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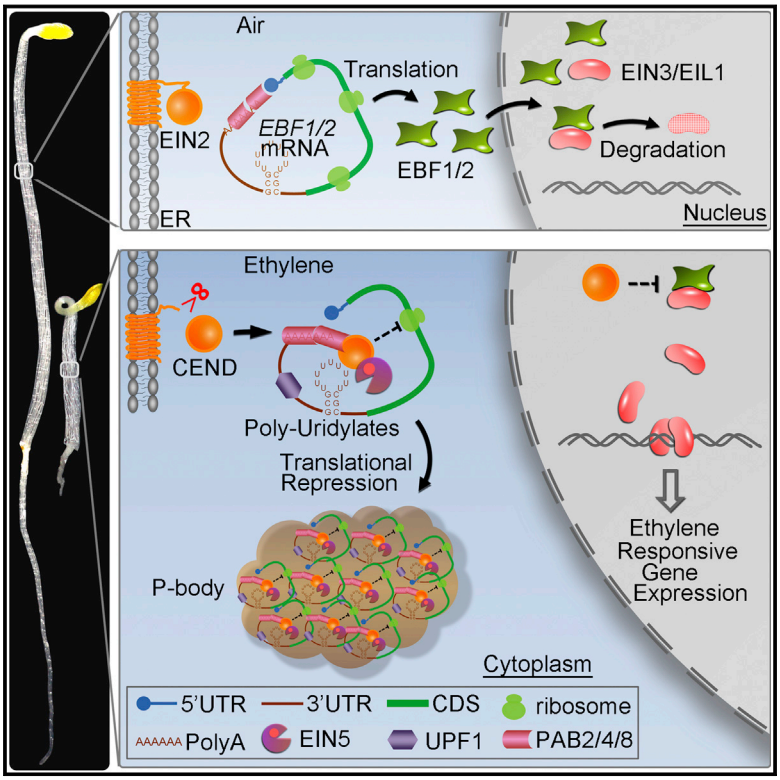
EIN2-Directed Translational Regulation of Ethylene Signaling in *Arabidopsis*

Graphical Abstract



Highlights

- Ectopic expression of *EBF1/2* 3′ UTR fragment leads to ethylene insensitivity
- 3′ UTR mediates ethylene-induced translational repression in an EIN2-dependent way
- PolyU motifs within 3′ UTR are critical for EIN2-directed translational inhibition
- EIN2 targets *EBF1* 3′ UTR to cytoplasmic P-body via interacting with EIN5 and PABs

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In Brief

This study reports a novel translational repression mechanism during ethylene signaling in which 3′ UTRs of mRNAs function as signal transducers.

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EIN2-Directed Translational Regulation of Ethylene Signaling in *Arabidopsis*

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SUMMARY

Ethylene is a gaseous phytohormone that plays vital roles in plant growth and development. Previous studies uncovered EIN2 as an essential signal transducer linking ethylene perception on ER to transcriptional regulation in the nucleus through a "cleave and shuttle" model. In this study, we report another mechanism of EIN2-mediated ethylene signaling, whereby EIN2 imposes the translational repression of *EBF1* and *EBF2* mRNA. We find that the *EBF1/2* 3′ UTRs mediate EIN2-directed translational repression and identify multiple poly-uridylates (PolyU) motifs as functional *cis* elements of 3′ UTRs. Furthermore, we demonstrate that ethylene induces EIN2 to associate with 3′ UTRs and target *EBF1/2* mRNA to cytoplasmic processing-body (P-body) through interacting with multiple P-body factors, including EIN5 and PABs. Our study illustrates translational regulation as a key step in ethylene signaling and presents mRNA 3′ UTR functioning as a "signal transducer" to sense and relay cellular signaling in plants.

INTRODUCTION

Ethylene is a gaseous phytohormone produced by plants in response to various internal and environmental stimuli, which triggers a wide range of physiological and morphological responses (Johnson and Ecker, 1998). During the past decades, a relatively linear ethylene signaling pathway has been established through the application of molecular and genetic approaches (Guo and Ecker, 2004). In *Arabidopsis*, ethylene is perceived by a group of ER-located receptors (Chang and Stadler, 2001). In the absence of ethylene signal, the hormone-free receptors activate a Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) (Gao et al., 2003; Kieber et al., 1993). Activated CTR1 and the receptors cooperatively inhibit an ER-located membrane protein ETHYLENE INSENSITIVE 2 (EIN2) through physical interaction and protein phosphorylation (Alonso et al., 1999; Bisson and Groth, 2011; Ju et al., 2012).

EIN2 is a key component in ethylene signaling pathway, evidenced by completely ethylene-insensitive phenotypes of the *ein2* null mutants (Ji and Guo, 2013). It is encoded by a single-copy gene in *Arabidopsis*, and is conserved from charophyte green algae to land plants (Ju et al., 2015). While the function of its N-terminal membrane-spanning domain is not clear, the C-terminal end of EIN2 (CEND) is thought to participate in signaling output, as ectopic expression of this domain alone can partially activate ethylene responses (Alonso et al., 1999; Wen et al., 2012). Recent studies reported that CEND can be phosphorylated by the receptors-activated CTR1 in the absence of ethylene (Ju et al., 2012; Qiao et al., 2012). Upon ethylene application, inactivation of the receptors and CTR1 abolishes the phosphorylation state of CEND, leading to its proteolysis from the ER-tethered N terminus, followed by shuttling into the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). However, this "cleave and shuttle" mode might represent part of the EIN2 actions, as induced nuclear localization of CEND only partially activates ethylene signaling (Ji and Guo, 2013; Wen et al., 2012). Meanwhile, ethylene also induces CEND to form discrete and prominent foci in the cytoplasm (Qiao et al., 2012; Wen et al., 2012), but the function of such cytoplasmic portion remains unexplored.

In the nucleus, components working downstream of EIN2 are two master transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and its homolog EIN3-LIKE 1 (EIL1), which regulate the vast majority of ethylene-directed gene expression (Chang et al., 2013; Chao et al., 1997). One of the key regulatory mechanisms of ethylene signaling is the stabilization of EIN3/EIL1 proteins, wherein ethylene acts to repress the proteasomal degradation of EIN3/EIL1 mediated by two F-box proteins, EIN3-BINDING F-BOX 1 (EBF1) and EBF2, in an EIN2-dependent manner (An et al., 2010; Guo and Ecker, 2003; Potuschak et al., 2003). However, the molecular mechanism of how ethylene or EIN2 represses the function of *EBF1/2* is still elusive.

ETHYLENE INSENSITIVE 5 (*EIN5*), encoding a cytoplasmic 5′-3′ exoribonuclease (AtXRN4), is another component positively modulating ethylene responses (Olmedo et al., 2006; Potuschak et al., 2006). Currently, little is known about how EIN5 modulates ethylene signaling, except for the genetic evidence suggesting its participation in the regulation of *EBF1/2* function (Olmedo et al., 2006; Potuschak et al., 2006). Notably, small RNA fragments corresponding to *EBF1* and *EBF2* mRNA 3′ UTR were processed and accumulated in *ein5* (Olmedo et al., 2006; Potuschak et al., 2006; Souret et al., 2004). Our recent work uncovered that

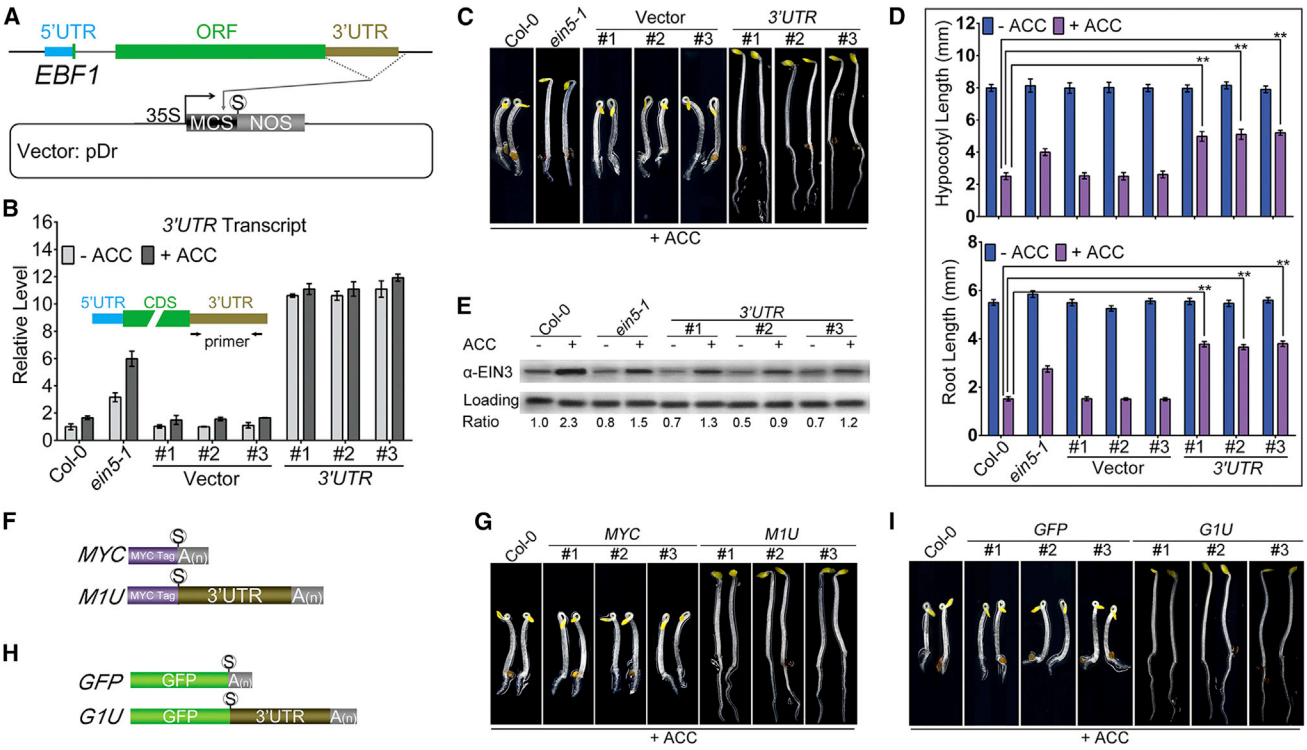

Figure 1. Overexpression of *EBF1 3′ UTR* Results in Reduced Ethylene Sensitivity

(A) Schematic diagrams of the gene structure of *EBF1* and the *3′-UTR*-overexpressing construct. Full-length *EBF1 3′ UTR* (643 bp after stop codon) plus a 66-bp flanking sequence was inserted into the multiple cloning site (MCS) prior to the NOS terminator in pDr vector. S in open circle, stop codon.

(B) Quantification of *3′ UTR* transcripts in etiolated seedlings of three independent transgenic lines grown on MS medium with (+) or without (–) ACC (an ethylene biosynthetic precursor). Vector means pDr-expressing transgenic plants while *3′ UTR* means *3′-UTR*-overexpressing transgenic lines. Arrows denote the primers used for qRT-PCR to detect the levels of *3′ UTR*.

(C) The triple response phenotypes of seedlings corresponding to (B).

(D) Quantification of hypocotyl lengths and root lengths of the seedlings in (C). **$p < 0.01$. Mean ± SD, $n > 10$.

(E) Immunoblot assays showing EIN3 protein levels of seedlings corresponding to (B). A nonspecific band served as a loading control. The numbers indicate the relative EIN3 protein levels as calculated from three biological replicates.

(F and H) Schematic maps of *M1U* (*MYC-EBF1 3′ UTR*) and *G1U* (*GFP-EBF1 3′ UTR*), as well as two control transcripts *MYC* and *GFP*. A(n) represents the poly(A) tail. Of note, all these transcripts are driven by CaMV 35S promoters.

(G and I) The triple response phenotypes of etiolated seedlings of wide-type Col-0 as well as three independent lines of indicated transgenic plants.

See also Figure S1.

EIN5, in combination with 3′-5′ RNA decay pathway, is responsible for the removal of many defective coding transcripts as well as the cleavage fragments of miRNA targets, including 3′ UTRs, which are otherwise subjected to posttranscriptional gene silencing (Zhang et al., 2015). However, genetic evidence disfavored the possibility that 3′ UTR fragments of *EBF1/2* mRNA are processed and targeted to small RNA-mediated gene silencing pathway (Potuschak et al., 2006). Interestingly, ectopic expression of a 3′ UTR-truncated *EBF2* gene resulted in a stronger ethylene insensitive phenotype than that of the *EBF2* full-length gene (Konishi and Yanagisawa, 2008), implying a negative role of 3′ UTR on the *EBF2* function.

In this study, we sought to investigate the regulatory mechanisms of how ethylene signal is relayed from cytoplasm to nucleus, and how EIN2 and EIN5 participate in this signaling process. Strikingly, we found that ectopic expression of either *EBF1* or *EBF2* 3′ UTR fragments confers strong ethylene-insensitivity phenotypes through promoting the translation of endogenous *EBF1/2* mRNAs. Furthermore, we found that ethylene induces EIN2 to target *EBF1* 3′ UTR to cytoplasmic processing-body (P-body) through interacting with EIN5 and other P-body factors to repress *EBF1/2* translation. Our study uncovers another branch of ethylene signaling pathway mediated by cytoplasmic EIN2 in translational control.

RESULTS

Overexpression of *EBF1 3′ UTR* Leads to Reduced Ethylene Sensitivity

Previous studies revealed that the *ein5* mutant accumulated *EBF1/2* mRNA 3′ UTR fragments (Olmedo et al., 2006; Potuschak et al., 2006; Souret et al., 2004). We thus speculated that the over-accumulated 3′ UTR fragments could contribute to the ethylene insensitivity of *ein5*. To test this speculation, we overexpressed the *EBF1* 3′ UTR region (*1U*) in wild-type Col-0 plants (Figures 1A and 1B). The so-called "triple response"

phenotype is commonly used as an ethylene-specific growth response in *Arabidopsis*, which refers to exaggerated apical hooks, shortened hypocotyls and roots of dark-grown seedlings exposed to ethylene or treated with ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Bleecker et al., 1988; Ecker, 1995). Overexpression of *1U* conferred significant attenuation of triple response phenotypes to Col-0, resulting in elongated hypocotyls and roots compared with control seedlings (Figures 1C and 1D). Consistently, we found that the levels of EIN3 protein were lower in *1U* transgenic plants than that in Col-0 (Figure 1E).

Furthermore, we fused *1U* to the *MYC* tag and *GFP* coding sequence (referred to as *M1U* and *G1U*), respectively (Figures 1F and 1H), and overexpressed these fusion genes in wild-type Col-0 (Figures S1A–S1C and S1F–S1H). Similar to *1U*-overexpressing seedlings, *M1U*- and *G1U*-overexpressing plants displayed reduced ethylene sensitivity and impaired EIN3 protein accumulation compared with control plants (Figures 1G, 1I, S1D, S1E, S1I, and S1J). Together, these results demonstrate that overexpression of *1U*, alone or in fusion with unrelated transcripts, reduces ethylene sensitivity.

Overexpression of *EBF1 3′ UTR* Promotes the Translation of Endogenous *EBF1/2* mRNAs

Interestingly, we found that ethylene hyposensitivity resulting from *1U*-overexpression was partially restored by a defect in either *EBF1* or *EBF2* (Figure 2A). Due to the fatal effect of over-accumulated EIN3 in *ebf1 ebf2* double mutant, we next overexpressed *M1U* in *β-estradiol-inducible EIN3-Flag/ein3 eil1 ebf1 ebf2* (*iEIN3/qm*) (An et al., 2010), which was used as a substitution of the lethal *ebf1 ebf2* double mutant (Figure S2A). We found that *M1U* no longer affected the triple response phenotypes (Figure 2B), and the abundance of EIN3 protein was comparable between *iEIN3/qm* and *M1U iEIN3/qm* (Figure S2B). Together, these results demonstrate that the presence of *EBF1/2* is required for the *1U*-overexpression-induced repression of ethylene responses, implying that exogenous *3′ UTR* expression modulates the function of *EBF1/2*.

We found that the levels of both *EBF1* and *EBF2* transcripts were not evidently affected by *1U* overexpression (Figure S2C), excluding the modulation of *EBF1/2* at the level of transcription or RNA decay. We next examined whether the translation of *EBF1/2* mRNAs is under the regulation. Without good antibody against EBF1 or EBF2 available, two experiments were conducted for this purpose. Using polysome profiling assays, we found that the translation of *EBF1* and *EBF2* mRNAs was repressed by ethylene, as the portion of high-density polysome-associated *EBF1/2* mRNAs was decreased upon ethylene application (Figure 2C). Notably, *1U* overexpression recovered the drop of the portion of polysome-associated *EBF1/2* mRNAs (Figure 2C). Therefore, *1U* overexpression augments the translation of endogenous *EBF1/2* mRNAs, which is subjected to repression by ethylene.

Furthermore, we constructed transgenic plants harboring *GFP-EBF1* followed by *1U* or not (*G1F* and *G1C*, respectively) (Figure 2D), and expressed an inducible *1U* (*iEBF1U*) in these plants to examine the effect of exogenous *1U* expression on *G1F* or *G1C* translation. We found that, while *GFP-EBF1* mRNA levels were comparable, GFP-EBF1 protein levels were downregulated by ethylene and upregulated by *1U* overexpression in *G1F* plants (Figures 2E and 2F). By contrast, in *G1C* plants, the GFP-EBF1 protein levels were virtually unchanged upon *1U* expression regardless of ethylene application (Figures 2E and 2F). Collectively, these results suggest that the over-accumulation of *1U* transcripts boosts the function of *EBF1/2* by enhancing their translation.

Based on these observations, we propose a translational interference model, in which ectopically expressed *1U* transcripts interferes with the endogenous *EBF1/2 3′ UTRs* that supposedly exert a repressive role on the translation of *EBF1/2* mRNAs. Such translational interference could arise from the competition and/or titration of translational repressors binding to the endogenous 3′ UTR regions (Figure 2G).

The 3′ UTRs Impart Translational Inhibition to *EBF1/2* mRNAs in Response to Ethylene

We next tested the translational interference model (Figure 2G) by examining the effect of *EBF1* 3′ UTR on *GFP* mRNA translation (Figures 1H, 1I, and S1F–S1J). We found that, with the comparable transcript levels (Figure S1G), seedlings expressing *G1U* accumulated much lower GFP fluorescence or protein abundance than those expressing *GFP* alone, particularly when treated with ACC (Figures 3A–3D). Ethylene caused over 80% of decrease in the translational efficiency of *G1U* whereas had no effect on *GFP* alone (Figures 3C and 3D). The ACC-promoted reduction in GFP protein abundance was restored by the application of ethylene inhibitor silver ions (Ag^+) (Figure 3E). Taken together, these results indicate that *EBF1* 3′ UTR confers translational repression to its fusion mRNA in response to ethylene.

Next, we determined the biological significance of the *EBF1* mRNA 3′ UTR–mediated translational repression in ethylene signal transduction. We constitutively expressed *M1C* (*MYC-EBF1, MYC* tag fused with the *EBF1* coding sequence) and *M1F* (*MYC* fused with the *EBF1* full-length transcript including coding sequence and 3′ UTR) (Figure 3F). Compared with control plants, *M1F* expression resulted in reduced ethylene sensitivity, whereas *M1C* expression conferred nearly complete ethylene insensitivity (Figure 3G). In agreement with the triple response phenotype, the amount of MYC-EBF1 protein was nearly constant in *M1C* but progressively decreased in *M1F* upon treatment with increasing doses of ACC (Figure 3H). Given the comparable mRNA abundance between *M1F* and *M1C* (Figures S3A and S3B), we concluded that translational repression of *EBF1* mRNA via its 3′ UTR is critical for *EBF1* function in ethylene signaling.

We further found that the overexpression of *EBF2 3′ UTR* (*2U*) also led to reduced ethylene sensitivity in *GFP-EBF2 3′ UTR* (*G2U*) transgenic plants (Figures S3C and S3D). Like *EBF1* 3′ UTR, *EBF2* 3′ UTR also conferred translational repression to the *GFP* mRNA fused with it (Figure S3E). Thus, the 3′ UTRs of both *EBF1* and *EBF2* act similarly to impose translational repression to their respective mRNAs in response to the ethylene signal.

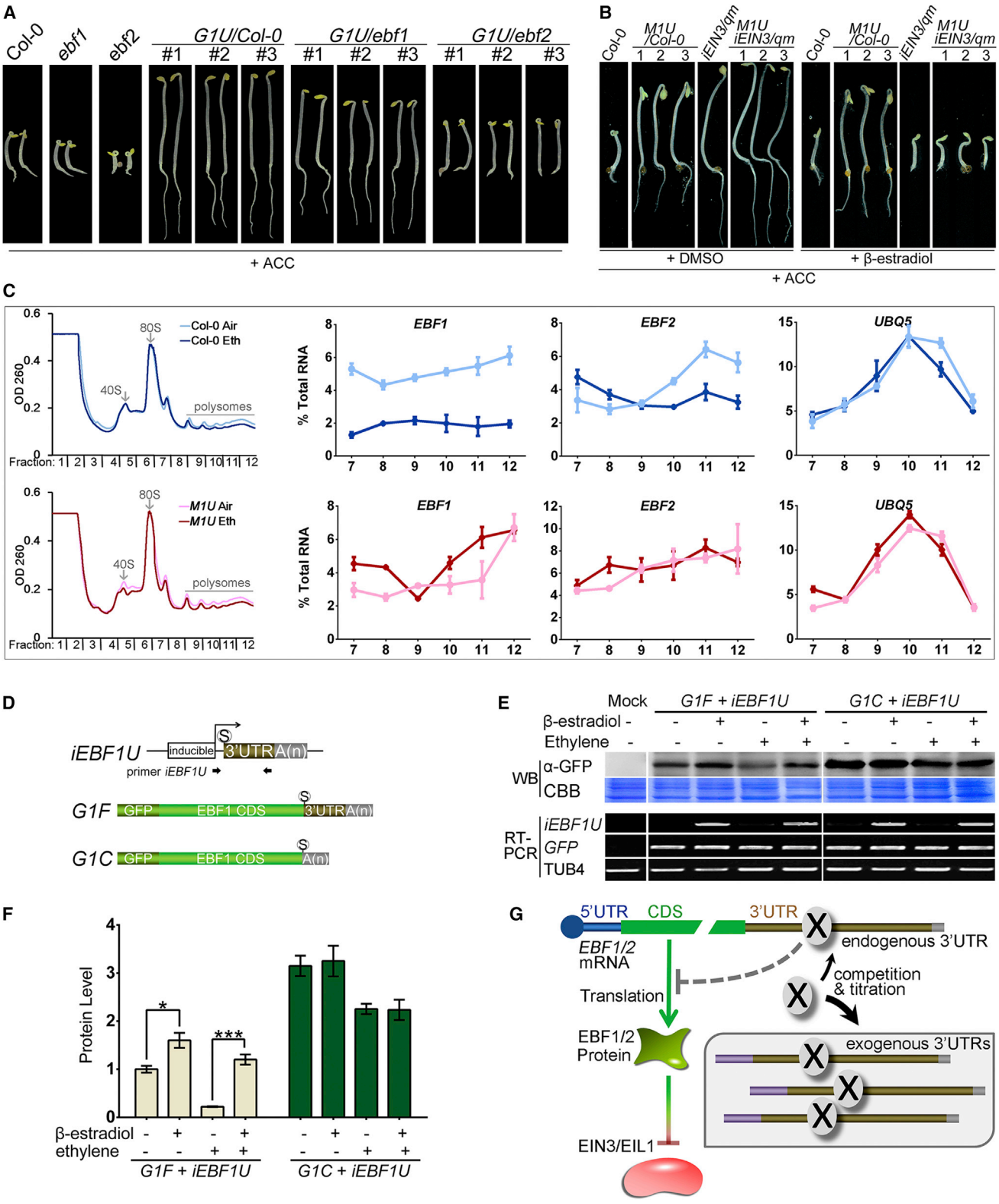

Figure 2. Overexpression of *EBF1 3′ UTR* Enhances the Translation of Endogenous *EBF1/2* mRNAs

(A and B) Triple response phenotypes of etiolated transgenic seedlings expressing *G1U* (*GFP-EBF1 3′ UTR*) treated with ACC (A), and seedlings expressing *M1U* (*MYC-EBF1 3′ UTR*) treated with ACC in combination with DMSO or β-estradiol (B). *iEIN3/qm* is the β-estradiol-induced *EIN3-Flag* in the *ein3 eil1 ebf1 ebf2* quadruple mutant background, which was used to substitute for the lethal *ebf1 ebf2* double mutant (An et al., 2010).

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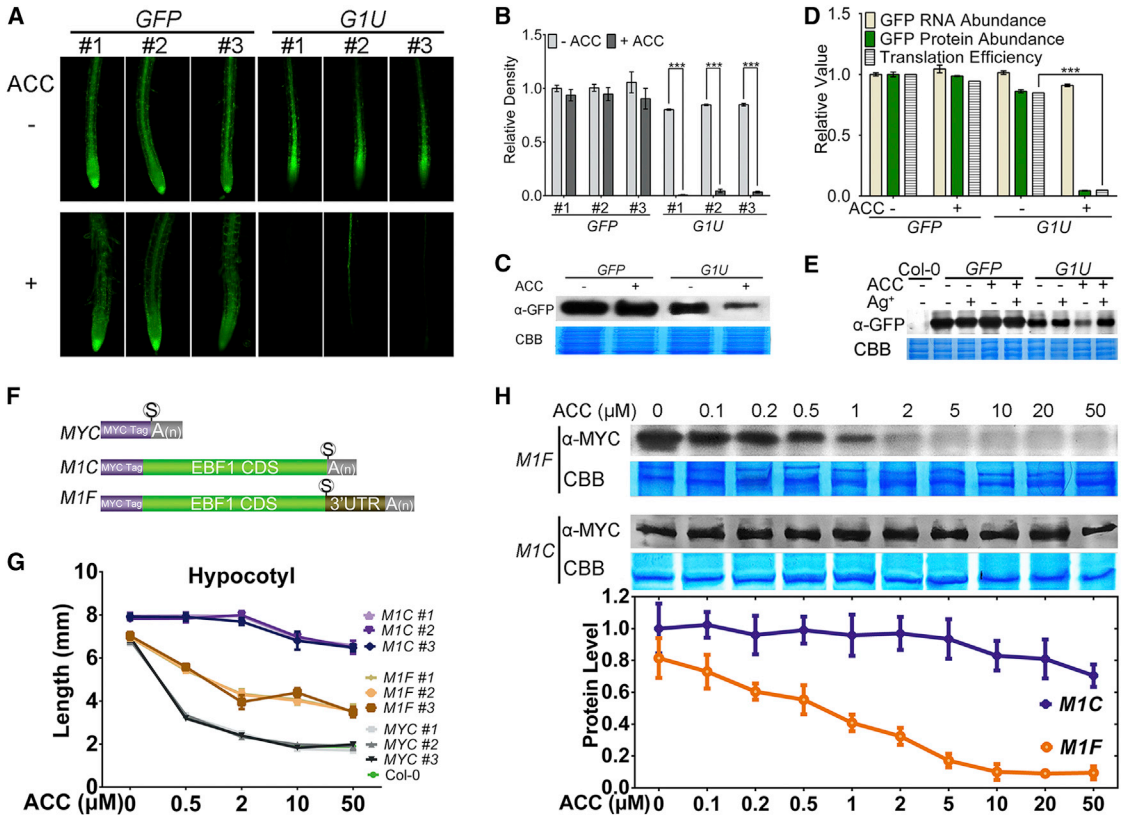

Figure 3. *EBF1* 3′ UTR Confers Translational Repression to Its Fusion Transcripts in Response to Ethylene

(A and B) GFP fluorescence in the roots of three independent transgenic seedlings expressing *GFP* or *G1U* (*GFP-EBF1 3′ UTR*) with (+) or without (–) ACC treatment (A) and the relative quantifications of GFP fluorescence (B). ***p < 0.001. Mean ± SD, n > 20 roots.

(C) Immunoblot assays showing GFP protein abundance in whole etiolated seedlings with (+) or without (–) ACC treatment.

(D) qRT-PCR analysis of *GFP* mRNAs and quantification of GFP proteins in (C). The ratio of protein to mRNA abundance was defined as the translation efficiency. ***p < 0.001; calculations based on three biological repeats.

(E) Immunoblot assays showing GFP protein abundance in etiolated seedlings treated with (+) or without (–) ACC and/or silver ion.

(F) Structures of *MYC*, *M1C* (*MYC-EBF1* CDS), and *M1F* (*MYC-EBF1* full length containing CDS and 3′ UTR) transcripts.

(G) Hypocotyl lengths of etiolated seedlings of three independent transgenic lines expressing *MYC*, *M1C*, and *M1F*. Mean ± SD, n > 20.

(H) Immunoblot assays indicating MYC-EBF1 protein abundances (top) and their relative quantifications (bottom) in seedlings treated with increasing doses of ACC. Calculations were based on three biological repeats.

See also Figure S3.

EIN2 Is Essential for 3′-UTR-Mediated Translational Repression of *EBF1* mRNA

We next investigated the role of key ethylene signaling components in 3′-UTR-mediated translational regulation. The ethylene-induced repression of *G1U* mRNA translation, manifested by reduced GFP fluorescence, was similarly observed in Col-0 and *ein3 eil1*, but not in *ein2* and a receptor mutant *etr1* (Figures 4A and S4A), suggesting that the upstream signaling components including the receptors and EIN2 are required for 3′-UTR-mediated translational repression, whereas EIN3/EIL1 are not. Expression of a β-estradiol-inducible version of *EIN2* was sufficient to restore such translation inhibition in *ein2*, and

(C) Polysome profiling assays with sucrose density gradient accompanied by qRT-PCR to analyze translational status of *EBF1/2* mRNAs. A_{254} absorption was monitored together with fractionation (left). The fractions containing 40S, 80S of ribosome, and polysomes are indicated. The abundance of *EBF1* and *EBF2* mRNA in each fraction was detected by qRT-PCR and quantified as a percentage relative to their total amount (right). *UBQ5* mRNA was used as a reference.

(D) Structures of *iEBF1U* (β-estradiol-inducible *EBF1 3′ UTR*) transcript, *G1F* (*GFP-EBF1* full length containing CDS and 3′ UTR) and *G1C* (*GFP-EBF1* CDS). Arrows indicate the primer pair used to analyze the expression of *iEBF1U*.

(E) Coexpression of *G1F* or *G1C* together with *iEBF1U* in etiolated seedlings treated with or without ethylene and β-estradiol for 4 hr before RT-PCR and western blotting analysis. Protein loading was manifested by Coomassie brilliant blue (CBB) staining.

(F) Quantitative measurements of GFP-EBF1 proteins in (E) based on three biological repeats. *p < 0.05; ***p < 0.001.

(G) A translational interference model proposes that the exogenously overexpressed *3′ UTRs* enhance the translation of endogenous *EBF1/2* mRNAs by competing with their inherent 3′ UTRs and thus titrating unknown repressor X bound to 3′ UTRs.

See also Figure S2.

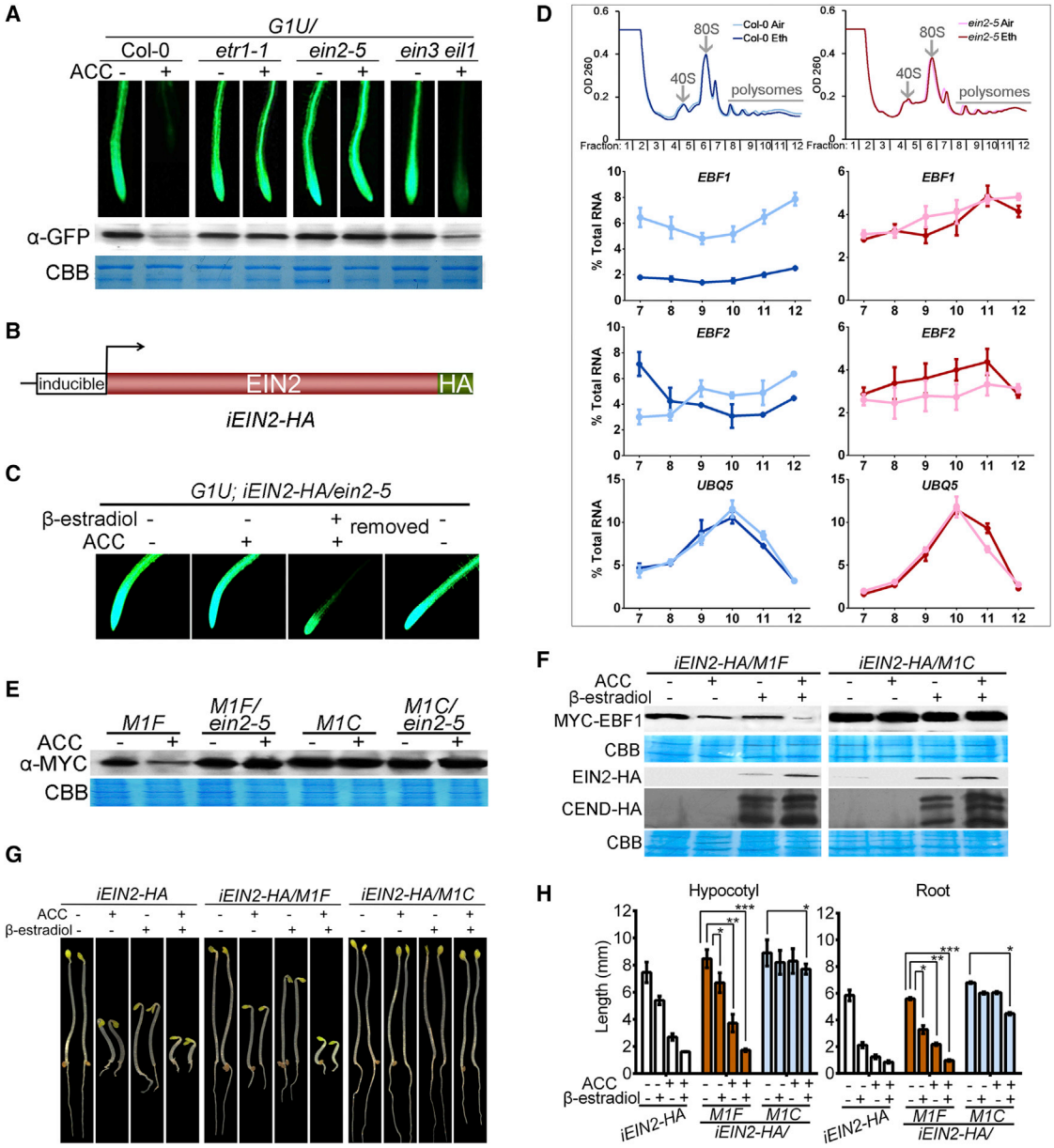

Figure 4. EIN2 Is Required for *EBF1* 3′-UTR-Mediated Translational Repression

(A) GFP fluorescence in the roots of etiolated seedlings expressing *G1U* (*GFP-EBF1 3′ UTR*) in different genotype backgrounds (top). Immunoblot assays showing GFP protein abundance in whole seedlings (bottom).

(B) Structure of the β-estradiol-inducible *EIN2-HA* gene (*iEIN2-HA*).

(C) GFP fluorescence in the roots of etiolated seedlings transiently treated with or without ACC and β-estradiol for 6 hr. "Removed," removal of both ACC and β-estradiol.

(D) Profiles of polysome-associated *EBF1*, *EBF2*, and *UBQ5* mRNAs in Col-0 and *ein2-5*.

(E) Immunoblot assays showing MYC-EBF1 protein abundance in etiolated seedlings of transgenic plants expressing *M1F* (*MYC-EBF1 CDS+3′UTR*) or *M1C* (*MYC-EBF1 CDS*).

(F) Immunoblot assays showing MYC-EBF1 and EIN2-HA protein abundance in transgenic plants expressing *iEIN2-HA* together with *M1F* or *M1C*. Note that multiple processed C-terminal fragments of induced EIN2-HA (CEND-HA) were also shown.

(G) Triple response phenotypes of etiolated seedlings corresponding to (F).

(H) Quantitative measurements of hypocotyls (left) and roots (right) of etiolated seedlings in (G). *p < 0.05; **p < 0.01; ***p < 0.001. Mean ± SD, n > 20.

See also Figure S4.

the removal of β-estradiol led to the efficient translation of *G1U* again (Figures 4B and 4C). A similar scenario was observed with transiently expressed β-estradiol-inducible *EIN2* and *G1U* in tobacco (Figure S4B), supporting that EIN2 is essential for *EBF1* 3′ UTR-directed translational repression.

To gain further evidence for EIN2-regulated *EBF1/2* mRNA translation, we compared the polysome profiles of *EBF1/2* mRNAs between Col-0 and *ein2* (Figure 4D). The polysome profiles of *EBF1/2* mRNAs remained virtually unchanged in *ein2* when treated with ethylene, in contrast with the apparent ethylene-induced polysome profile shifts observed in Col-0 (Figures 2C and 4D). Meanwhile, we found that the ethylene-evoked translational repression of *M1F* (*MYC-EBF1* full-length transcript) was abolished in *ein2* (Figures 4E and S4C), but exacerbated by addition of *EIN2* function (Figure 4F). By contrast, the translation of *M1C* (*MYC-EBF1 CDS*) remained unaffected upon depletion or addition of *EIN2* (Figures 4E, 4F, and S4C). Furthermore, the partial ethylene-insensitivity phenotype of *M1F* transgenic plants was largely suppressed by the overexpression of *EIN2*, whereas the strong ethylene insensitivity of *M1C* was hardly affected (Figures 4G and 4H). Taken together, these results indicate that 3′ UTR is a critical ethylene-responsive element to repress *EBF1* translation, and EIN2 is necessary and sufficient for directing such translational repression.

EIN2-Directed Translational Repression Is Mediated by PolyU Motifs of *EBF1/2* 3′ UTRs

We next dissected the functional *cis* elements within the *EBF1/2* 3′ UTRs by utilizing a dual-construct translation analysis system in tobacco leaves, in which a *3′ UTR* fragment of interest was fused with the *GFP* coding region, together with *mCherry* as the internal control in the same reporter construct (Figures 5A and S5A). The GFP intensities relative to mCherry intensities were calculated to indicate the translation efficiency of *GFP* mRNA (Figure 5B). Whereas the translation of *GFP* alone was not altered by introduction of *EIN2* and/or ACC application, the translation of *G1U* and *G2U* (*GFP* fused with *EBF1/2* 3′ UTR, respectively) was remarkably repressed by either expression of *EIN2* or ACC application (Figures S5B–S5D and S5K), and to a further extent when combining these two treatments (Figure S5C). As a control, expression of EIN3 protein had no effect on the translation of *G1U* (Figures S5E–S5H). These results confirmed the inhibitory effect of *EBF1/2* 3′ UTRs on translation in an EIN2-dependent manner.

EBF1 3′ UTR was arbitrarily segmented into five fragments ranging from 98 to 150 nt in length (Figure 5C). Three fragments, including *1Ua*, *1Ub* and *1Ud*, were able to mediate EIN2-induced translational repression (Figure 5D). Using the computation algorithm MEME and RNAfold, we identified a total of 7 poly-uridylates motifs in the predicted stem-loop structure within these three fragments (Figure 5E). These sequences were designated as Ethylene Responsive RNA elements containing Poly-Uridylates (*ERR-PolyU*, or *EPU* for short) (Figure 5E). Deletion of *EPUs* in each fragment or all seven *EPUs* in *1U,* which did not change their overall predicted secondary structures (Figure S5I), eliminated EIN2-directed translational repression (Figure 5F). Similarly, five *EPUs* were found in *EBF2* 3′ UTR (Figure S5J), and they were all required for *2U* to mediate EIN2-induced translational inhibition (Figure S5K). Sequence alignment of *EBF* 3′ UTRs from different plant species revealed that PolyU motifs are among the most conserved regions (Figures S5L and S5M), suggesting the 3′-UTR-mediated translational regulation as a well-preserved mechanism of ethylene signaling.

To further investigate the role of *EPUs* in relaying ethylene signaling, we generated the transgenic plants expressing either the *GFP-EBF1* full-length transcript driven by its own promoter (*pEBF1::G1F*) or seven *EPUs*-depleted version (*pEBF1::G1FΔ7U*) in *ebf1* mutant background. While expression of *pEBF1::G1F* rescued *ebf1* to the wild-type level, the *pEBF1::G1FΔ7U/ebf1* seedlings exhibited nearly complete ethylene insensitivity, phenocopying *pEBF1::G1C/ebf1* plants (*GFP-EBF1 CDS* driven by its own promoter) (Figure 5G). Consistent with the ethylene-response phenotype, the levels of EIN3 protein were much lower in both *pEBF1::G1FΔ7U/ebf1* and *pEBF1::G1C/ebf1* than that in Col-0 or *pEBF1::G1F/ebf1*, whereas the GFP-EBF1 protein was more abundant in the former two lines, particularly under ethylene treatment (Figure S5N). These results suggest that *EPU*-mediated translational inhibition plays a key part in regulating EBF1 protein abundance as well as ethylene signal transduction.

From *1Ud*, we selected a region harboring two *EPUs* that is predicted to form a hairpin structure (Figure 5H), and repeated it three times to construct an artificial *3′ UTR* that possessed six *EPUs* (*6x EPU*) (Figure 5H). Similar to *G1U*, the translation of *GFP-6x EPU* mRNA was highly reduced upon *EIN2* induction (Figure 5I). Furthermore, transgenic overexpression of *GFP-6x EPU* but not *GFP-1UΔ7U* conferred ethylene insensitivity phenotype (Figure S5O). Together, these results demonstrate that *EPUs* mediate the EIN2-directed translational repression of *EBF1/2*, which represents a crucial mechanism of ethylene signaling.

We also examined the functional domain of EIN2 in translational repression. By taking advantage of the tobacco system, we narrowed down the C-terminal end of EIN2 fragments (CEND) to amino acids (aa) 654–1272 that were required for translational repression (Figures 5J, 5K, and S5P). Within this region, a predicted nuclear localization signal (NLS, aa 1262–1269, LKRYKRRL) was previously identified to be required for the nuclear translocation as well as the functionality of CEND (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). We found that deletion or mutation of this NLS region also disrupted the function of CEND in translational repression (Figures 5J and 5K). Interestingly, replacement of the NLS with a distinct K/R-rich NLS sequence (NLS': KPKKKRKV) was able to relocate CEND into the nucleus but failed to restore its translational repression ability (Figures 5K and S6G). Together, these results suggested that the short motif (aa 1262–1269) was also critical for the translational repression function of EIN2 independent of its being a nuclear localization signal.

Association and Co-localization of EIN2 with *EBF1 3′ UTR* in Cytoplasmic Foci

We next investigated how EIN2 imposes translational repression of *1U/2U*-containing mRNAs. We first examined whether EIN2 associates with *1U in vivo*. RNA-immunoprecipitation assays (RNA-IP) in tobacco leaves indicated that EIN2 preferentially associated with mRNAs containing *1U* (*G1U, M1U*), but not

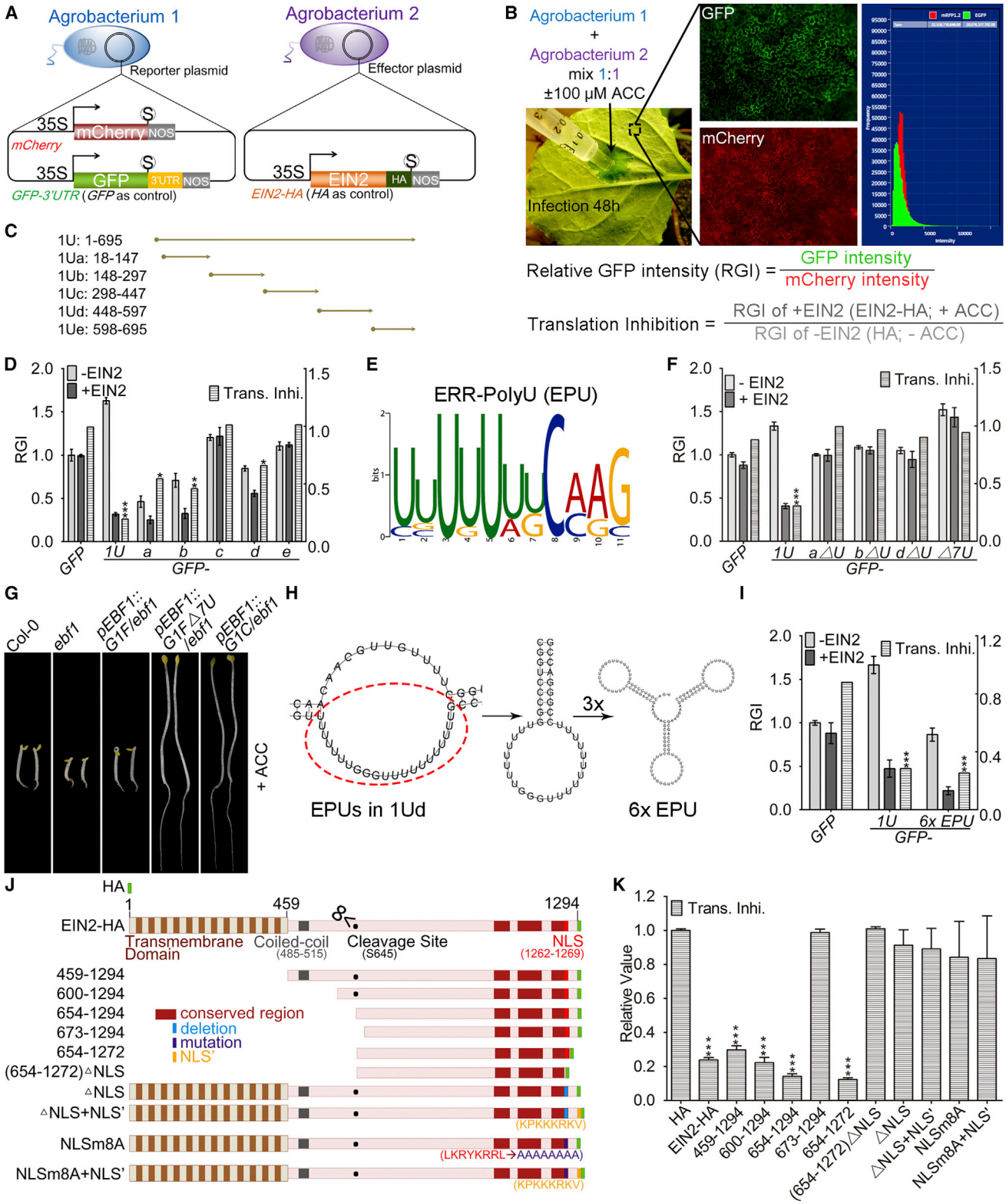

Figure 5. PolyU Motifs in *EBF1* 3′ UTR Are Necessary and Sufficient for EIN2-Directed Translational Inhibition

(A and B) Plasmids used in the dual-construct translation analysis system (A) as well as the workflow (B). The reporter plasmid harbors the reference gene *mCherry* and the reporter gene *GFP-3′UTR* (*GFP* as control). The effector plasmid possesses *EIN2-HA* (*HA* as control) (A). ACC application was used to further activate the

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with *GFP* mRNA alone, and ethylene enhanced the association between EIN2 and *G1U* mRNA (Figures 6A and S6A).

Next, we sought to examine the subcellular localization and dynamics of *1U*-containing mRNAs and EIN2. We adopted the MS2 system (Bertrand et al., 1998) to directly visualize the subcellular localization of *1U*-containing mRNAs. In this system, YFP was fused to the C terminus of MS2 coat protein (MY), and six tandem repeats of MS2 binding sites (6X MS2bs) were inserted into *M1U* to produce a reporter RNA *MYC-6X MS2bs-1U* (*M6U*), while a reporter RNA *MYC-6X MS2bs* (*M6*) served as a negative control (Figures 6B, S6B, and S6C). RNA-IP assay revealed the association of EIN2 and *M6U in vivo* (Figure S6A), and transgenic plants overexpressing *M6U* showed ethylene-insensitivity phenotypes (Figure S6D), demonstrating the functionality of this fusion RNA. In the absence of ethylene, *M6U* was observed to spread in the cytoplasm and concentrate in the nucleus, similar to the distribution pattern of *M6* (Figure 6C). Notably, ethylene treatment specifically induced *M6U* but not *M6* to form granules in the cytoplasm (Figures 6C and 6E).

Meanwhile, we found that ethylene treatment can also induce a proportion of EIN2 to form cytoplasmic foci in addition to its nuclear accumulation (Figures S6E and S6F; Movies S1 and S2). In the presence of ethylene, a portion of EIN2 protein and *M6U* mRNA were co-localized in cytoplasmic foci (Figure 6D). Furthermore, the cytoplasmic foci formation of *M6U* was abolished in *ein2* (Figure 6E), suggesting the requirement of EIN2 for foci formation of *1U*-containing mRNA. Taken together, these results suggest that ethylene promotes the association of EIN2 with *1U*, which in turn is targeted to cytoplasmic foci.

We further found that EIN2 CEND (aa 459–1294) as well as the minimal functional fragment of EIN2 (aa 654–1272) were also able to form cytoplasmic foci under ethylene treatment, whereas all the translation-dysfunctional fragments of EIN2, including aa 673–1294, deletion or mutation of NLS, failed to form foci in the cytoplasm (Figure S6G). It is noteworthy that the addition of another functional NLS sequence (NLS') could not restore the cytoplasmic foci formation of NLS-deleted or -mutated EIN2 (Figure S6G). Together with the observations made in Figure 5K, these results demonstrate that the NLS sequence of EIN2 is critical for its cytoplasmic foci formation as well as translational regulation function.

P-Body Is Involved in *EBF1/2* 3′-UTR-Mediated Translational Repression by EIN2

Given that *EBF1/2* RNAs are subjected to the regulation by EIN5, an exoribonuclease associated with processing body (P-body) (Decker and Parker, 2012; Xu and Chua, 2011), and that *1U*-containing mRNA forms cytoplasmic foci, we next determined whether *1U* directs its fusion mRNA to P-body. Upon ethylene treatment, both *M6U* mRNA and EIN2 protein were partly co-localized with EIN5 in cytoplasmic foci (Figures 7A and 7B), indicative of their P-body localization. Additionally, yeast-two-hybrid (Y2H) and luciferase complementation imaging (LCI) assay indicated that EIN2 CEND interacted with EIN5 (Figures 7C and 7D). Co-immunoprecipitation (Co-IP) assays revealed that EIN5 associated with EIN2 mainly in the presence of RNA, as treatment with RNase largely diminished EIN5-EIN2 association (Figure 7E). Furthermore, we found that several other P-body components, such as PAB2, PAB4 and PAB8 (Decker and Parker, 2012), also interacted with EIN2 CEND in yeast and plant cells in a RNA-dependent manner (Figures 7C, 7D, and S7A–S7D). In keeping with these biochemical results, knockout mutants of P-body component genes, such as *EIN5*, *PAB2*, *PAB8*, and *UPF1*, exhibited reduced ethylene sensitivity manifested by compromised triple response phenotypes, target gene expression, and EIN3 protein accumulation (Figures 7F and S7E–S7G). The combinations of these mutants led to increasing severity of ethylene-insensitivity phenotypes, particularly for the *ein5 upf1 pab2 pab8* quadruple mutant, which exhibited strong insensitivity to ethylene (Figures 7F and S7E). Therefore, several P-body components act cooperatively to repress the translation of mRNAs harboring *1U*.

Although UPF1 was not detected to physically interact with EIN2, we observed the binding of UPF1 to *1U* (Figure S7H), consistent with its function as a non-selective RNA binding protein (Hogg and Goff, 2010). The comparable mRNA levels of *EBF1* and *EBF2* between the P-body mutants and wild-type plants further supported a control of translation rather than transcription or RNA decay of *EBF1/2* by P-body (Figure S7F). Taken together, we proposed that after activation by ethylene, EIN2 CEND associates with the 3′ UTR of *EBF1/2* mRNAs and targets them to P-body via interacting with multiple P-body components, thus repressing the translation of *EBF1* and *EBF2*, resulting in EIN3/EIL1 accumulation and ethylene responses (Figure 7G).

DISCUSSION

A Cytoplasmic Mode of EIN2 Action in Ethylene Signaling

Recently, three groups have uncovered a "cleave and shuttle" mode of EIN2 action, wherein its C-terminal end (CEND) is

EIN2-HA protein. Translational inhibition was calculated by relative GFP intensity in the presence of EIN2-HA and ACC application (RGI of +EIN2) normalized with that without EIN2-HA and ACC (RGI of -EIN2) (B).

(C and D) Fragments of *1U* (*EBF1 3′ UTR*) and their effects on the translation of *GFP* mRNA with or without EIN2 function. *p < 0.05, **p < 0.01, ***p < 0.001. Calculations of translational inhibition were based on three biological repeats and the value of *GFP* control was set as 1.

(E) The PolyU ethylene responsive RNA elements (termed as *ERR-PolyU* or *EPU*) shared in the fragments *1Ua*, *1Ub*, and *1Ud*.

(F) The effects of *EPUs* on the translational inhibition. *ΔU*, deletion of *EPU*. *Δ7U*, deletion of all seven *EPUs* in full-length *1U*. ***p < 0.001.

(G) Triple response phenotype of etiolated seedlings in the presence of ACC. *G1F* (*GFP-EBF1* full length containing CDS and 3′ UTR), *G1FΔ7U* (*G1F* with all seven *EPUs* deleted), and *G1C* (*GFP-EBF1* CDS) were all driven by native *EBF1* promoter (*pEBF1*) and expressing in *ebf1* mutant.

(H and I) An engineered *6x EPU* fragment and its effect on translational inhibition upon EIN2 activation. G and C bases were added to the two *EPUs* in *1Ud* to produce a stem-loop structure, which was repeated three times to generate *6x EPU*.

(J and K) Scheme for different EIN2 fragments and their inhibitory effect on *G1U* (*GFP-EBF1 3′ UTR*) translation. ΔNLS indicates the deletion of the predicted nuclear localization signal. NLS′ represents a distinct NLS sequence. NLSm8A means the substitution of the NLS motif with eight alanine residues. ***p < 0.001. See also Figure S5.

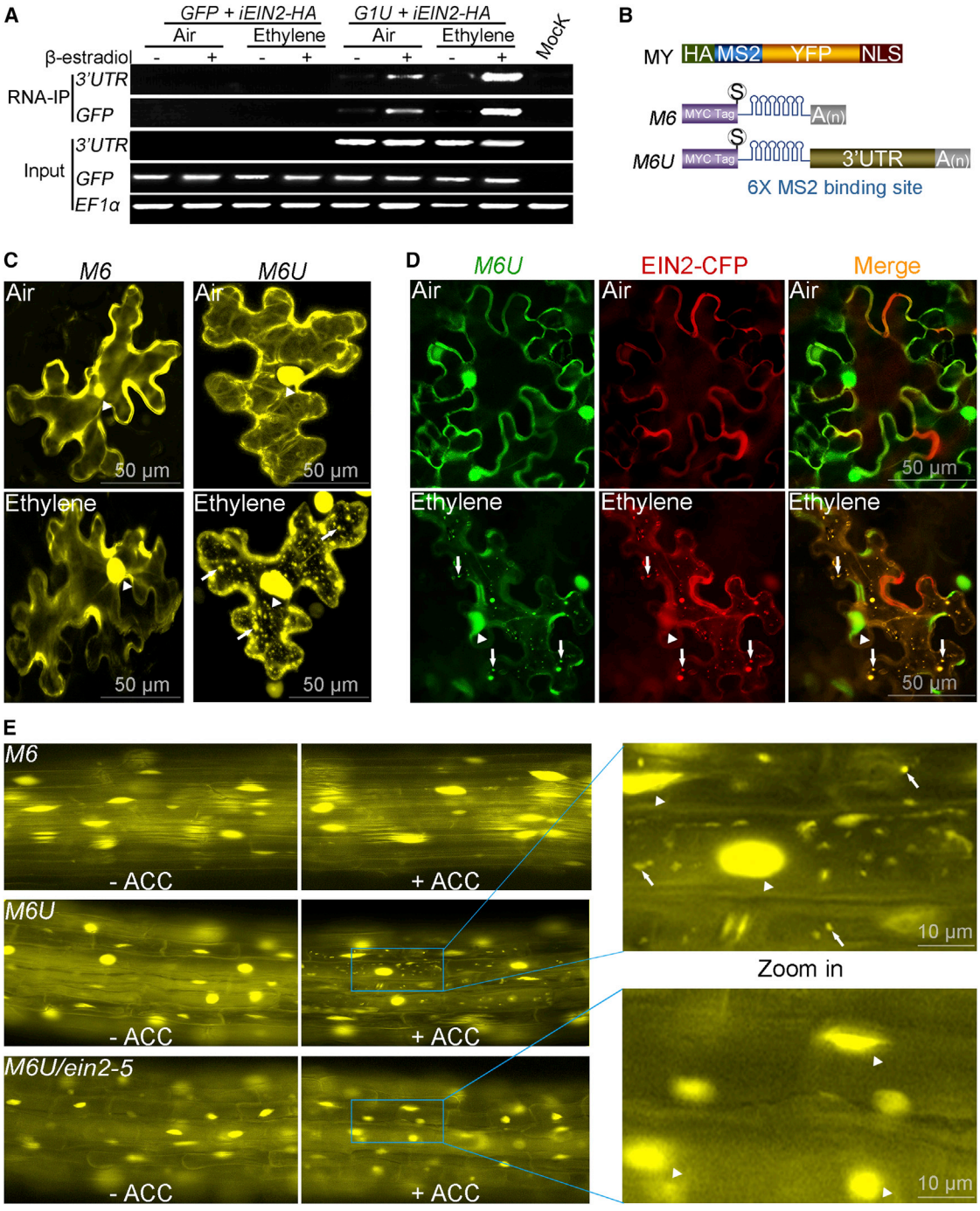

Figure 6. Ethylene Induces the Association and Co-localization of *EBF1 3′ UTR* with EIN2 in Cytoplasmic Foci

(A) RNA-IP assays indicating the association between EIN2 and *G1U* (*GFP-EBF1 3′ UTR*) in tobacco leaves. *GFP* acts as a negative control. (*iEIN2-HA*: β-estradiol-inducible *EIN2-HA*).

(B) Schematic diagrams of the MS2/RNA-MS2bs system. MY means MS2 coat protein linked with YFP; *M6* and *M6U*, *MYC-6X MS2 binding site* and *MYC-6X MS2 binding site -EBF1 3′ UTR*, respectively. S in a circle, stop codon.

(C) YFP fluorescence revealing the subcellular localization of *M6* and *M6U* RNAs in tobacco leaves treated with or without ethylene. Arrows mark cytoplasmic foci, while triangles indicate nuclei.

(D) Co-localization of EIN2-CFP and *M6U* in tobacco leaves upon ethylene treatment.

(E) The subcellular localization of *M6* or *M6U* in transgenic *Arabidopsis* seedlings treated with or without ACC. Right panels are zoom-in images of the boxed areas in left.

See also Figure S6.

processed and translocated into the nucleus to activate ethylene signaling (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). Here, we report another mechanism of EIN2-mediated ethylene signaling, whereby EIN2 imposes translational repression of *EBF1/2* mRNA in cytoplasmic P-body compartments. This cytoplasmic mode of EIN2 action was revealed by several lines of evidence: (1) EIN2 and ethylene treatment inhibit the translation of *EBF1/2* mRNAs. (2) EIN2 is both necessary and sufficient for the translational repression of *1U*-containing mRNAs. (3) EIN2 is colocalized and associated with *1U*. (4) *1U*, EIN2 and the EIN5 are co-localized in P-bodies upon ethylene treatment. (5) EIN2 interacts with several P-body factors including EIN5, PAB2/4/8. (6) Mutations in P-body protein genes led to evident ethylene insensitivity, particularly in combinations. Together with previous studies, our discovery illustrates that EIN2 guarantees the accumulation of key transcription factors EIN3/EIL1 in response to ethylene through at least two parallel mechanisms (Figure 7G). The cytoplasmic function of EIN2 is critical for quickly shutting down the protein synthesis of *EBF1/2*, leading to rapid depletion of EBF1/2 proteins due to its proteasomal degradation (An et al., 2010). Meanwhile, a subset of CEND is translocated into the nucleus to further stabilize and/or activate EIN3/EIL1 directly or indirectly (Ji and Guo, 2013).

It has been previously reported that ethylene application causes polysome prevalence during the ripening of pear and avocado fruits, suggesting a positive regulation of translation by ethylene (Drouet and Hartmann, 1979; Tucker and Laties, 1984). In an accompanying study, Merchante et al. (2015) used a plant-optimized genome-wide ribosome footprinting technique and successfully identified a group of mRNA targets that are upregulated or downregulated by ethylene at translational level. Of these targets, *EBF1* and *EBF2* are prominent as ethylene-repressed mRNAs that are dependent on EIN2 but not EIN3/EIL1, as observed also in our study. Thus, the EIN2-dictated translational control represents an early signaling event that operates in the cytoplasm either in parallel with or prior to the nuclear signaling cascade. Interestingly, this research together with previous studies (Qiao et al., 2012; Wen et al., 2012) revealed that a predicted NLS motif in the very C terminus of EIN2 is essential for its functions in both cytoplasm and nucleus. Given the recent finding that NLS is also critical for the association between EIN2 and the ethylene receptor ETR1 on ER (Bisson and Groth, 2015), it remains to be addressed how such short motif is involved in seemingly distinctive subcellular signaling events.

EBF1/2 3′ UTRs Function as Critical Ethylene-Responsive and Signal-Relaying Elements

In mammals, 3′ UTRs targeted by microRNAs are critical for the regulation of proto-oncogenes and tumorgenesis (Mayr and Bartel, 2009). Recent efforts were taken to systematically analyze human 3′ UTRs, and dozens of novel *cis*-regulatory elements were identified that affect mRNA stability and translation (Oikonomou et al., 2014; Zhao et al., 2014). Our study revealed that the 3′-UTR-mediated translational repression of *EBF1/2* is vital for relaying the ethylene signal in plants. The biological significance of this repression was demonstrated by the findings that deletion of *EBF1* 3′ UTR or the *EPU* motifs greatly enhanced the translation of *EBF1* mRNA and led to nearly complete ethylene insensitivity (Figures 4G and 5G). We further identified multiple PolyU motifs in the loop of predicted stem-loop structures (*EPUs*) as functional *cis* elements shared in *EBF1* and *EBF2* 3′ UTRs (Figure 5). Considering the conservation of EIN2 from green algae to land plants (Ju et al., 2015), and of PolyU motifs in the *EBF* 3′ UTR sequences from different plant species (Figures S5L and S5M), we believe that the 3′-UTR-mediated translational regulation might be an evolutionarily widespread mechanism of ethylene signaling.

Furthermore, our study indicates that the ethylene-induced *EBF1/2* translational repression is likely to be achieved by targeting *EBF1/2* transcripts into P-body in an EIN2-dependent manner. Although our initial in vitro pull-down assays failed to detect their direct binding, RNA IP experiment revealed the association of EIN2 with *EBF1* 3′ UTR in vivo (Figure 6A). It raises the possibility that some unidentified RNA binding proteins, which could specifically recognize PolyU motifs of 3′ UTRs, directly or indirectly interact with EIN2 and tether it to *EBF1/2* mRNAs (Figure 7G). EIN2, therefore, may act as a hormone-activated switch to target *EBF1/2* mRNAs (and probably other mRNAs as well) to P-bodies via interaction with P-body proteins.

Cytoplasmic foci, including P-body and stress granules, have been observed in plant cells under myriad stress conditions (Maldonado-Bonilla, 2014). The importance of P-body in ethylene signaling was manifested as mutants of several P-body components led to reduced ethylene sensitivity (Figure 7F). Therefore, ethylene, well known as a stress hormone, might adopt the translational repression mechanism via P-body to quickly shut down gene expression under adverse stress conditions.

Utilizing the Translational Interference Effect of 3′ UTR to Modulate Gene Function

Overexpression of the coding sequence of a gene had been widely utilized as a powerful genetic tool to study the gene functions in animals and plants (Prelich, 2012). In this study, we demonstrated that overexpression of *3′ UTR* could also result in remarkable interference with the function of their cognate genes as well as the signaling output. Several lines of evidence supported that the exogenous expression of *3′ UTR* leads to the enhancement or de-repression of the endogenous *EBF1/2* mRNA harboring the same or related 3′ UTR in a *trans*-acting manner. Given the strong phenotype of *3′-UTR*-overexpressing transgenic plants, our study offers an alternative tool to study and regulate the function of genes in vivo.

The translational interference effect of 3′ UTR illustrated in this work is reminiscent of the action of microRNA sponges (Ebert et al., 2007) as well as competitive endogenous RNA (*ceRNA*) in mammals (Denzler et al., 2014; Salmena et al., 2011), and microRNA target mimics in plants (Franco-Zorrilla et al., 2007), all of which share a common underlying mechanism referred to as molecular titration (Bosson et al., 2014; Buchler and Louis, 2008). As such, the accumulation of *3′ UTR* fragments, as observed in *ein5* (Souret et al., 2004), might hold biological importance, such as to coordinate or buffer the translational regulation of related mRNAs. In the future, a more systematic identification and study of 3′ UTRs in plants and animals would

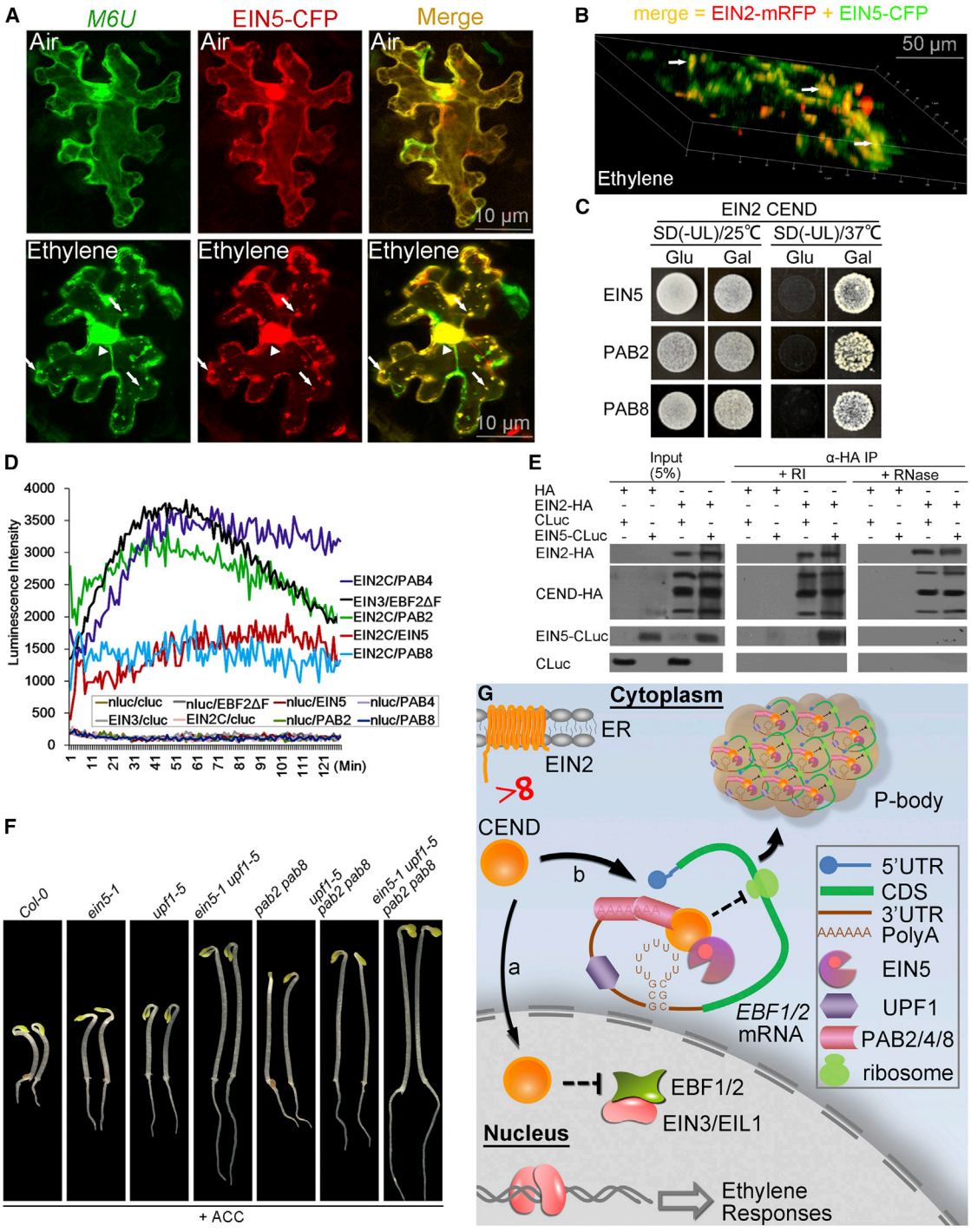

Figure 7. EIN2 Co-localizes with *EBF1 3′ UTR* in P-Body and Interacts with Multiple P-Body Components

(A) Co-localization of *M6U* (*MYC-6X MS2 binding site-EBF1 3′ UTR*) (green) and EIN5 (red) in P-bodies of tobacco leaves. Arrows mark cytoplasmic foci (P-bodies), while triangles indicate nuclei.

(B) A 3D image showing partial co-localization of EIN2 (red) and EIN5 (green) in P-bodies (arrow).

(C) Yeast-2-hybrid assays indicating the interactions between EIN2 CEND (889–1294) and EIN5 as well as PAB2/8.

(D) Luciferase complementation imaging (LCI) assays manifesting the interaction between EIN2 CEND and P-body components in *Arabidopsis* protoplasts. Combinations in the right list show strong interaction, while the others in the bottom box are either negative controls or exhibit no interaction.

(E) Co-immunoprecipitation assays indicating the association between EIN2 and EIN5 in the presence of RNA. Immunoblot assays showing the amount of expressed proteins in tobacco leaf extracts (input) and after IP with anti-HA antibody. HA and CLuc were used as negative controls. RI, RNase inhibitor.

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provide new information about gene functions as well as their regulatory mechanisms.

EXPERIMENTAL PROCEDURES

Arabidopsis Materials and Growth Conditions

The ecotype Columbia (Col-0) was the parent line for all mutants and transgenic plants used in this study. Transgenic lines in different genetic backgrounds were constructed by genetic crosses. Unless otherwise stated, all *Arabidopsis* seedlings were grown on MS medium supplied with or without 10 μM ACC, or other chemicals, for 3–4 days. For transient treatments, 100 μM ACC or 10 ppm ethylene was used for seedlings, and 100 μM ACC or 100 ppm ethylene was used for tobacco leaves.

Polysome Profiling

Arabidopsis polysomes were fractionated over sucrose gradients as described (Missra and von Arnim, 2014) with minor modifications. 3-day-old etiolated seedlings were treated with 10 ppm ethylene for 4 hr and then ground in liquid nitrogen followed by resuspension in polysome extraction buffer. Supernatant was loaded onto a 15%–60% sucrose gradient and spun in a Beckman SW40Ti rotor at 40,000 rpm for 4 hr at 4°C. We collected 12 fractions by a gradient fractionator. Total RNA in each fraction was isolated using TRIZOL reagent (Life Technologies) and then subjected to reverse transcription and real-time PCR analysis.

RNA Immunoprecipitation

4-week-old tobacco leaves were infected with the mixture of two agrobacterium strains. Two days after agroinfiltration, the tobacco leaves were treated with air or ethylene for 4 hr and subsequently collected to be ground in liquid nitrogen, and protein/RNA complexes were extracted using two volumes of IP buffer. After removal of insoluble debris by centrifugation, cell extracts were incubated with anti-HA antibody (Sigma) for 2 hr on ice with occasional gentle mixing. The anti-HA-decorated extracts were incubated with pre-washed protein G agarose beads. The co-immunoprecipitated RNA was isolated by TRIZOL reagent (Life Technologies) and analyzed by qRT-PCR.

Co-Immunoprecipitation

One-month-old tobacco leaves were infected with the mixture of three agrobacterium strains. Protein samples prepared from tobacco leaves 48 hr after Agrobacterium-mediated infiltration were homogenized in ice-cold IP buffer with the volume ratio of 1/2. After centrifugation, lysates were supplemented extemporaneously with RNase inhibitor (Promega) or RNase (Promega) and then incubated for 2 hr at 4°C under gentle agitation in the presence of EZview anti-HA affinity gel (Sigma). Antibody-coupled agarose beads were washed and subsequently denatured to detect the IPed proteins using western blot.

See Supplemental Experimental Procedures for details on the above-described materials and methods, as well as additional methods and procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, one table, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.09.037.

AUTHOR CONTRIBUTIONS

W.L., M.M., and H.G. designed all experiments, analyzed data, and wrote the manuscript. W.L. and M.M. performed most of the experiments and prepared data. W.L. and Y.F. conducted the polysome profiling assays. W.L. and Y.W. performed microscopy. H.L., M.L., and F.A. contributed to the generation and analysis of myriad transgenic plants. M.M. and Y.M. conducted LCI, yeast, and tobacco dual-construct translational assays. Y.F. and M.M. helped prepare the manuscript.

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(F) Triple response phenotypes of etiolated seedlings of indicated genotypes.
(G) A schematic model depicting two branches of ethylene signaling pathway relaying from EIN2 on the ER membrane into nucleus to regulate EIN3/EIL1 protein stability. The cytoplasmic mode of EIN2 action revealed in this study is highlighted as the formation of P-bodies containing CEND, *EBF1/2* 3′ UTRs, and several P-body proteins including EIN5, PABs, and UPF1.
See also Figure S7.

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