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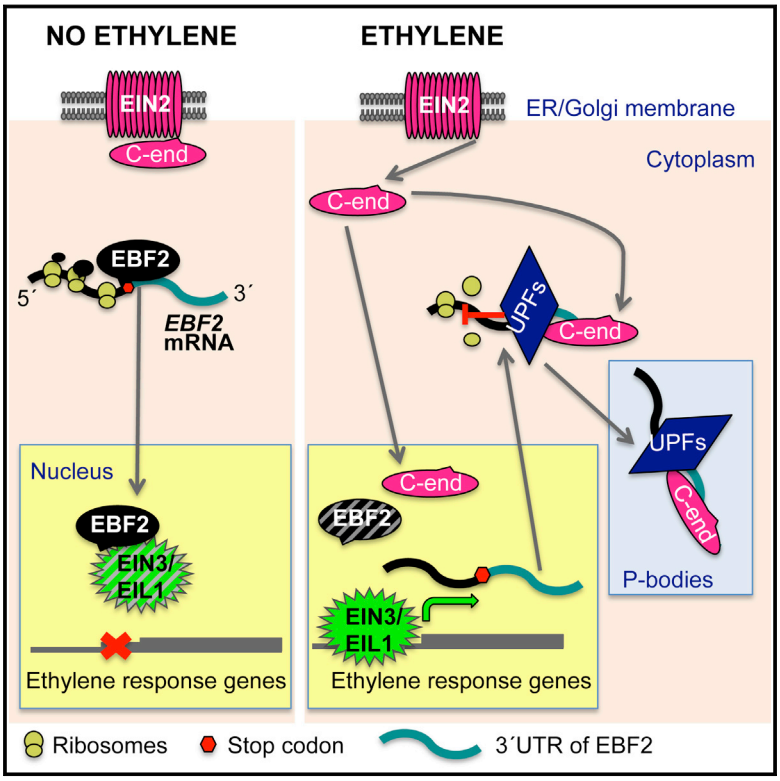
Gene-Specific Translation Regulation Mediated by the Hormone-Signaling Molecule EIN2

Graphical Abstract



Highlights

- Ribosome footprinting uncovers a role of translation in the ethylene response
- The *EBF2* 3′UTR is sufficient to confer translational control
- Regulation of *EBF2* translation is required for proper ethylene responses
- *EBF2* translation control depends on functional EIN2 and UPFs, but not EIN3/EIL1

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In Brief

Ribosome footprinting unveils gene-specific regulation of translation by the hormone ethylene involving the 3′UTR of the transcript of a known negative regulator, as well as a key ethylene signaling protein and the components of the nonsense-mediated decay machinery.

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Gene-Specific Translation Regulation Mediated by the Hormone-Signaling Molecule EIN2

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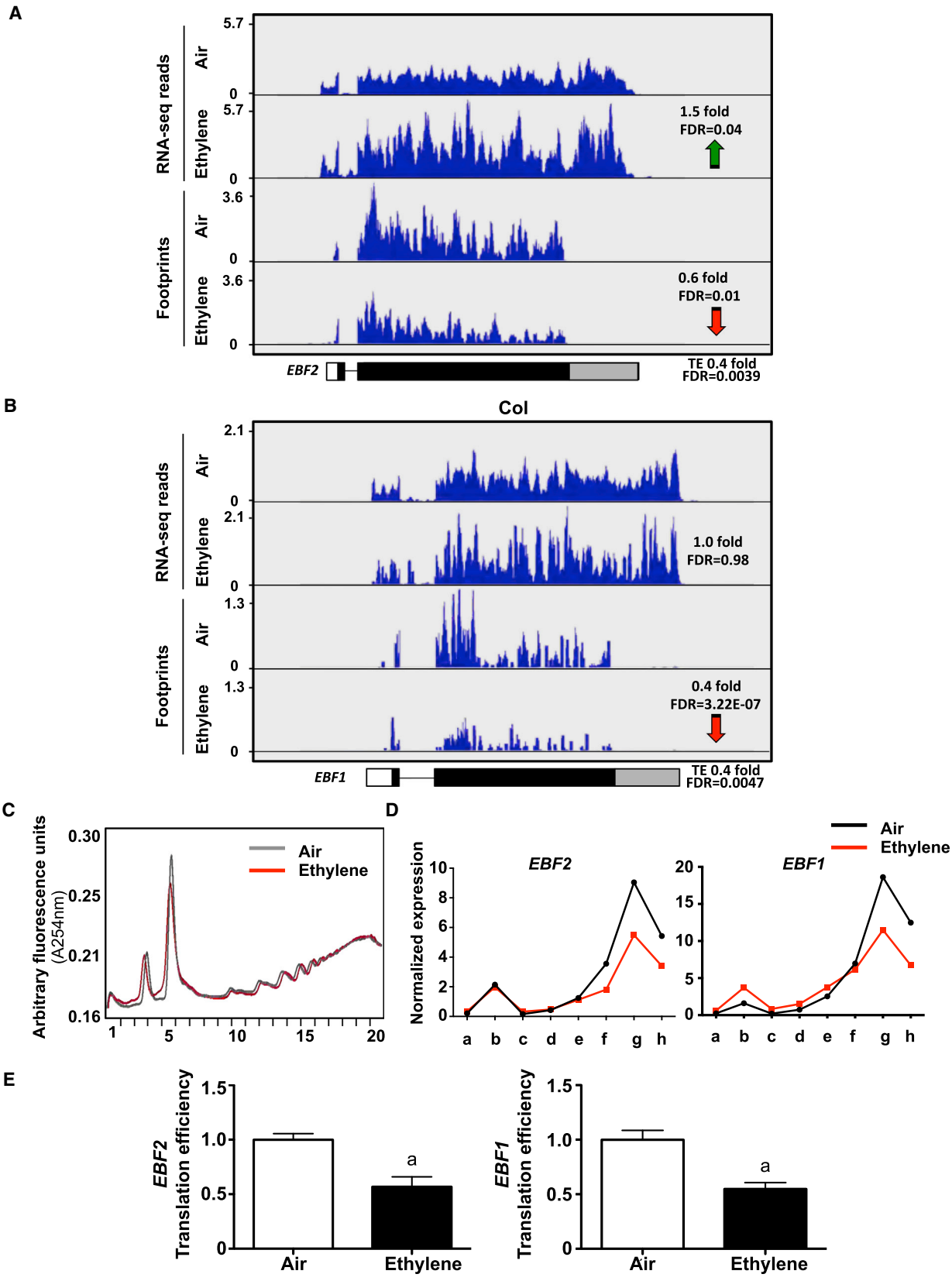
SUMMARY

The central role of translation in modulating gene activity has long been recognized, yet the systematic exploration of quantitative changes in translation at a genome-wide scale in response to a specific stimulus has only recently become technically feasible. Using the well-characterized signaling pathway of the phytohormone ethylene and plant-optimized genome-wide ribosome footprinting, we have uncovered a molecular mechanism linking this hormone's perception to the activation of a gene-specific translational control mechanism. Characterization of one of the targets of this translation regulatory machinery, the ethylene signaling component *EBF2*, indicates that the signaling molecule EIN2 and the nonsense-mediated decay proteins UPFs play a central role in this ethylene-induced translational response. Furthermore, the 3′UTR of *EBF2* is sufficient to confer translational regulation and required for the proper activation of ethylene responses. These findings represent a mechanistic paradigm of gene-specific regulation of translation in response to a key growth regulator.

INTRODUCTION

The plant hormone ethylene plays a central role in coordinating the multitude of molecular processes underlying developmental programs and environmental responses critical for plant survival (Abeles et al., 1992). The plant's response to ethylene is initiated by the binding of this hormone to its cognate receptors—in *Arabidopsis*, a small family of five proteins (ETR1, ETR2, ERS1, ERS2, and EIN4) with sequence similarity to the bacterial two-component histidine kinases (Bleecker et al., 1988; Hua and Meyerowitz, 1998). Although some specialization has been recognized for the receptors, they are all thought to function primarily by modulating the activity of the rapidly accelerated fibrosarcoma (RAF)-like kinase CTR1 (Clark et al., 1998). Inactivation of this kinase in the presence of ethylene results in a reduction in the phosphorylation levels of the endoplasmic-reticulum-localized transmembrane protein EIN2 and cleavage and translocation of the unphosphorylated C terminus of EIN2 (EIN2C) to the nucleus (Ju et al., 2012; Qiao et al., 2012). Downstream of EIN2, two different responses have been characterized. On the one hand, there is a rapid inhibition of growth that takes place within minutes of exposure to the hormone and does not involve the key transcriptional regulators EIN3 and EIL1 (Binder et al., 2004). On the other hand, there are many other, and possibly slower, changes induced by this hormone, including transcript level alterations in hundreds of genes that do require the function of these two transcriptional regulators (Binder et al., 2004; Chang et al., 2013). In contrast with the lack of information on the molecular mechanism behind the fast growth-inhibition response, all EIN3/EIL1-dependent responses are activated by the aforementioned translocation of the unphosphorylated EIN2C to the nucleus. Preventing this translocation stops the activation of EIN3/EIL1 (Qiao et al., 2012). The F-box proteins ETP1/ETP2 and EBF1/EBF2 control EIN2 and EIN3 protein abundance, respectively (Guo and Ecker, 2003; Potuschak et al., 2003; Qiao et al., 2009). Interestingly, *EBF2* itself is a direct transcriptional target of EIN3 (Konishi and Yanagisawa, 2008), suggesting the existence of a feedback regulatory loop that quickly dampens EIN3 activity shortly after activating this signaling cascade. The critical importance of the EIN3 regulation by the EBFs is further substantiated by the observation that EBF2 protein levels are also modulated by an unknown EIN2-dependent mechanism (He et al., 2011). Finally, a P-body-localized 5′-3′ exoribonuclease EIN5 (also known as XRN4) has also been implicated in the regulation of the *EBF2* activity (Olmedo et al., 2006; Potuschak et al., 2006; Souret et al., 2004; Weber et al., 2008).

Using a plant-optimized ribosome footprinting approach, we show that ethylene affects translation of several genes, among them the *EBF*s. The translational regulation of *EBF2* is mediated by its long 3′ UTR and requires the activity of the ethylene signaling components *EIN2* and *EIN5* and the nonsense-mediated decay proteins *UPF*s, but not that of the ethylene transcriptional master regulators *EIN3*/*EIL1*. EIN2C can interact with the 3′UTR of *EBF2* and localizes to P-bodies. These findings not only provide direct evidence for the translation regulation of



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specific genes in response to this hormone but also the conceptual framework to decipher the molecular mechanism of a previously proposed branch of ethylene signaling.

RESULTS

Ribosome Footprinting Unveils a New Translation-Based Branch of the Ethylene Response

To probe the effects of ethylene on translation at the whole-genome level, we implemented the ribosome footprinting technology, Ribo-seq, which allows for capturing the ribosomal load of expressed genes in the genome at a single-codon resolution (Ingolia et al., 2009). Using Ribo-seq, we looked for ethylene-triggered changes in translation rates that could not be explained by changes in transcript levels.

Total mRNA and ribosome footprint analyses were carried out in parallel to identify changes in translation efficiency in response to ethylene (Figure S1A) (see the Supplemental Experimental Procedures). A 4 hr ethylene treatment was selected to capture robust early responses and to avoid secondary long-term effects of this hormone. The high quality of the Ribo-seq data (Ingolia et al., 2009) is evidenced by the abrupt appearance of a footprint signal 14–15 nt upstream of the start codon, a rapid decline in signal around 14–15 nt upstream of the stop codon, low density of footprints in the 5′ and 3′ UTR, and a strong 3 nt periodicity (Figures S1B–S1D), which represents the codon-long stepwise movement of the ribosome along the mRNA. None of these features were observed in the RNA sequencing (RNA-seq) libraries (Figures S1B–S1D).

Ethylene induced global mRNA level changes (Figure S1E and Table S1) that were followed by concomitant alterations in the levels of translation (Figures S1E and S1F and Table S1). However, in agreement with previous comparisons between protein and RNA levels (de Godoy et al., 2008; Ingolia et al., 2009), the correlation between the changes in transcript accumulation and translation levels was relatively poor, with an r^2 value of 0.22 (Figure S1F), suggesting the existence of a layer of regulation at the translational level. In fact, we identified several mRNAs affected by ethylene in their translational efficiency (Table S1). Importantly, two key ethylene signaling genes, *EBF1* and *EBF2*, were found in this list of translationally regulated genes (Table S1). *EBF1* and *EBF2* encode F-box proteins involved in the degradation of EIN3/EIL1 in the absence of ethylene. In prior studies, the EBF protein levels have been shown to decrease after ethylene treatment (Guo and Ecker, 2003; Potuschak et al., 2003), although the transcript levels of at least *EBF2* are known to increase in response to this hormone (Konishi and Yanagisawa, 2008). After 4 hr of exposure to ethylene, and coinciding with previous reports, we observed an ~1.5-fold increase in the *EBF2* mRNA, yet a surprising 2.8-fold decrease in its translation efficiency (TE) (Figure 1A and Table S1). Likewise, we observed a reduction in the TE of *EBF1* (Figure 1B) and several other genes (Figure S2 and Table S1). These ethylene effects on the translation of *EBF1* and *EBF2* were further supported by the reduction of the relative levels of these mRNAs in the heavy fractions of a polysome profile (Figures 1C and 1D). To further validate these findings, the ethylene effects on TE of six selected genes, including *EBF1*, *EBF2*, and a negative control, *RTE1*, were evaluated by calculating the ratio between the expression level of these genes in polysomal and total mRNA (Figures 1E and S2D). Although this approach is not as sensitive at detecting changes in the ribosomal load of an mRNA as are Ribo-seq or ribosome profiling, it can accurately quantify alterations in the ratio of the mRNA subpopulations that are actively engaged in translation versus those populations that are non-translating. The TE of *EBF1* and *EBF2* in ethylene decreased nearly to half of that in air (Figure 1E), confirming the results of Ribo-seq (Figures 1A and 1B) and polysome profiling (Figures 1C and 1D). Similarly, the TE of the other three selected genes was also repressed by ethylene, whereas no effect was detected for *RTE1*, a transcriptionally induced negative control (Figure S2D and Table S1). Together, these results suggest that the multitude of responses triggered by the hormone ethylene is the result of regulation of gene expression not only at the transcriptional level as shown previously (Chang et al., 2013) but also at the translational level. These changes in translation are likely due to shifts in the equilibrium of translated and non-translated populations of target mRNAs rather than quantitative alterations in the translation rates of individual transcripts. These findings also reveal that, as in the case of the transcriptional regulation, some of the components of the ethylene signal transduction pathway are themselves subject to ethylene-triggered translational regulation, raising the possibility of intricate feedback regulatory loops functioning in this signaling pathway.

The 3′UTR of *EBF2* Is Sufficient to Confer Ethylene-Mediated Regulation of Translation and Is Required for Proper Plant Responses to This Hormone

Since EBF2 is a key negative regulator of ethylene signaling (Guo and Ecker, 2003; Potuschak et al., 2003), we reasoned that the observed translational repression of this gene may have a significant physiological effect. Although translation regulatory

Figure 1. Translation of *EBF2* and *EBF1* Is Quickly Downregulated by Ethylene

(A and B) Normalized distribution of RNA-seq and Ribo-seq reads in air and in ethylene along the *EBF2* (A) and *EBF1* (B) genes.

5′ UTR, coding DNA sequence (CDS), 3′ UTR, and introns are marked as white, black, and gray boxes and a line, respectively. The fold change and the associated false discovery rate (FDR) for the ethylene effect on transcript and footprint levels, as well as the fold change in the footprint levels given the levels of mRNA (TE) and the corresponding FDR, are shown.

(C) 10%–50% sucrose gradient absorbance (A_{254}) profiles of ribosome complexes obtained from *Arabidopsis* seedlings grown in air and/or 4 hr ethylene.

(D) Polysomal distribution of *EBF* transcripts in air and 4 hr ethylene. a–h correspond to fractions 4+5 through 18+19 shown in (C) pooled in pairs. *EBF* mRNA levels were normalized against *At4g34270*. *EBF* expression in each polysomal fraction was calculated as the percentage of its expression in total RNA.

(E) TE of the *EBF2* and *EBF1* mRNAs, calculated as their relative expression in polysomal/total RNA fractions, in seedlings grown in air or treated with 10 ppm of ethylene for the last 4 hr of the experiment. Expression levels of *EBFs* were normalized against *At4g34270*. (a) indicates a significant difference of the ethylene effect on the *EBF* TE (t test, $p < 0.05$). Bars represent means ± SEM for three biological replicates.

3-day-old etiolated seedlings were used in all of the experiments.

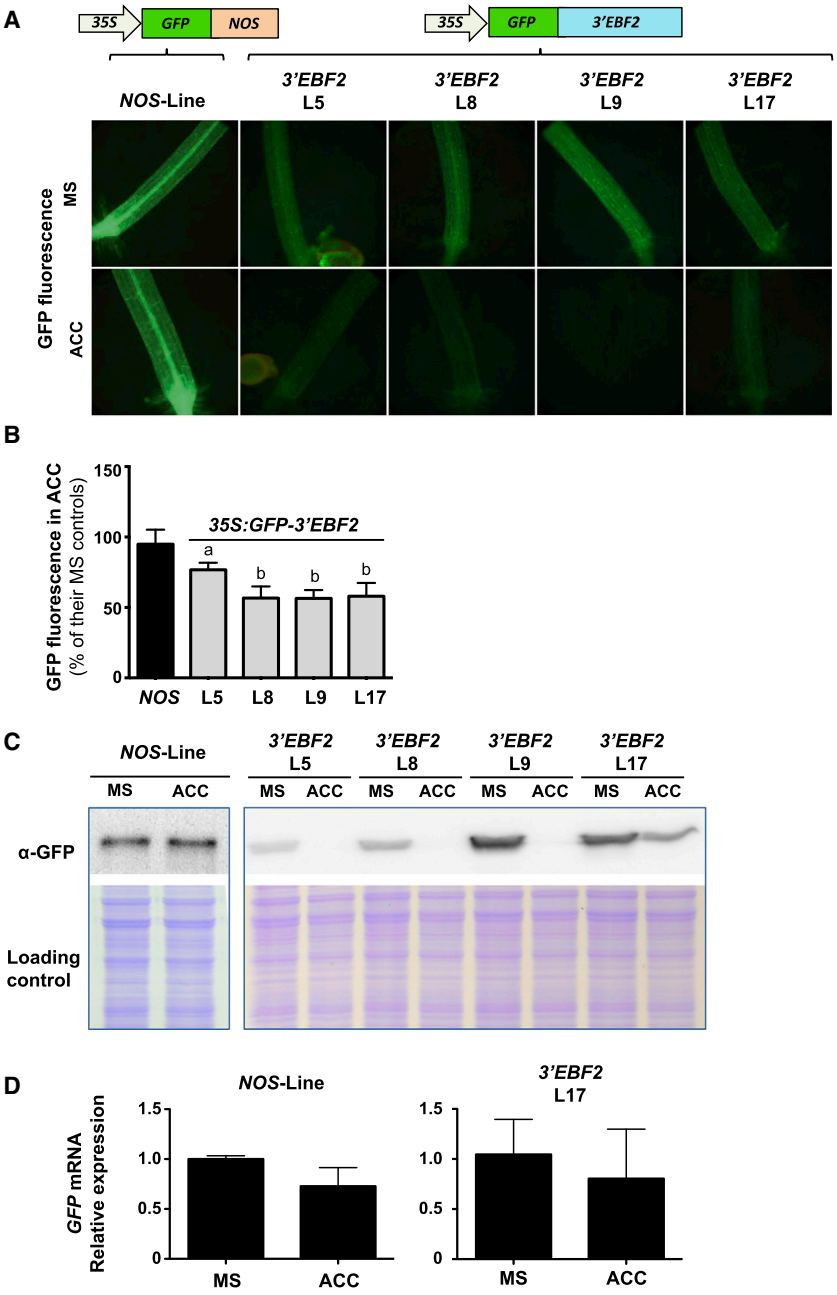

Figure 2. The *3′EBF2* Is Sufficient to Confer Ethylene-Mediated Regulation of Translation

(A and B) (A) Hypocotyl fluorescence and (B) its quantification (n = 15) in 3-day-old etiolated seedlings grown in the presence (ACC) or absence (MS) of the ethylene precursor ACC and harboring either the *35S:GFP-NOS* or the *35S:GFP-3′EBF2* constructs as depicted on top of the photos. GFP fluorescence is expressed as the % of fluorescence in ACC compared to that in MS controls. Bars represent means ± SD. a and b indicate a significant effect of the ethylene treatment on the levels of fluorescence (t test, $p < 0.005$ and $p < 0.0001$, respectively).

(C) Anti-GFP western blot of total protein extracts from the transgenic lines shown in (A).

(D) Relative expression of *GFP* mRNA from two selected lines from (A). Bars represent means ± SEM for three biological replicates. Expression levels of the *EBF2* transgenes were normalized against *At5g44200*.

3-day-old etiolated seedlings were used in all of the experiments.

elements can be located in both 5′ and 3′ UTRs (Szostak and Gebauer, 2013), we decided to investigate the potential regulatory role of the atypically large 590-bp-long 3′ UTR of *EBF2* (*3′EBF2*) first, as it has previously been implicated in modulating the activity of this gene and prior efforts to determine the mechanism of such regulation via changes in *EBF2* mRNA stability were not conclusive (Potuschak et al., 2006). *GFP* reporter was fused to either *3′EBF2* or the *NOS* terminator and placed under the control of the constitutive *35S* promoter (Figure 2A). The effect of ethylene on the GFP fluorescence of stably transformed wild-type plants was examined under the standard ethylene triple response assay conditions. While the transgenic lines with the *NOS* terminator alone were equally fluorescent in control media and in media supplemented with the ethylene precursor ACC, the *3′EBF2* lines showed a strong reduction in the levels of fluorescence in ACC (Figures 2A and 2B). Western blot with an anti-GFP antibody confirmed that the observed decrease in fluorescence in the latter was due to a reduction in the amount of GFP protein (Figure 2C), whereas the *NOS* terminator line showed equal amounts of GFP protein in the presence and absence of ACC. As expected, we observed a range of GFP protein levels in different transgenic lines, and in general, lower levels were found in the *3′EBF2* lines even in the absence of exogenous ethylene, observations that likely resulted from positional effects and endogenous ethylene. To further demonstrate that this ethylene effect in the *3′EBF2* lines was due to changes at the level of protein translation rather than transcription or mRNA stability, the *GFP* mRNA was quantified by qRT-PCR. The differences in the GFP protein accumulation could not be explained by an ethylene-mediated effect on the mRNA levels (Figure 2D), which is consistent with previous reports that did not detect an effect of ethylene on the mRNA stability of *EBF*s (Potuschak et al., 2006).

To better understand the role of *3′EBF2* in mediating the observed ethylene effect on translation, we took a complementary approach utilizing previously generated transgenic lines (Konishi and Yanagisawa, 2008). In these lines, the *ebf2* mutant is complemented with either a native genomic construct of *EBF2* or a similar construct in which *3′EBF2* was replaced by the *NOS* terminator (Figure 3A). Transgenic lines complemented with the native genomic construct showed a clear reduction in the TE of

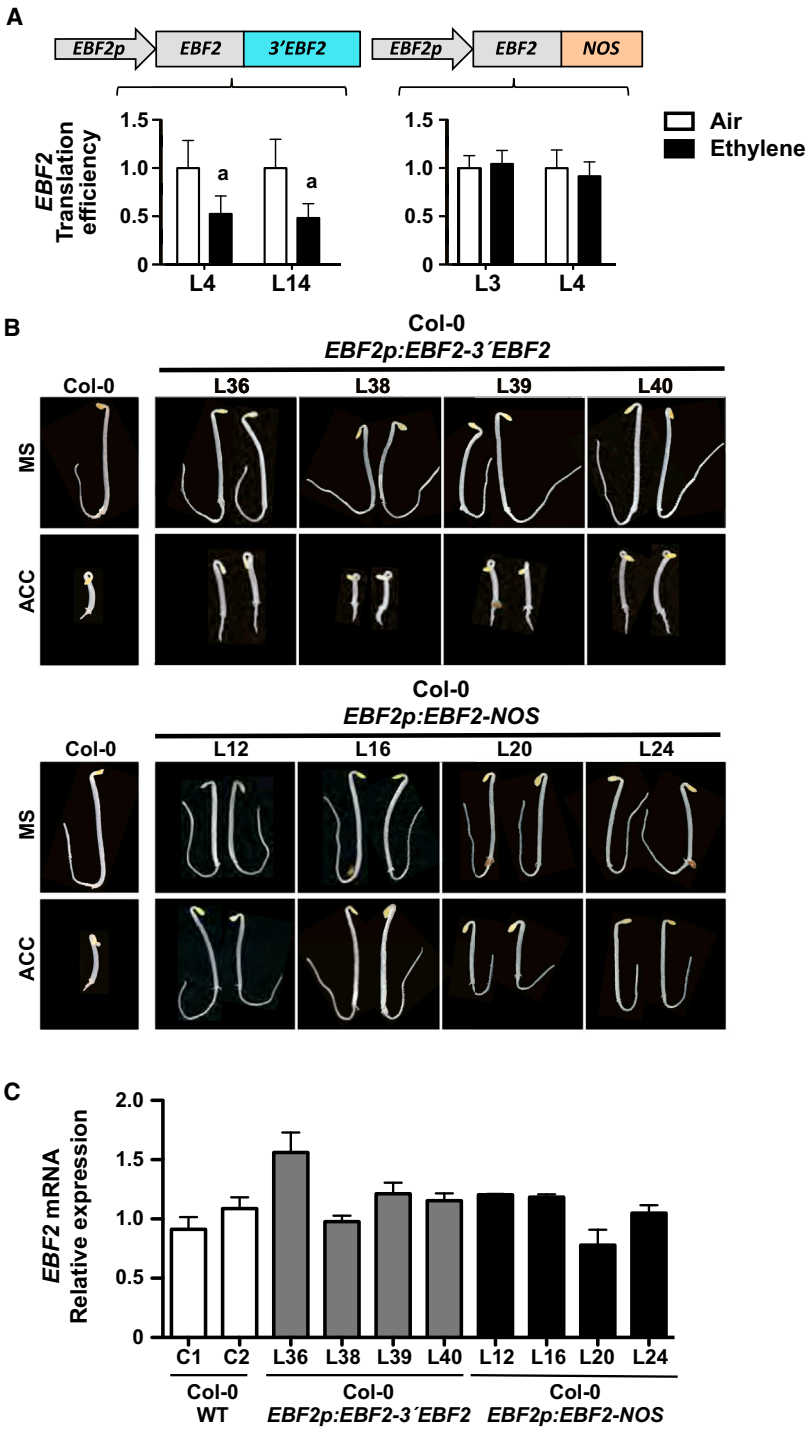

Figure 3. The *3′EBF2* Is Required for the Proper Translation of *EBF2* and Plant Response to Ethylene

(A) TE of the *EBF2* mRNA, calculated as the relative expression in polysomal/total RNA fractions, in *ebf2* seedlings complemented with *EBF2p:EBF2-3′EBF2* and *EBF2p:EBF2-NOS* constructs grown in air or treated with 10 ppm of ethylene for the last 4 hr of the experiment. (a) indicates a significant difference of the ethylene effect in the different genotypes (ANOVA, $p < 0.01$). Bars represent means ± SEM for three biological replicates. Expression levels of the *EBF2* transgenes were normalized against *At4g34270*.

(B) Representative images of 3-day-old etiolated seedlings of the indicated genotypes grown in control media (MS) or in the presence of 10 μM ACC (ACC).

(C) *EBF2* mRNA expression levels in 3-day-old etiolated seedlings of the same genotypes as shown in (B) normalized against *At5g44200* and expressed relative to the average of Col-0 plants.

Error bars represent means ± SEM for three technical replicates.

EBF2 (Figure 3A), equivalent to that of the native *EBF2* in wild-type plants (Figure 1). However, ethylene had no effect on the TE of *EBF2* in the *NOS* terminator lines (Figure 3A). Taken together, these results demonstrate that *3′EBF2* is sufficient to confer ethylene-mediated translational regulation.

We re-examined the ethylene response of the aforementioned *ebf2* lines expressing either the native *EBF2* genomic construct or the corresponding control with the *3′EBF2* replaced by the *NOS* terminator (*EBF2-NOS* lines). As previously reported (Konishi and Yanagisawa, 2008), the lines with the native *3′EBF2* showed normal ethylene response in the classical triple response assay, whereas the *NOS* terminator lines showed strong ethylene insensitivity (Figure S3A). Although previously these phenotypes were attributed to the slightly elevated levels of *EBF2* mRNA in the *NOS* terminator lines (Konishi and Yanagisawa, 2008), our results suggested that the ethylene insensitivity of these lines could also be due to the loss of the 3′ UTR-mediated translational repression of *EBF2* by ethylene (Figure 3A). To distinguish between these two possibilities, we generated additional transgenic lines in a wild-type genetic background using either the native *EBF2* or the *EBF2-NOS* terminator constructs. As shown in Figures 3B and 3C, no correlation between the ethylene phenotype and the levels of *EBF2* mRNA was observed (Figures 3B and 3C). The biological significance of the regulatory role of *3′EBF2* was further supported by the observation that plants expressing *GFP-3′EBF2* under the strong *35S* promoter displayed mild ethylene insensitivity (Figure S3B). These results suggest that the presence of high levels of *3′EBF2* can interfere with the molecular machinery responsible for the translational repression of the endogenous *EBF2* mRNA. Taken together, the findings described above strongly support the idea that the translational regulation conferred by *3′EBF2* is critical for the proper function of the ethylene signaling pathway and the plant response to this hormone.

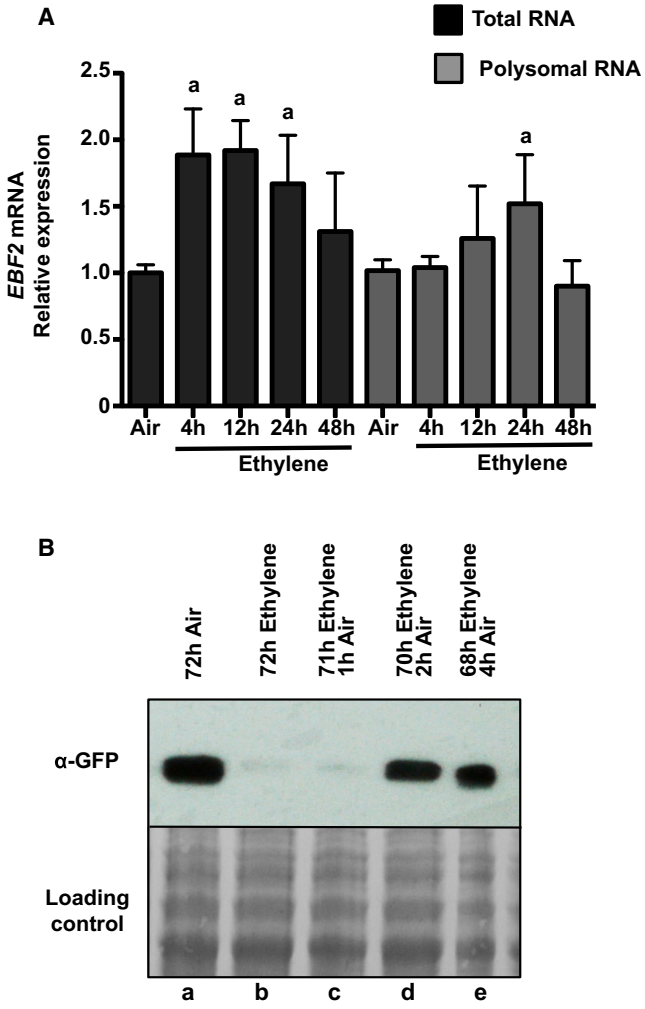

Figure 4. *EBF2* Displays Complex Transcriptional and Translational Dynamics in Response to Ethylene

(A) Relative expression levels of *EBF2* mRNA in total and polysomal RNA fractions from 3-day-old etiolated Col-0 seedlings during a time-course treatment using 10 ppm of ethylene. (a) indicates significant difference between that time point and the corresponding "Air" control (t test, $p < 0.05$). Bars represent means ± SEM for three biological replicates. Expression levels of *EBF2* were normalized against *At4g34270*.

(B) Anti-GFP western blot in *35S:GFP-3′EBF2* of total protein extracts from 3-day-old etiolated seedlings during a time-course ethylene withdrawal experiment.

The Dynamics of Transcriptional and Translational Regulation of *EBF2* Shed New Light on Molecular Mechanisms of the Ethylene Response Kinetics

Our results regarding the opposite effects of a short ethylene exposure on *EBF2* expression at the transcriptional and translational level, together with the known role of *EBF2* in the control of *EIN3* activity, suggest existence of a regulatory mechanism involved in the dynamic aspects of the ethylene response. To investigate the role of transcriptional and translational regulation of *EBF2* in the observed kinetics of the ethylene response, we examined by qRT-PCR the levels of *EBF2* mRNA in both total and polysomal RNA fractions at different times after initiating the ethylene treatment. In agreement with previous reports (Chang et al., 2013), the levels of *EBF2* mRNA quickly increased, reaching the highest expression in the total RNA sample 4 hr after the beginning of the ethylene treatment, staying high at the 12 hr time point and slowly decreasing thereafter (Figure 4A). In contrast, polysomal *EBF2* mRNA remained low for the first 4 hr (Figure 4A), despite the high levels of total *EBF2* mRNA. Lack of efficient translation of *EBF2* (a negative regulator of ethylene responses that targets EIN3 for degradation) thus allows for a full-scale ethylene response in early stages of exposure of plants to ethylene. Interestingly, this period of low EBF2 accumulation coincides with the previously reported maximum in EIN3 activity (Chang et al., 2013). Only after a prolonged ethylene exposure (12 hr to 24 hr) did we observe an increase in *EBF2* mRNA accumulation in the polysomal fraction (Figure 4A), which correlates with the previously described decrease in EIN3 activity (Chang et al., 2013) and coincides with a parallel decline in the total mRNA levels of *EBF2* (Figure 4A) (Chang et al., 2013). Thus, the attenuation of the ethylene response under continuous exposure to this hormone is preceded by an increase in the mRNA levels of *EBF2* in the polysomal fraction (Figure 4A), suggesting that the dynamic balance between transcriptional and translational activity of *EBF2* plays a critical role in diminishing the ethylene response upon prolonged exposure to the hormone.

To examine the reversibility of the ethylene effect on translation, we performed a time-course recovery experiment using the *p35S:GFP-3′EBF2* lines (Figure 4B) that can monitor the ethylene effect specifically on translation—i.e., in the absence of transcriptional regulation. We compared the accumulation of the GFP protein in seedlings grown in air, exposed to ethylene for the entire duration of the experiment (72 hr), or exposed to ethylene for 71, 70, or 68 hr and then allowed to recover in the absence of the hormone for the last 1, 2, or 4 hr of the total 72-hr-long experiment, respectively (Figure 4B). As shown in Figure 4B, in spite of the attenuation process described above, ethylene was able to nearly completely suppress the translation of the *3′EBF2*-containing mRNA expressed under a strong constitutive promoter even after 72 hr of constant exposure to the hormone. Importantly, the protein levels of GFP rapidly increased after ethylene was removed, reaching maximum levels just 2 hr after the withdrawal of ethylene (Figure 4B). These results support the idea that the translation regulation conferred by *3′EBF2* plays a role in re-establishing homeostasis upon removal of ethylene. Importantly, the analysis of the *ebf2* mutant has previously implicated this gene in the resumption of growth after ethylene withdrawal (Binder et al., 2007), further supporting the physiological significance of the observed translation dynamics of this gene.

The Ethylene-Triggered Regulation of Translation of *EBF2* mRNA Is *EIN2* Dependent but *EIN3/EIL1* Independent

To determine which canonical components of this hormone signaling pathway are required to mediate the translational regulation of *EBF2* mRNA, we examined the expression of the *35S:GFP-3′EBF2* construct in the strong ethylene signaling mutants *ein2-5* and *ein3-1 eil1-1* (Figure 5). The GFP fluorescence of

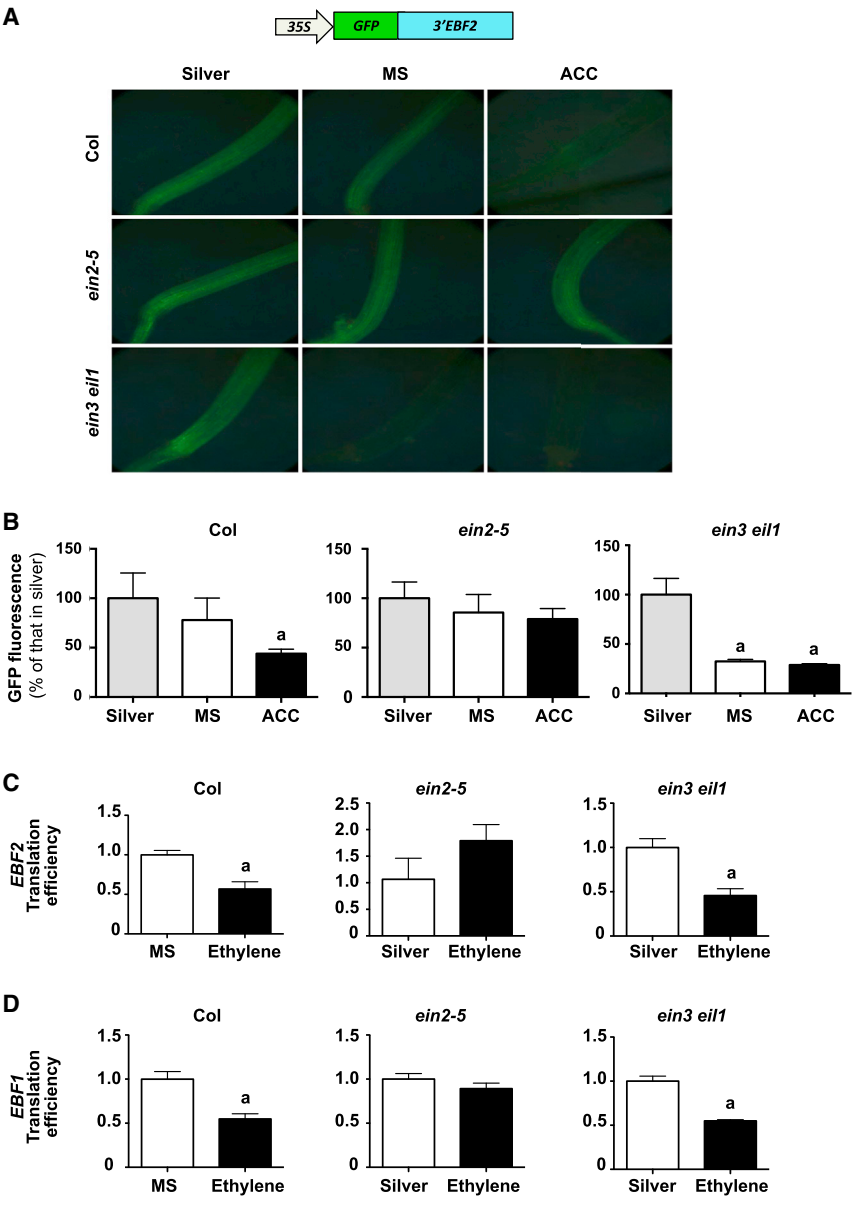

Figure 5. The Ethylene-Dependent Regulation of Translation of *EBF2* and *EBF1* Is *EIN2* Dependent but *EIN3/EIL1* Independent

(A–D) (A) Hypocotyl fluorescence, (B) its quantification, and (C and D) TE of the endogenous *EBF2* (C) and *EBF1* (D) mRNA in 3-day-old etiolated Col-0, *ein2-5*, and *ein3-1 eil1-1* seedlings harboring *35S:GFP-3′EBF2* and grown in 5 mg/l silver, air, or in 10 μM ACC.

(B and C) GFP fluorescence (B) was quantified across multiple seedlings (n = 7) and expressed as the percentage of fluorescence in ACC compared to that in silver controls. Error bars in (B) represent means ± SD. (a) indicates a significant effect of the ethylene treatment on the levels of fluorescence (one-way ANOVA, $p < 0.0001$). TE was calculated as the relative expression in polysomal/total RNA fractions. Col graphs are the same as those shown in Figure 1, plotted here again to facilitate their comparison with the mutants. (a) indicates a significant difference of the ethylene effect on the *EBF2* mRNA TE (t test, $p < 0.05$). Error bars in (C) and (D) represent means ± SEM for three technical replicates. Expression levels of the *EBF* transgenes were normalized against *At4g34270*.

wild-type, *ein2*, and *ein3 eil1* seedlings homozygous for the transgene was examined (Figures 5A and 5B). A silver-treated control was included to mitigate the effect of elevated endogenous levels of ethylene in *ein2* and *ein3* mutants (Guzmán and Ecker, 1990; Vandenbussche et al., 2012). While ethylene had a dramatic effect on the levels of GFP fluorescence in the wild-type plants, we did not observe any changes in the GFP intensity in the *ein2* plants treated either with silver or with the ethylene precursor ACC (Figures 5A and 5B). Surprisingly, the levels of fluorescence in the *ein3 eil1* double mutant were clearly affected by ethylene (Figures 5A and 5B). GFP fluorescence in this mutant was high in silver (where the effect of endogenous ethylene was suppressed) but dramatically decreased in plants grown in ACC-supplemented or un-supplemented media (where the high levels of endogenous ethylene were sufficient to trigger a full response). These results indicate that, while the function of *EIN2* is required for the *3′EBF2*-mediated ethylene-triggered regulation of translation, *EIN3/EIL1* are not.

Next, we investigated the effects of the *ein2* and *ein3 eil1* mutants on the TE of the endogenous *EBF2* and *EBF1* in plants grown in silver- or ACC-supplemented media (Figures 5C and 5D). In full agreement with the results obtained for the GFP fluorescence of the *35S:GFP-3′EBF2* reporter construct, the TE of *EBF*s did not significantly change in the presence of silver or ACC in the *ein2* mutant, but a robust reduction in TE typically observed in wild-type plants in response to ethylene was also seen in the *ein3 eil1* mutant (Figures 5C and 5D).

To determine the molecular mechanism by which *EIN2* mediates translational repression of the *EBF2* mRNA, both a miRNA-based and a protein/RNA interaction-based mechanisms were considered. A negative outcome of prior efforts to elucidate the possible regulation of *EBF2* by miRNA (Souret et al., 2004), together with the lack of known or predicted miRNA in the *Arabidopsis* genome likely to target *EBF2* (Alves et al., 2009), made us disfavor the miRNA possibility. Nevertheless, we decided to examine the ethylene response of the strong small RNA biogenesis mutant *dcl2-1 dcl3-1 dcl4-2* (Henderson et al., 2006). Consistent with the idea that small RNAs are not involved in the regulation of translation of *EBF2*, the mutant displayed wild-type level of ethylene sensitivity in the standard triple

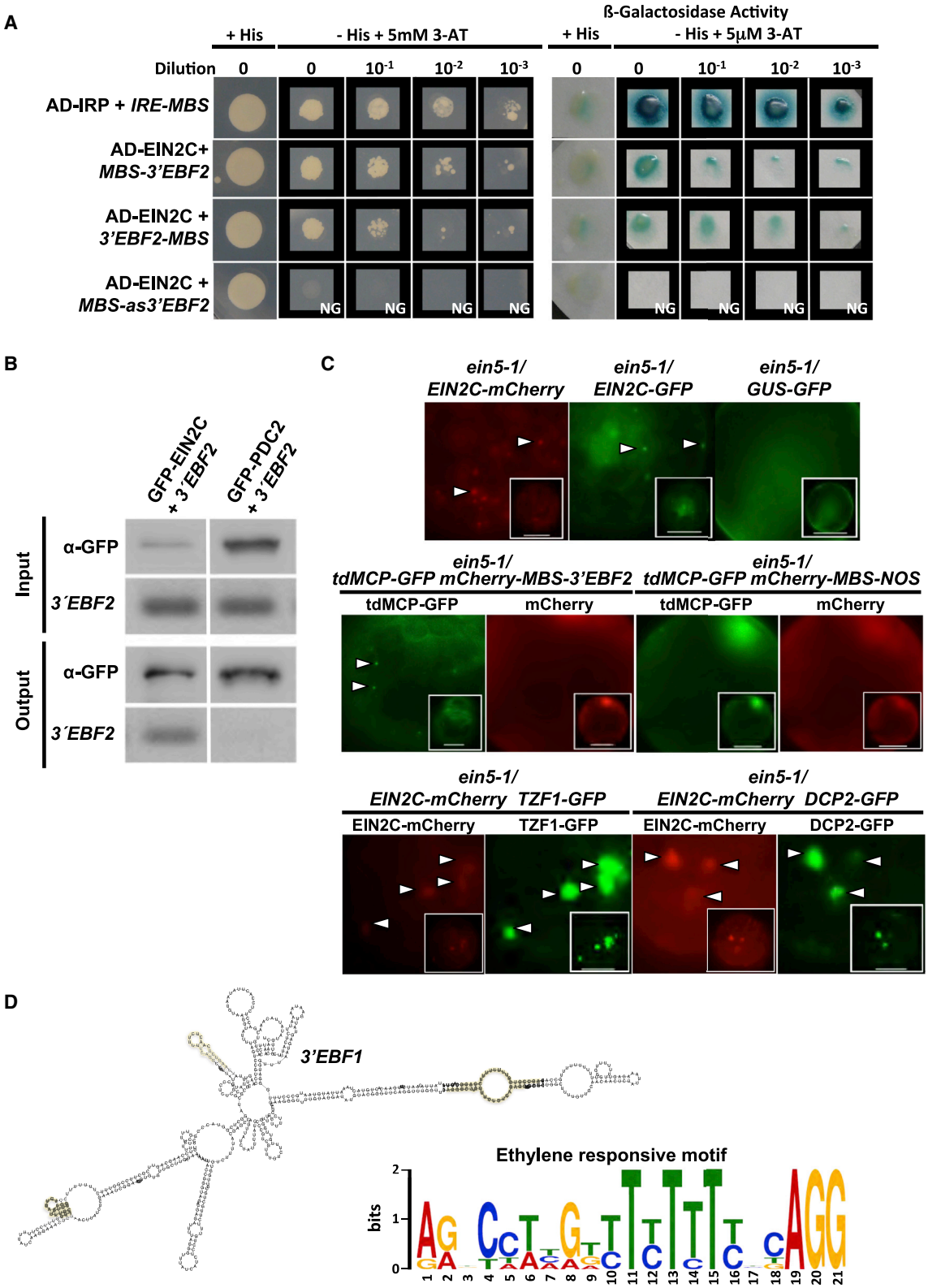

Figure 6. The EIN2C Interacts with the *3′EBF2* mRNA and Localizes to P-Bodies

(A) Yeast three-hybrid assay of the interaction between *3′EBF2* and EIN2C. Activity of the reporter genes for interaction between the RNA bait and the protein prey is shown (HIS3 activity [growth] on His- media, left, and β-galactosidase [blue color] in X-gal, right). All yeast strains employed harbor the DNA binding domain of

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response assay (Figure S3C). These results are in agreement with the previous observation that *hen1*, *rdr2*, *dcl2*, *dcl3*, *sde1*, and *dcl4* mutants are not impaired in their response to ethylene (Potuschak et al., 2006).

Since *EIN2* is the most downstream known signaling component required for the translational regulation of *EBF2*, we decided to examine the interaction between the signal transducer—i.e., EIN2C (Alonso et al., 1999)—and the *3′EBF2* mRNA. Using the yeast three-hybrid system (SenGupta et al., 1996), we were able to detect interaction of EIN2C with two different RNA hybrids of *3′EBF2*, but not with the antisense version of this 3′ UTR (Figure 6A). Next, we investigated if EIN2C from plant extracts could bind its target *EBF2* mRNA in vitro. Using *Nicotiana benthamiana*, we transiently expressed *35S:GFP-EIN2C* or the negative control *35S:GFP-PDC2* and measured the capacity of the tagged proteins to bind to the in-vitro-transcribed *3′EBF2* RNA in an RNA-immunoprecipitation assay (Figure 6B). While we could detect the RNA for *3′EBF2* in the samples with EIN2C, we were not able to detect its presence in the PDC2 control samples. These results suggest that EIN2C could bind to mRNAs in the cytoplasm and regulate their translation. An obvious implication of this mechanistic model is that EIN2C should be localized not only in the nucleus, as previously reported (Qiao et al., 2012), but also in the cytosol. To test this possibility, we reexamined the subcellular localization of transiently expressed GFP- or mCherry-tagged EIN2C in *Arabidopsis* protoplasts and/or tobacco leaves. As reported previously, EIN2C was nuclear localized (Figures S4A and S4B). We reasoned that, perhaps, under standard conditions, only a small fraction of EIN2C and/or only transiently is localized in the cytosol. Two different approaches were used to enhance the activity of EIN2C in the cytosol. First, we examined the subcellular localization of (1) GFP-tagged EIN2C in tobacco leaves co-transfected with a construct expressing *mCherry-3′EBF2* under the strong *35S* promoter (Figure S4B) and of (2) mCherry-tagged EIN2C in *Arabidopsis* protoplasts co-transfected with a construct expressing *CFP-MBS-3′EBF2* under the strong *35S* promoter (Figure S4C). While, in *Arabidopsis* protoplasts, we were not able to consistently detect a significant alteration in the EIN2C subcellular distribution, with EIN2C detected mainly in the nucleus (Figure S4C), in tobacco leaves, EIN2C was consistently localized both in the nucleus and in the cytoplasm where it formed distinct fluorescent foci (Figure S4B). Next, we examined the subcellular localization of mCherry- or GFP-tagged EIN2C in protoplasts obtained from the *Arabidopsis ein5-1* mutant known to accumulate high levels of *3′EBF2* (Potuschak et al., 2006). As shown in Figure 6C, the subcellular distribution of tagged EIN2C in *ein5* dramatically shifted from nuclear to dual nuclear/cytoplasmic localization. As in tobacco, EIN2C in *ein5* was not uniformly distributed in the cytosol but rather formed punctate aggregates. In contrast, localization of the GFP fusion protein expressed from the control *35S:GUS-GFP* construct was not affected by the *ein5* mutation (Figures 6C and S4D). The EIN2C aggregates were found to correspond to P-bodies by co-localization experiments between EIN2C-mCherry and the P-body markers TZF1-GFP and DCP2-GFP (Goeres et al., 2007; Pomeranz et al., 2010) (Figure 6C). Furthermore, *3′EBF2* localized to similar cytoplasmic granules both in *ein5-1* protoplasts (Figure 6C) and wild-type tobacco leaves (Figure S4E). These results not only support the idea that EIN2C is part of an RNA-protein complex localized to the P-bodies but also suggest that the ethylene defects of *ein5* could be the consequence of an overload of the translation regulation machinery similar to what is observed in plants overexpressing *GFP-3′EBF2* under the strong *35S* promoter (Figure S5). Alternatively, or perhaps in addition to this effect, the ethylene insensitivity of *ein5* could also be caused by the disruption of the normal trafficking of EIN2C to the nucleus. Consistent with the idea that, in *ein5* the translation regulatory machinery involved in the control of *EBF2* mRNA translation is overloaded and, therefore, defective, we observed that, in the *ein5* mutant, the effect of ethylene on the GFP fluorescence level of *35S:GFP-3′EBF2* and on the TE of the endogenous *EBF2* mRNA was significantly reduced compared with the responses observed in the corresponding wild-type controls (Figure S5).

Having established that the ethylene responsive element mediating the EIN2-dependent translation regulation is located in the 3′UTRs of *EBF2* and *EBF1* mRNAs, we searched for a conserved sequence motif. Using the MEME motif finder (Bailey and Elkan, 1994), a conserved motif present multiple times in these two genes was identified (Figure 6D). Importantly, using the AME package (McLeay and Bailey, 2010), this motif was shown to be significantly enriched (p value = 2.63e−3) among the 3′ UTRs of the genes translationally regulated by ethylene (Table S1).

Translation Regulation and Ethylene Responses Are Disrupted in the *upf2* Mutants

In a parallel approach to identify additional genes involved in the ethylene response, five mutants were found and ordered in three complementation groups (Figures 7A, and S6A, and S6B). Map-based cloning and identification of additional insertional alleles showed that the causal mutations resided in the three core components of the nonsense-mediated RNA decay machinery, *UPF1*, *UPF2*, and *UPF3* (Figure S6B and Table S3). Based on

LexA fused to the bacteriophage MS2 coat protein (MCP). The additional constructs specific for each strain are the positive control prey protein (AD-IRP), the positive control RNA bait (*IRE-MBS*), the GAL4-activation domain fused to EIN2C (AD-EIN2C), and three different *3′EBF2* RNA baits with the *MS2 binding site* (*MBS*) fused in the sense orientation to the 5′ (*MBS-3′EBF2*) and 3′ (*3′EBF2-MBS*) ends or in the antisense orientation (*MBS-as3′EBF2*). NG indicates no growth.

(B) RNA immunoprecipitation of GFP-EIN2C and the control protein GFP-PDC2 purified from transfected tobacco leaves and incubated with the in-vitro-transcribed *3′EBF2* mRNA. The protein and RNA levels in the input and those retained in the anti-GFP column are shown.

(C) Representative images of *ein5-1* mesophyll protoplasts transfected with the indicated constructs. TZF1 and DCP2 were used as P-body markers. tdMCP is the tandem version of MCP. *MBS* corresponds to 24 copies of the MCP binding sequence. The white scale bar represents 25 μm.

(D) Predicted ethylene-responsive translation *cis*-regulatory element. MEME motif finder identified a consensus sequence present in the 3′ UTRs of *EBF1* and *EBF2* mRNAs and significantly enriched in the 3′UTRs of genes regulated at the translational level by ethylene. The sequence of the ethylene responsive motif (inner panel) and the secondary structure of the 3′UTR of *EBF1* (with the motif-matching sequences highlighted in yellow) are shown.

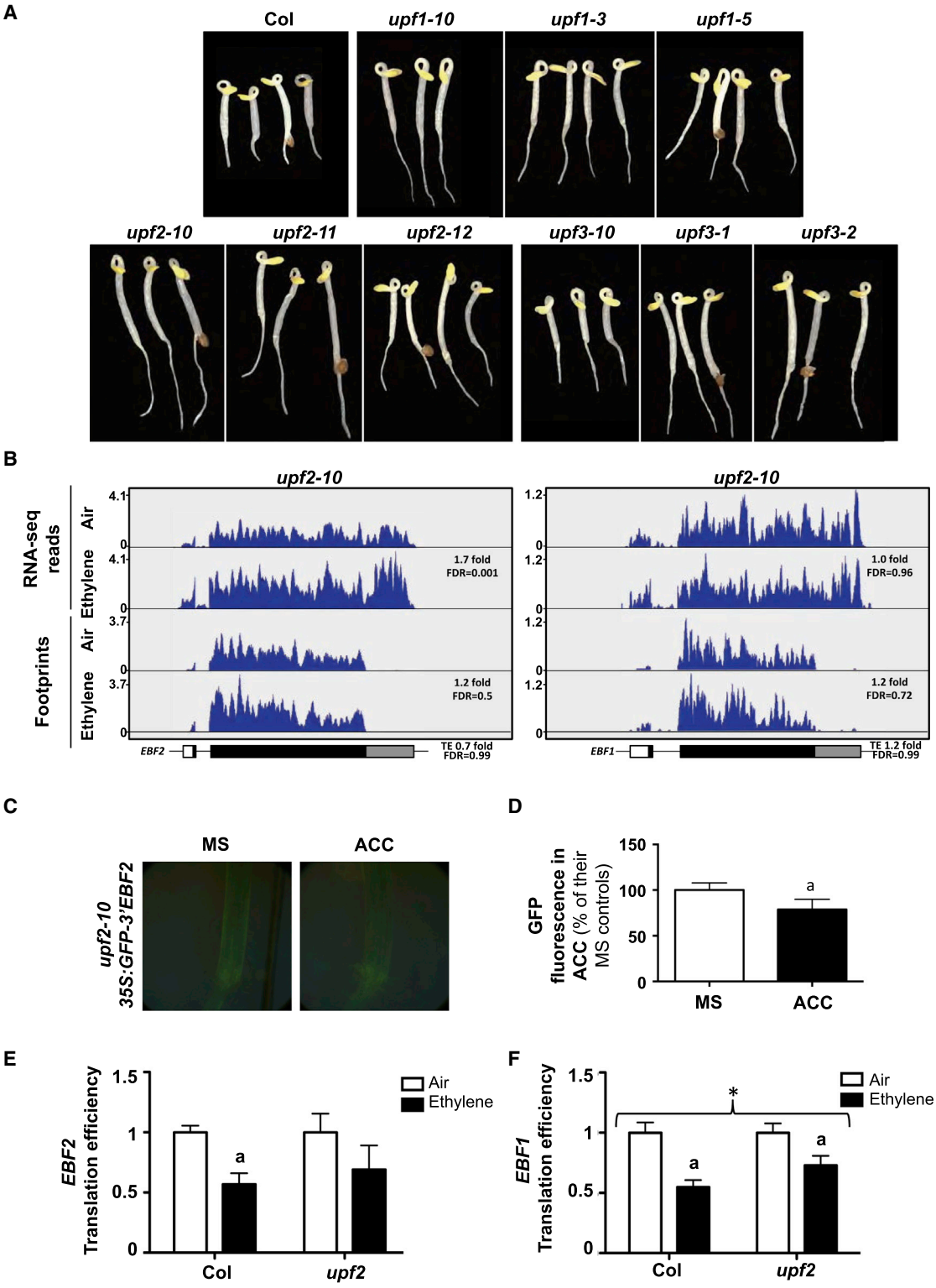

Figure 7. Ethylene-Triggered Root Growth Inhibition and Translational Regulation of *EBF*s Are Disrupted in the *upf2* Mutant

(A) Representative images of 3-day-old etiolated seedlings of the indicated genotypes grown in the presence of 10 μM ACC.

(B) Normalized distribution of RNA-seq and Ribo-seq reads in air and in ethylene along the *EBF2* (left) and *EBF1* (right) genes in *upf2-10*. 5′ UTR, CDS, 3′ UTR, and intron are marked as white, black, and gray boxes and a line, respectively. The fold change and the associated FDR for the ethylene effect on transcript and footprint levels, as well as the fold change in the footprint levels given the levels of mRNA (TE) and the corresponding FDR, are shown.

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our findings on the role of ethylene in the regulation of translation of *EBF2* mRNA through its atypically long 3′ UTR, the lack of ethylene effects on the *EBF* mRNA stability (Potuschak et al., 2006), and the known role of the UPFs in inhibiting translation and targeting to the P-bodies of mRNAs with long 3′ UTRs, we decided to investigate the function of the UPFs in the translational regulation of *EBF*s. Ribo-seq experiments in the hypomorphic allele of *UPF2*, *upf2-10*, clearly show that the translational regulation by ethylene of *EBF2*, *EBF1* (Figure 7B and Table S2), and several other ethylene-regulated mRNAs (Figure S2) was dramatically attenuated. Similarly, the analysis of GFP fluorescence from the *35S:GFP-3′EBF2* construct (Figures 7C and 7D) and the quantification of TE of the endogenous *EBF* mRNA in response to ethylene (Figures 7E and 7F) also show that the *upf2-10* mutation attenuates the ethylene-induced translational regulation of the *EBF* mRNA. These findings, together with our results that translational regulation mediated by EIN2 does not require functional EIN3/EIL1, suggest that the function of UPFs needed for proper ethylene response is also required upstream of EIN3/EIL1. Importantly, we observed that, as in the case of EIN2C, the subcellular localization of UPF1 is also altered in the *ein5* background (Figure S4F), changing from a uniform nuclear/cytosolic to markedly punctuated foci. This subcellular localization of UPF1 partially overlaps with that of EIN2C (Figure S4G), suggesting P-body localization. Finally, we analyzed the kinetics of the ethylene response and recovery of both *upf2* and the mild ethylene insensitive transgenic plants expressing *35S:GFP-3′EBF2*. This analysis shows that both the *upf2* mutant and the *35S:GFP-3′EBF2* transgenic lines show similar defects during the recovery process after ethylene exposure (Figures S3D and S6C).

DISCUSSION

The response of plants to the hormone ethylene has been extensively studied, and a linear signaling pathway responsible for triggering the multitude of responses to this hormone has been identified. Importantly, all known gene-expression changes triggered by this hormone require the entirety of the pathway, including *EIN2* and the master transcriptional regulators *EIN3* and *EIL1* (Chang et al., 2013; Olmedo et al., 2006). Other facets of the ethylene response, however, have been shown to require the activity of *EIN2*, but not of *EIN3* or *EIL1* (Binder et al., 2004). Thus, non-transcriptional responses to ethylene have been postulated to exist and originate from a signaling pathway diverging at the level or downstream of *EIN2* and, therefore, not including *EIN3*/*EIL1*. Although the existence of this parallel pathway was proposed more than 10 years ago (Binder et al., 2004), the mechanistic understanding of such signaling process has remained obscure. Our finding that ethylene alters the TE of specific genes provided missing evidence to start to uncover the molecular nature of the postulated parallel pathway. The detailed characterization of the ethylene-mediated translational regulation of *EBF2* has revealed that this non-transcriptional ethylene effect was indeed *EIN3*/*EIL1* independent and *EIN2* dependent. Hence, the ethylene-triggered changes in translation fulfilled all the pre-requisites of a long-anticipated branch of the ethylene-signaling pathway diverging at the *EIN2* level. Furthermore, we were able to show that this translation-based signaling branch plays a significant physiological role in the ethylene response. For example, removal of *3′EBF2* resulted in the loss of translational responsiveness of this gene to ethylene, and consequently, dramatic alterations of the plant response to this hormone. In particular, our results indicate that the translational regulation of *EBF2* by ethylene plays a role in the still poorly understood process of plant recovery upon withdrawal of the hormone.

Our results also implicate the key signaling component *EIN2* in the translational regulation of gene expression in response to ethylene. Previous studies have shown that EIN2C moves to the nucleus in the presence of ethylene and that this translocation is required for the activation of the *EIN3*/*EIL1*-dependent transcriptional changes (Qiao et al., 2012). Here, we have shown that EIN2 must also function in a cytosolic process of translational control. Although EIN2 has been implicated in the plant response to a variety of stimuli (Gazzarrini and McCourt, 2003), conclusive evidence for an EIN2 role beyond ethylene signaling is still missing. The finding that EIN2C regulates translation opens new opportunities to investigate the full functional spectrum of this enigmatic protein. An additional mechanism can now be envisioned by which other signals impinge on ethylene signaling—i.e., by altering the translational regulatory activity of EIN2. We also found that the EIN2C localizes to cytoplasmic P-bodies under certain circumstances, such as in *ein5* mutants lacking the 5′-3′ XRN4 exoribonuclease activity (Potuschak et al., 2006; Souret et al., 2004). The observation that EIN2C is retained in the cytosol of *ein5* protoplasts suggests a possible mechanistic explanation for the ethylene insensitivity of this classical ethylene signaling mutant.

In addition to uncovering the EIN2C accumulation in P-bodies, we also showed that EIN2C has the capability to interact, directly or indirectly, with the *3′EBF2* mRNA, as also suggested by the results from the accompanying paper by Li et al. (2015) in this issue of *Cell*. In either case, these results, together with the finding that the *EIN2* function is required for the translational regulation of *EBF2*, raised the question of how EIN2 influences translation activity of its RNA targets. It is possible that the 3′ UTR-bound EIN2C directly or indirectly modulates the activity

(C and D) (C) Representative image and (D) GFP fluorescence of multiple seedlings (n = 7) expressed as the percentage of fluorescence in ACC compared to that in the MS controls. Error bars represent means ± SD. (a) indicates a significant effect of ethylene on the levels of fluorescence (t test, $p < 0.05$).
(E and F) TE of *EBF2* (E) and *EBF1* (F) mRNA, calculated as the relative expression in polysomal/total RNA fractions, in 3-day-old etiolated Col-0 and *upf2-10* seedlings grown in air (Air) or treated with 10 ppm of ethylene for the last 4 hr of the experiment (Ethylene).
The asterisk (*) indicates a significant difference of the ethylene effect on the *EBF* TE between Col and *upf2-10* (two-way ANOVA, $p < 0.05$). (a) indicates a significant difference of the ethylene effect on the *EBF* TE in the indicated genotypes (t test, $p < 0.05$).
The Col measurements in (E) and (F) are the same as in Figure 1D, plotted here again to facilitate the comparison between Col and *upf2-10*. Expression levels of the *EBF* transgenes were normalized against *At4g34270*.

of a component of the general translational machinery, thus selectively inhibiting the translation of its targets. In fact, some of the best-documented examples of gene-specific translation regulation involve the direct interaction of an RNA-binding protein with particular 3′ UTR sequences and a subsequent recruitment of general translation regulators (Szostak and Gebauer, 2013). For example, the *Drosophila* Bicoid protein directly binds to the 3′ UTR of the embryo-patterning mRNA *caudal*. This interaction, however, is not sufficient to repress the translation of *caudal*, and Bicoid has to recruit the CAP-binding protein 4EHP that (due to its low affinity for the translation initiation factor eIF4G) attenuates the rates of translation of the Bicoid targets by failing to recruit the eIF3-containing 43S translation initiation complex (Cho et al., 2005). It is interesting to note here that UPF1 has also been shown to repress translation initiation by directly interacting with eIF3 and, thus, to prevent the formation of the 43S translation initiation complex (Isken et al., 2008). We have provided experimental evidences linking *UPF* function not only with the ethylene response, but also, more specifically, with the translational repression of *EBF2* by this hormone. Furthermore, we show that, under certain experimental conditions, such as in plants lacking functional *EIN5*, the *3′EBF2*-binding protein EIN2C and UPF1 co-localize in P-bodies. Based on this, we propose a mechanistic model (Figure S7) in which the binding of EIN2C to *3′EBF2* triggers the recruitment of the UPFs to this mRNA, which in turn results in the inhibition of translation initiation by interfering with the formation of the 43S complex. Although our initial attempts to show a direct interaction between EIN2C and the UPFs by means of the yeast two hybrid have failed, it is still possible that EIN2C directly or indirectly recruits the UPFs, perhaps, as it has been suggested by Li et al. (2015) in the accompanying paper, via a yet-uncharacterized RNA-binding protein that recruits EIN2 to its target mRNAs. It is also important to point out that the relatively weak ethylene defects observed in the *upf* mutants are likely the result of the hypomorphic nature of the alleles identified in our screen, as well as the fact that the function of UPFs is required for the translational effect of EIN2C but not necessarily for its activation of the EIN3/EIL1 activity. We have focused here on the regulation of *EBF2*, but it would be interesting to study other translationally regulated genes identified herein and explore their role in ethylene-related processes, including transcription-independent fast growth inhibition response (Binder et al., 2004). Finally, additional studies on the temporal dynamics of the transition of the translationally regulated mRNAs from polysomes to P-bodies in ethylene and back to polysomes upon ethylene withdrawal will be necessary to extend the mostly static single-time-point studies described herein.

EXPERIMENTAL PROCEDURES

Plant Growth and Ribosome Footprinting

Plant growth conditions and hormonal treatments of *Arabidopsis* seedlings were as described (Stepanova et al., 2005). Ribosome footprinting (Ingolia et al., 2009) was carried out using pelleted polysomes (Mustroph et al., 2009) with the following modifications. Polysomes were isolated in Extraction Buffer (100 mM Tris-HCl [pH 9], 10 mM Tris-HCl [pH 7.4], 100 mM sucrose, 100 mM KCl, 75 mM NaCl, 20 mM $MgCl_2$, 12.5 mM EGTA [pH8], 3 mM DTT, 6.25 μl/ml detergent mix [20% (w/v or v/v) of each of the four detergents in water: Brij-35, Triton X-100, Igepal CA 630 and Tween 20], 25 μl/ml Triton X-100, 37.5 μg/ml cycloheximide, 25 μg/ml chloramphenicol), and the digestion with the RNase I was carried out in a volume of 4.5 ml. After digestion, monosomes were re-pelleted and purified by sucrose gradient fractionation. RNA fragments corresponding to the ribosome footprints were recovered from the purified monosomes and sequenced as described (Ingolia et al., 2009). Data processing was performed using a combination of custom-made Perl scripts, as well as R and Bioconductor programs.

Immunoblot and qRT-PCR

Protein samples were prepared by homogenizing the liquid nitrogen-ground tissues in 2× SDS-PAGE sample buffer (Laemmli, 1970) and boiling the homogenate for 5 min. Proteins were separated through a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and hybridized to anti-GFP antibodies (Living Colors A.v. Monoclonal Antibody, Clontech).

Total RNA was extracted as previously described (Reuber and Ausubel, 1996). Polysomal RNA was isolated by pelleting polysomes (Mustroph et al., 2009) and then extracting the RNA by the SDS/acid phenol method (Ingolia et al., 2009). Reverse transcription and qPCR (Applied Biosystems) were performed according to manufacturer's recommendations. Primer sequences are listed in the Supplemental Experimental Procedures.

Yeast Three-Hybrid and RNA Immunoprecipitation

The yeast three-hybrid system (Bernstein et al., 2002) was used to test the interaction between the EIN2C fragment (amino acids 459 to 1278) and *3′EBF2* RNA. Interaction was inferred based on the activity of LacZ and HIS3 reporters as described (Deplancke et al., 2006).

RNA immunoprecipitation assay was performed as described (Nicaise et al., 2013). Protein extracts from *Nicotiana benthamiana* leaves expressing *35S:GFP-EIN2C-pGWB6* or a negative control *35S:GFP-PDC2-pGWB6* (Stepanova et al., 2011) were incubated with anti-GFP-TRAP-A beads (Chromotek) and 50 μg of *3′EBF2* RNA synthesized in vitro using RiboMAX Large Scale RNA Production System-T7 (Promega). After extensive washes, RNA-protein complexes were eluted from the beads by incubating at 60°C for 15 min in 200 μl of Elution Buffer (1% SDS, 0.1 M NaHCO3) and treated for 1 hr at 60°C with 40 μg Proteinase K, followed by SDS/Phenol RNA extraction, reverse transcription (Applied Biosystems), and 30 cycles of qPCR (Power SYBR green Master Mix, Applied Biosystems).

Protoplast and Tobacco Transient Expression Assays

Protoplasts were isolated using the tape-*Arabidopsis* sandwich method (Wu et al., 2009) and transfected according to a published protocol (Yoo et al., 2007). Transient expression in *Nicotiana benthamiana* leaves was performed as described elsewhere (Wang et al., 2015).

Imaging was done using a Leica DFC365 FX camera attached to a compound microscope DM5000 with the following filters: GFP filter cube (EX 470/40 EM 525/50), CFP filter cube (Ex 436/20 Em 480/40), and TX2 filter cube (Ex 560/40 Em BP645/75). The Objective HCX PLAPO 40×/0.10 was used.

A more detailed description of the materials and methods is provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the sequencing data reported in this paper is NCBI SRA: SRP056795.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.09.036.

AUTHOR CONTRIBUTIONS

C.M., A.N.S., and J.M.A. designed and carried out the experiments and wrote the manuscript. Q.H. and S.H. performed the bioinformatic analysis. B.M.B.

did the kinetic analysis of the ethylene responses. J.B. performed qRT-PCR, yeast, protoplast, and tobacco studies. K.R.S., P.E., and J.Y. assisted in the identification and/or cloning of the mutants.

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