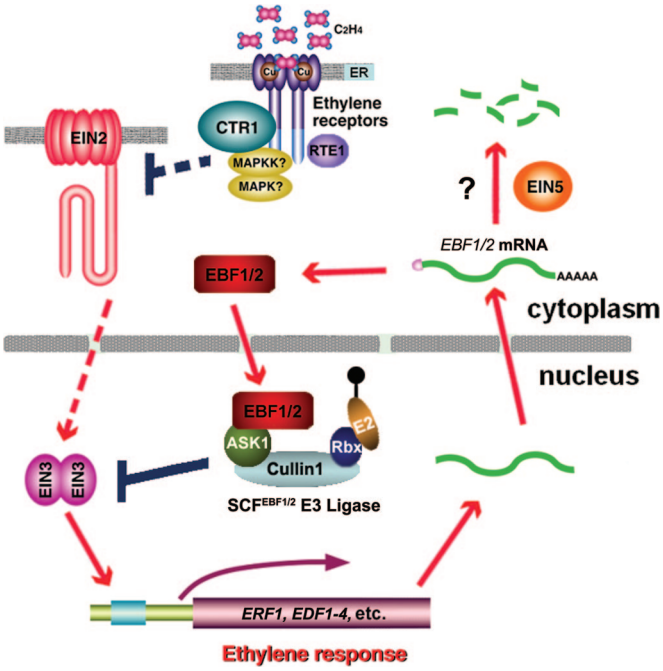

Fig. 6. A model of the role of EIN5 in the ethylene signal transduction pathway. Ethylene (C_2H_4) is perceived by repressing the action of receptor complexes including ETR/ERS/EIN4 receptors, RTE1, and Raf-like protein kinase CTR1, which negatively regulates downstream signaling component EIN2. Upon ethylene treatment, EIN2 is derepressed and could thus transmit the signal into the nucleus to activate a number of transcription factors, including EIN3 and EIL1. EIN3 directly binds to the regulatory elements of target genes and induces the expression of yet other transcription factors (i.e., ERFs and EDFs) that would ultimately regulate a series of ethylene responses. In the absence of ethylene signal, a Skp1-Cullin1-F-box complex consisting of one of two F-box proteins, EBF1 and EBF2, targets EIN3 protein for degradation via an ubiquitin/proteasome pathway. Interestingly, *EBF1*/*EBF2* gene expression is induced by ethylene in an EIN3-dependent manner, which forms a negative feedback regulation on the EIN3 function. EIN5, a 5′→3′ exoribonuclease, is involved in facilitating the turnover of *EBF1*/*EBF2* mRNA through a yet unknown mechanism. Therefore, EIN5 is proposed to antagonize the negative feedback regulation on EIN3 by promoting *EBF1* and *EBF2* mRNA decay, which consequently allows the accumulation of EIN3 protein to trigger the ethylene response. Red arrows and blue bars represent positive and negative regulations, respectively. The dotted lines represent regulatory steps in which a direct physical link between upstream and downstream components has yet to be demonstrated.

marginal induction of these genes. It is interesting to notice that the profile of expression coincides with the phenotypic appearance of the plants with the wild type exhibiting the full triple response, and *ein2* being the extreme, with none of the triple-response features evident (Fig. 1). In *ein5* seedlings there is an intermediate Ein$^-$ phenotypic response to ethylene gas (Fig. 1). Upon cloning *EIN5* we were surprised to find that it codes for the previously described 5′→3′ exoribonuclease XRN4. XRN4/EIN5 has previously been shown to degrade mRNA in a 5′→3′ direction (18, 29). Therefore, this enzyme is likely involved in the mRNA decay pathway in which deadenylation is followed by the hydrolysis of the 5′ cap structure and processive degradation of the mRNA body (30). Specific to the ethylene signaling pathway, we find that XRN4/EIN5 targets for decay the mRNAs of the EIN3-regulating F-box proteins EBF1 and EBF2 through a yet unknown mechanism (Fig. 6). We propose that the function of XRN4/EIN5 in the ethylene signal transduction pathway is likely to antagonize the negative feedback regulation on EIN3 by promoting *EBF1* and *EBF2* mRNA decay, which results in the accumulation of EIN3 protein, triggering the ethylene response (Fig. 6). Analysis of the ethylene-insensitive mutant *ein5* has led us to uncover a previously unknown aspect of the regulation of the ethylene response, namely the participation of an enzyme with known functions in mRNA turnover and RNA interference, XRN4/EIN5 (24, 29, 31).

Although XRN1 is required for many processes in yeast, it was observed that in yeast strains lacking this protein (xrn1Δ) only a small fraction of transcripts are over-accumulated, thus suggesting that 3′→5′ decay, not 5′→3′ decay, may be the major mRNA decay activity in yeast cells (32). In concordance, our analysis carried out with gene expression arrays suggests that 3′→5′ decay, not 5′→3′ decay, may be the major mRNA decay activity in plants. A comparison of the expression data from air-treated seedlings of wild-type Col-0 and *ein5* mutant plants did not reveal a global effect on the abundance of transcripts expressed in plants (data not shown). Additionally, whole-genome tiling array expression profiling also identified a specific class of EIN5 substrates (Fig. 9 and data not shown), including *EBF1* and *EBF2* mRNAs (Fig. 5 *A* and *B*). In concordance with these microarray results, a previous analysis of *ein5* mutant plants versus wild-type Col-0 plants carried out on 8,000 *Arabidopsis* genes identified only 14 genes that seemed to accumulate in *ein5* mutant plants (29). Furthermore, *ein5* mutant plants do not manifest gross developmental defects, but have only slightly serrated leaf edges (Fig. 1*D*). Taken together, these results suggest that XRN4/EIN5 is likely an exonuclease required for the decay of specific target mRNAs, whereas a 3′→5′ activity suffices for general mRNA decay in *Arabidopsis*.

XRN4/EIN5 is a 5′→3′ exoribonuclease with homologs in many eukaryotic organisms (Figs. 3*D* and 7). Interestingly, the closest homolog of XRN4/EIN5 in *Caenorhabditis elegans*, *xrn-1*, is required for proper embryonic development. Loss of this key protein results in embryos that fail to complete ventral enclosure, where the outer layer of cells normally closes over the mesoderm in a purse-string movement (33). Furthermore, *S. cerevisiae* strains containing mutation in *XRN1* manifest pleiotropic defects including meiotic arrest, reduced spore viability, and slow growth (25). Recently, *XRN1* has also been demonstrated to play a role in yeast iron homeostasis (34). In plants, XRN4/EIN5 has already been demonstrated to degrade selected mRNAs including the 3′ products of the endonucleolytic cleavage directed by selected microRNAs (29). Here we further demonstrate that the XRN4/EIN5 exonuclease is a required component of the ethylene signal transduction pathway. Taken together, these results suggest that within plants and other eukaryotic organisms XRN4/EIN5 and its homologs are likely active in many other pathways in which target mRNAs are required for specific function.

Materials and Methods

Plant Lines and Growth Conditions. The Columbia ecotype of *Arabidopsis* was used in this study. Seeds were surface-sterilized as described previously (35), resuspended in a suitable volume of top agar (0.3% low-melt agarose), and spread onto Murashige and Skoog agar [Murashige and Skoog salts (Invitrogen, Carlsbad, CA) and 0.8% agar (pH 5.7)]. Seeds were cold-treated for 4 days (4°C) and then were covered with aluminum foil and moved to an incubator at 24°C. Adult plants were grown in potting soil (Metro Mix 250; Grace-Sierra, Boca Raton, FL) under continuous illumination at 23°C under a 16-h light/8-h dark cycle. The *ein5-2* and *ein5-7* (formerly *ein*7) mutants were previously identified (23). The *ein5-1*, *ein5-2*, and *ein5-7* alleles were isolated from an x-ray-mutagenized population (ecotype Columbia) (17). *ein5-3* and *ein5-4* were screened out from a fast neutron mutagenized population. *ein5-5*, *ein5-6*, and *ein5-11* were recovered from the T-DNA collection of The Salk Institute for Biological Studies (36). *ain1-2* and *ain1-7* come from an ethyl methanesulfonate (EMS)-mutagenized population. Only *ain1-2* was derived from a C24 ecotype. Ethylene treatment of *Arabidopsis* seedlings grown on Petri plates was performed in containers by flowing through hydrocarbon-free air supplemented with 10 ppm ethylene or were treated with hydrocarbon-free air alone (17).

Genetic Analysis of Double Mutants. Double mutants were generated by genetic crosses, and homozygous lines were identified by PCR-based genotyping. *Arabidopsis* seeds were surface-sterilized and plated on the surface of MS medium supplemented with different concentrations of ACC. After 4 days at 4°C the plates were wrapped in foil and kept in a 24°C incubator for 3 days before the phenotypes of seedlings were analyzed.

Northern Blot Analysis. Total RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA), fractionated by electrophoresis on 1.2% agarose/6% formamide gels, and transferred to Hybond nylon membranes (Amersham Biosciences, Piscataway, NJ). Each DNA probe was ^{32}P-labeled by the multiprime labeling system (Amersham Biosciences), and hybridization, stripping, and reprobing were carried out as described (19).

Microarray Experiments. Cold-treated seeds in MS plates were placed in chambers at 24°C in the dark with hydrocarbon-free airflow for 3 days, after which some of the chambers were connected to ethylene gas at 10 ppm while the others remained on air treatment. Four hours later the seedlings of each plate were quickly collected and frozen in liquid nitrogen. Total RNA was prepared from both air-treated and ethylene-treated etiolated seedlings of *Arabidopsis* Col-0, *ein2-5*, and *ein5-1* by using the RNeasy Plant Mini Kit. Biotinylated target RNA was prepared from 16 μg of total RNA by using the procedure described by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, a primer encoding a T7 RNA polymerase promoter fused to (dT)24 (Genset Oligos, La Jolla, CA) was used to prime double-stranded cDNA synthesis using the SuperScript Choice System (Invitrogen). The resulting cDNA was transcribed *in vitro* by using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY) in the presence of biotinylated UTP and CTP to produce biotinylated target cRNA. The labeled target cRNA was purified, fragmented, and hybridized to *Arabidopsis* microarrays (Affymetrix ATH1 gene expression arrays) according to protocols provided by the manufacturer in hybridization oven model 640 (Affymetrix). The arrays were washed and stained with streptavidin–phycoerythrin by using a GeneChip Fluidics Station model 400 and then scanned with a Gene Array Scanner (Hewlett-Packard, Palo Alto, CA). Scanned images were processed and quantified by using GeneChip Suite 3.2. Genespring software (Silicon Genetics, Redwood City, CA) was used to manage and filter the array data. Each measurement was divided by the 50.0th percentile of all measurements in that sample. The percentile was calculated with all normalized measurements above 10. For samples where the bottom 10th percentile was less than the negative of the 50.0th percentile, it was used as a background and subtracted from all of the other genes first. Experiments were carried out in duplicate and hybridized to two sets of expression chips. Expression values were analyzed by using GeneSpring software version 4.2.

For the oligonucleotide tiling array experiments 10-day-old light-grown *Arabidopsis* Col-0 and *ein5-1* seedlings were transferred to a Petri plate containing incubation buffer (1 mM Pipes, 1 mM sodium citrate, 1 mM KCl, and 15 mM sucrose) and maintained in agitation at 75 rpm for 30 min covered with foil. At this point vacuum was applied for exactly 15 s and cordycepin (Sigma Chemical, St. Louis, MO) was added to a final concentration of 150 μg/ml. The seedlings were then incubated for two more hours in the dark at room temperature and kept in agitation at 75 rpm in Petri plates. The seedlings were then briefly blotted on filter paper to dry and kept in liquid nitrogen. Total RNA was extracted from the samples by using the RNeasy plant kit (Qiagen, Valencia, CA). Biotinylated target RNA was prepared from 120 μg of total RNA from each sample by using the GeneChip Expression Analysis system (Affymetrix). Each

sample of the purified and fragmented labeled target cRNA was hybridized to one set of 12 custom-made genome tiling arrays (Affymetrix) that span the entire *Arabidopsis* genome at high resolution (28). Hybridization, washes, and staining were carried out as described above for the Affymetrix array ATH1. Cell files were obtained by using GCOS software (Affymetrix), and ChipViewer software was used to visualize and analyze the tiling chip data (28).

Immunoblot Assays. Immunoblot assays were performed as described (5). In brief, 3-day-old dark-grown seedlings were treated with 10 ppm ethylene or hydrocarbon-free air for 4 h before protein samples were extracted for Western blot analysis. The protein extracts were fractionated by 4–12% gradient Tris-glycine Novex precast gels (Invitrogen) blotted onto a nitrocellulose filter. The blot was probed first with anti-EIN3 antibody and was subsequently stripped with 0.2 N glycine (pH 2.5) three times and reprobed with anti-CRY1 antibody (37).

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1. Johnson, P. R. & Ecker, J. R. (1998) *Annu. Rev. Genet.* **32,** 227–254.
2. Wang, K. L., Li, H. & Ecker, J. R. (2002) *Plant Cell* **14,** Suppl., S131–S151.
3. Ecker, J. R. (1995) *Science* **268,** 667–675.
4. Chen, Y. F., Etheridge, N. & Schaller, G. E. (2005) *Ann. Bot. (London)* **95,** 901–915.
5. Guo, H. & Ecker, J. R. (2003) *Cell* **115,** 667–677.
6. Resnick, J. S., Wen, C. K., Shockey, J. A. & Chang, C. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 7917–7922.
7. Larsen, P. B. & Chang, C. (2001) *Plant Physiol.* **125,** 1061–1073.
8. Larsen, P. B. & Cancel, J. D. (2003) *Plant J.* **34,** 709–718.
9. Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C. & Genschik, P. (2003) *Cell* **115,** 679–689.
10. Gagne, J. M., Smalle, J., Gingerich, D. J., Walker, J. M., Yoo, S. D., Yanagisawa, S. & Vierstra, R. D. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 6803–6808.
11. Nehring, R. & Ecker, J. R. (2004) in *Plant Hormones: Biosynthesis, Signal Transduction, Action!*, ed. Davies, P. J. (Kluwer, Dordrecht), pp. 350–368.
12. Chang, C. & Stadler, R. (2001) *Bioessays* **23,** 619–627.
13. Chang, C. & Bleecker, A. B. (2004) *Plant Physiol.* **136,** 2895–2899.
14. Hirayama, T., Kieber, J. J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J. M., Dailey, W. P., Dancis, A. & Ecker, J. R. (1999) *Cell* **97,** 383–393.
15. Woeste, K. E. & Kieber, J. J. (2000) *Plant Cell* **12,** 443–455.
16. Barry, C. S. & Giovannoni, J. J. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 7923–7928.
17. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. (1993) *Cell* **72,** 427–441.
18. Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. & Ecker, J. R. (1999) *Science* **284,** 2148–2152.
19. Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. & Ecker, J. R. (1997) *Cell* **89,** 1133–1144.
20. Alonso, J. M., Stepanova, A. N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F. M. & Ecker, J. R. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 2992–2997.
21. Solano, R., Stepanova, A., Chao, Q. & Ecker, J. R. (1998) *Genes Dev.* **12,** 3703–3714.
22. Yanagisawa, S., Yoo, S. D. & Sheen, J. (2003) *Nature* **425,** 521–525.
23. Roman, G., Lubarsky, B., Kieber, J. J., Rothenberg, M. & Ecker, J. R. (1995) *Genetics* **139,** 1393–1409.
24. Kastenmayer, J. P. & Green, P. J. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 13985–13990.
25. Larimer, F. W. & Stevens, A. (1990) *Gene* **95,** 85–90.
26. Kastenmayer, J. P., Johnson, M. A. & Green, P. J. (2001) *Methods Enzymol.* **342,** 269–282.
27. Redman, J. C., Haas, B. J., Tanimoto, G. & Town, C. D. (2004) *Plant J.* **38,** 545–561.
28. Yamada, K., Lim, J., Dale, J. M., Chen, H., Shinn, P., Palm, C. J., Southwick, A. M., Wu, H. C., Kim, C., Nguyen, M., *et al.* (2003) *Science* **302,** 842–846.
29. Souret, F. F., Kastenmayer, J. P. & Green, P. J. (2004) *Mol. Cell* **15,** 173–183.
30. Meyer, S., Temme, C. & Wahle, E. (2004) *Crit. Rev. Biochem. Mol. Biol.* **39,** 197–216.
31. Gazzani, S., Lawrenson, T., Woodward, C., Headon, D. & Sablowski, R. (2004) *Science* **306,** 1046–1048.
32. He, F., Li, X., Spatrick, P., Casillo, R., Dong, S. & Jacobson, A. (2003) *Mol. Cell* **12,** 1439–1452.
33. Newbury, S. & Woollard, A. (2004) *RNA* **10,** 59–65.
34. Lee, A., Henras, A. K. & Chanfreau, G. (2005) *Mol. Cell* **19,** 39–51.
35. Vogel, J. P., Woeste, K. E., Theologis, A. & Kieber, J. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4766–4771.
36. Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., *et al.* (2003) *Science* **301,** 653–657.
37. Lin, C., Ahmad, M., Gordon, D. & Cashmore, A. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8423–8427.