Uncertainty of EIN2$^{\text{Ser645/Ser924}}$ Inactivation by CTR1-Mediated Phosphorylation Reveals the Complexity of Ethylene Signaling

Jingyi Zhang$^{1,2,3}$, Yuying Chen$^{1,2}$, Jian Lu$^1$, Ying Zhang$^1$ and Chi-Kuang Wen$^1$.*  
$^1$National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai 200032, China  
$^2$These authors contributed equally to this article.  
$^3$Present address: Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA  
*Correspondence: Chi-Kuang Wen (qwen@sibs.ac.cn)

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ABSTRACT

ETHYLENE INSENSITIVE2 (EIN2) is a key component of ethylene signaling whose activity is inhibited upon phosphorylation of Ser$^{645}$ and Ser$^{924}$ by the Raf-like CONSTITUTIVE TRIPLE-RESPONSE 1 (CTR1) in the absence of ethylene. Ethylene prevents CTR1 activity and thus EIN2$^{\text{Ser645/Ser924}}$ phosphorylation, and subcellular trafficking of a proteolytically cleaved EIN2 C terminus (EIN2-C) from the endoplasmic reticulum to the nucleus and processing bodies triggers ethylene signaling. Here, we report an unexpected complexity of EIN2-activated ethylene signaling. EIN2 activation in part requires ethylene in the absence of CTR1-mediated negative regulation. The ein2 mutant was complemented by the transgenes encoding EIN2, EIN2 variants with mutations that either prevent or mimic Ser$^{645}$/Ser$^{924}$ phosphorylation, or EIN2-C; and all the transgenic lines carrying these EIN2-derived transgenes responded to ethylene. Furthermore, we found that the fluorescence protein-tagged EIN2 and its variants were affected little by ethylene and exhibited similar subcellular distribution patterns: in the cytosolic particles and nuclear speckles. Of note, the subcellular localization patterns of EIN2 proteins fused with a fluorescence protein either at the N or C terminus were similar, whereas EIN2-C-YFP was primarily observed in the cytosol but not in the nucleus. Western blots and mass spectrum analyses suggested a high complexity of EIN2, which is likely proteolytically processed into multiple fragments. Our results suggested a nuclear localization of the full-length EIN2, weak association of the EIN2$^{\text{Ser645/Ser924}}$ phosphorylation status and ethylene signaling, and the complexity of ethylene signaling caused by EIN2 and its proteolytic products in different subcellular compartments. We propose an alternative model to explain EIN2-activated ethylene signaling.

Key words: ethylene signaling, EIN2 phosphorylation, EIN2 complexity, CTR1, ETR1

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INTRODUCTION

Ethylene, a gaseous plant hormone, plays pivotal roles in plant growth and development of various aspects. With use of Arabidopsis as a model plant, a model for ethylene signal transduction is proposed primarily based on genetic and molecular biology studies, which respectively propose and explain a signal transduction framework. The present model describes a pathway whereby the ethylene receptors interact with and activate the downstream Raf-like CONSTITUTIVE TRIPLE-RESPONSE 1 (CTR1) protein that phosphorylates ETHYLENE INSENSITIVE 2 (EIN2). Phosphorylated EIN2 is retained at the endoplasmic reticulum (ER) membrane and may undergo ubiquitination mediated by the F-box proteins EIN2 TARGETING PROTEIN 1 (ETP1) and ETP2, subject to the 26S proteasome-mediated degradation. Ethylene binding to the ethylene receptors prevents activity of the receptors and CTR1, and underphosphorylated EIN2 undergoes proteolytic cleavage by an unidentified mechanism(s). The resulting EIN2 carboxyl portion, designated EIN2-C, traffics to the nucleus and the processing bodies (P-bodies), thus facilitating expression of ethylene response genes by the transcription.
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factors EIN3 and EIN3-LIKE 1 (EIL1) and reduction of translatable EIN3-BINDING F-BOX PROTEIN 1 (EBF1) and EBF2 transcripts, respectively. Ethylene treatment reduces EBF1- and EBF2-mediated EIN3/EIL1 ubiquitination, and EIN3 and EIL1 levels are increased to induce the expression of ethylene response genes (Guo and Ecker, 2004; Bisson and Groth, 2010; Ju and Chang, 2012; Ju et al., 2012; Qiao et al., 2012; Li et al., 2015; Merchanter et al., 2015). EIN2-C may have a role in chromatin modifications to increase the expression of ethylene response genes by EIN3/EIL1 (Zhang et al., 2016, 2017). Besides the aforementioned activities revealed for EIN2 in ethylene signaling, EIN2-C associates with the ethylene receptors and is primarily localized at the ER membrane, and EIN2-C transgene complements the ein2-1 mutation (Bisson and Groth, 2010, 2015). There may be a mechanism by which the ethylene receptors directly mediate ethylene signaling to EIN2 (Wang and Wen, 2015).

Evidence for CTR1-mediated EIN2 phosphorylation is supported by in vitro phosphorylation studies and mass spectrum analyses. Yeast two-hybrid assay revealed a CTR1-EIN2 interaction, and heterogeneously expressed CTR1 but not its kinase-defective ctr1-1 version phosphorylates EIN2. Mass spectrum analyses revealed in vitro phosphorylation of EIN2 at the SerE45 and SerR24 residues, in line with the analyses that revealed in vivo EIN2 phosphorylation in the absence of ethylene treatment, as opposed to treatment (Chen et al., 2011; Ju et al., 2012), whereby ethylene treatment prevents CTR1 activation.

Relevance of EIN2 functionality and its phosphorylation status is implied from transformation studies. Ectopic overexpression of the underphosphorylated EIN2A variant, with the SerE45Ala replacement, results in constitutive ethylene responses, whereas the EIN2E variant, with the SerE45Asp replacement that mimics constitutive phosphorylation, does not complement the loss-of-function ein2-5 mutation nor affect ethylene signaling (Qiao et al., 2012). Immune assays and cell biology studies suggest a coupling of proteolytic cleavage of EIN2 and EIN2-C trafficking to the nucleus in response to ethylene. Fluorescence of the YELLOW FLUORESCENCE PROTEIN (YFP)-tagged EIN2E and EIN2A is localized in the nucleus/ cytosol and cytosol, respectively, and a putative EIN2-C of EIN2 and EIN2E can be immunologically detected. Immune assays detected only the full-length EIN2C variant but not its C terminus (Qiao et al., 2012). Consistently, overexpression of the non-phosphorylated EIN2AA (with the SerE45Ala and SerR24Ala replacements) also results in constitutive ethylene responses in ein2-5. Immune assays detected the full-length EIN2-YFP for proteins from the non-treated but not ethylene-treated plants. Thus, ethylene may prevent EIN2 phosphorylation and promote EIN2 cleavage, and the resulting EIN2-C could traffic to the nucleus and activate ethylene signaling (Ju et al., 2012). Functionality of EIN2-C in nuclear ethylene signaling activation is supported by studies involving EIN2-C overexpression that leads to elevated ethylene responses, and EIN2-C-YFP is associated with the nucleus (Qiao et al., 2012; Wen et al., 2012). Besides the reported results that propose EIN2 as being associated with the ER membrane, an independent study detected EIN2 in the nuclear but not cytosolic fraction by western blot analysis, and EIN2 may be proteolytically cleaved into multiple fragments in the cytosol and nucleus (Wen et al., 2012). These findings add uncertainty and complexity to our understanding of EIN2 functioning and the underlying mechanism.

Contrary to the present model that CTR1 is essential for EIN2 inactivation, our previous studies revealed the presence of a mechanism by which ethylene signal transduction can in part be independent of CTR1. Expression of the truncated ETR11-349 and the ethylene-insensitive etr1-1 cheF1 fragments greatly reversed the ctr1-1 and ctr1-2 mutant phenotype, requiring REVERSION-TO-ETHYLENE SUSCEPTIBILITY 1 and reduced ETHYLENE RESPONSE FACTOR 1 (ERF1) levels, which mark degrees of the ethylene response (Xie et al., 2006, 2012; Qiu et al., 2012). According to the present model, EIN2 is largely unphosphorylated and conceivably constitutively active in the two ctr1 mutants. The repression of ethylene responses by the truncated ETR11-349 and etr1-1 cheF1 indicates the presence of a mechanism by which the receptors may negate the activity of underphosphorylated EIN2 and perhaps that of EIN2-C. We do not exclude that ethylene signaling repression in ctr1 mutants by the ETR1 N terminus might result from gain of function because the expressed N terminus does not naturally exist. Nevertheless, the finding that the ctr1-1 ein2W308*/EIN2 sesquimutant shows a mild constitutive ethylene response phenotype and is responsive to ethylene treatment may support the presence of such a mechanism (Zhang et al., 2014). In other words, with the ctr1-1 allele, EIN2 is theoretically predominantly underphosphorylated and thus active, and the responsiveness to ethylene of ctr1-1 ein2W308*/EIN2 indicates activation of underphosphorylated EIN2 requiring ethylene. Moreover, results from independent studies show that the ctr1-1 mutant is ethylene responsive (Hua and Meyerowitz, 1998; Qiu et al., 2012), and the underphosphorylated EIN2 may thus be activated by ethylene.

In an effort to continuously investigate ethylene signaling involving EIN2 independent of CTR1, we studied the functionality of underphosphorylated EIN2A and EIN2AA variants, as well as the phosphomimetic EIN2D and EIN2EE variants (with the D and E replacements for the SerE45 and SerR24 residues, respectively) by transgenic expression. Expression of each of those transgenes complemented the ethylene-insensitive ein2-1 or ein2-5 mutation, and the resulting transgenic lines were ethylene responsive. Moreover, overexpression of EIN2-C, which is believed to confer constitutive ethylene signaling in the nucleus (Qiao et al., 2012), elevated a mild level of constitutive ethylene responses in ein2-1, and EIN2p: EIN2-C ein2-1 lines were responsive to ethylene. Multiple lines of evidence suggest an association of the constitutive ethylene response degrees with the transgene expression levels in these transgenic lines. The fluorescence protein-tagged EIN2 and those EIN2 variants were detectable in both the cytosol and nucleus, and changes in the cytosolic and nuclear fluorescence intensity did not conclude a coupled process of cytosolic EIN2 trafficking to the nucleus. GFP-EIN2 and EIN2-YFP shared the similar subcellular fluorescence pattern, and the full-length EIN2 is likely localized in the nucleus as reported (Wen et al., 2012). Immune assays detected a great complexity of EIN2-YFP signals, confirmed by mass spectrum analyses, in line with the previous report (Wen et al., 2012). EIN2 and EIN2-C overexpression may lead to gain-of-function effects, and their subcellular localizations and functions need to be carefully interpreted. We proposed an alternative model explaining ethylene signaling activation by EIN2.
RESULTS

EIN2 Responds to Ethylene in the Absence of CTR1-Mediated Negative Regulation

We previously reported that the ctr1-1 ein2*W308*/EIN2 sesquimutant has a reduced constitutive ethylene response phenotype and is responsive to ethylene treatment, suggestive of the presence of a mechanism by which EIN2 requires ethylene to activate ethylene signaling in the absence of CTR1 negative regulation (Zhang et al., 2014). Here, we characterized ethylene responsiveness of ctr1-1 ein2W308*/EIN2 seedlings and examined whether the observation can be repeated in the context of different alleles.

The F1 sesquimutant was obtained from the genetic crossing of ctr1-1 ein2W308*/EIN2 and ctr1-1, and the ethylene dose–response curves for ein2*W308*/EIN2 and ctr1-1 ein2W308*/EIN2 seedlings differed at low ethylene range. At high ethylene doses (1 and 10 μL L⁻¹ ethylene), degrees of the growth inhibition for ein2W308*/EIN2 and ctr1-1 ein2W308*/EIN2 seedlings were similar and slightly weaker than that of wild-type (Columbia-0 [Col-0]) seedlings (Figure 1A; see Supplemental Figure 1A for their phenotypes). Hypocotyl lengths were shorter for wild-type than for ein2W308*/EIN2 and ctr1-1 ein2W308*/EIN2 seedlings at high ethylene range (effect sizes presented as mean difference = 1.78 and 1.73 mm at 1 μL L⁻¹, and mean difference = 1.32 and 1.14 mm at 10 μL L⁻¹, respectively, Tukey’s multiple comparisons). The hypocotyl lengths for the two genotypes were similar, at 1 and 10 μL L⁻¹ ethylene, respectively (Sidak’s multiple comparisons, P > 0.05), which indicates that CTR1 activity is largely prevented at the ethylene concentration ≥1 μL L⁻¹, and the increase in degrees of the ethylene response may be in large part independent of CTR1. The ctr1-1 seedling revealed a differential response to ethylene of different concentrations and was shorter than ein2W308*/EIN2 and ctr1-1 ein2W308*/EIN2 seedings (Figure 1A and Supplemental Figure 1B).

As controls, ein2-5, ein2W308*, and ctr1-1 ein2W308*/EIN2 seedlings were insensitive to ethylene, with the seedling hypocotyl growth not inhibited (Figure 1B and Supplemental Figure 1A). This analysis involved two factors, the ethylene dose and genotype, and the seedling hypocotyl lengths at each ethylene dose were compared by two-way analysis of variance (ANOVA) (Lu and Wen, 2019; Zhang and Wen, 2019). No statistical significance was detected for the interaction between genotypes and ethylene doses (P = 0.868) nor for ethylene effects on the seedling growth (P = 0.809). Consistently, a posteriori Tukey multiple comparisons detected no statistical significance between each genotype at each ethylene dose. These results supported a complete ethylene insensitivity for ctr1-1 by ein2W308* and the same effects of ein2-5 and ein2W308* on ethylene response suppression.
Figure 2. Effect of the Non-phosphorylated EIN2A/EIN2AA and Phosphomimic EIN2DD/EIN2EE Variants on Ethylene Signaling.

(A) Hypocotyl lengths of the wild-type (Col-0) and 35S:EIN2A-YFP (in Col-0) seedlings. Data are mean and SE, and dots are individual measurements (n > 30). Numbers are mean difference (CI0.95) as the effect size, and power estimates the reproducibility of statistical significance.

(B and C) qRT–PCR measures of ERF1 level and EIN2 and EIN2A-YFP transcript copy numbers, respectively, of wild type (Col-0) and 35S:EIN2A-YFP (Col-0) plants. Data are mean and SE; symbols represent independent biological replicates.

(D) Hypocotyl lengths of 35S:EIN2A-YFP ein2-1 seedlings with and without ethylene treatment. Wild type (Col-0) and ein2-1 are controls. L2 and L8 are two independent transgenic lines. Data are mean and SE, n > 30 for each measurement, and dots are individual measurements. Numbers are mean differences (CI0.95) as the effect size.

(legend continued on next page)
The *ctr1-1* mutation results in D694E replacement that greatly impairs *in vitro* CTR1 kinase activity to an undetectable level, and the *ctr1-1* protein cannot phosphorylate EIN2 *in vitro* (Huang et al., 2003; Ju et al., 2012). We investigated whether our observations can be reproduced in other *ctr1* alleles, and the strong *ctr1-3* allele, resulting in early termination, was involved (Huang et al., 2003). For data transparency and integrity, measurements of each individual seedling were presented in dot plots, and effect sizes were reported to add estimation but without statistical significance between *ctr1-1* and *ctr1-3* seedling hypocotyl lengths (Figure 1D for the effect size), with adjusted *P* = 0.7051 for non-treated and *P* = 0.9734 for ethylene-treated *ctr1-1* and *ctr1-3*. The *ctr1-1* and *ctr1-3* alleles may have the same effects on ethylene signaling.

We next examined whether the *ctr1-3* mutation may completely prevent ethylene-induced seedling growth inhibition in *ein2*/*EIN2* plants. *CTR1* and *EIN2* are tightly linked, and obtaining *ctr1 ein2* mutants by genetic cross is challenging. With use of the CRISPR/Cas9 technique to edit the *EIN2* gene in *ctr1-3*, the *ctr1-3 ein2*1359A1360 mutation was obtained and confirmed by sequencing, and the Cas9 transgene for the CRISPR/Cas9 technique was crossed out by genetic crossing with *ctr1-3*. The *ein2*1359A1360 mutation, designated *ein2*265*, results in an insertion of an adenine nucleotide between nucleotides 1359 and 1360 of the *EIN2* coding sequence, causing a frameshift at residue 453 and early termination at residue 465. The *ctr1-3 ein2*265* mutant was crossed with *ctr1-3* to obtain the *ctr1-3 ein2*265*/EIN2* sesquimutant at the F1 generation. The *ctr1-3* allele did not completely prevent the ethylene-induced growth inhibition of *ein2*265*/EIN2* seedlings, and ethylene treatment inhibited the hypocotyl growth of *ctr1-3 ein2*265*/EIN2* seedlings (Figure 1E, one-tailed *t*-test, *P* < 0.05, power = 1; Supplemental Figure 1A and 1B) by 2.55 to 1.79 mm (95% confidence interval, CI0.95, of mean difference, power = 1). Consistent with the seedling growth inhibition phenotype, expression of *Ethylen RESPONSE FACTOR1* (*ERF1*), which marks degrees of the ethylene response, was slightly higher in 3SS:*EIN2*265*-YFP* seedlings than in wild-type seedlings (Figure 2B). The *EIN2*265*-YFP* transgene expression was determined by quantitative RT-PCR (qRT–PCR). In this study, the qRT–PCR primer pair matched the *YFP* sequence, and the *EIN2*--*YFP* transcript copy response, comprehensive comparisons of the seedling hypocotyl growth in response to ethylene treatment and between genotypes for data in Figure 1 are summarized in Supplemental Figure 1B.

The constitutive growth inhibition degrees in *ctr1-1* and *ctr1-3* did not differ, and ethylene further activated ethylene signaling in the absence of CTR1 negative regulation. Both *ctr1-1 ein2*265*/EIN2* and *ctr1-3 ein2*265*/EIN2* behaved similarly in response to ethylene, which implies EIN2 as a target of ethylene in the absence of CTR1-mediated negative regulation. We propose that at ethylene concentration >1 μL l−1, CTR1 activity may be largely impaired, and the increase in ethylene responses by higher ethylene levels may be independent of CTR1. At high ethylene concentrations, hypocotyl growth inhibition of *ein2*/*EIN2* and *ctr1 ein2*/*EIN2* seedlings was slightly weaker than that of wild-type seedlings, likely a result of haploinsufficiency.

### Expression of Non-phosphorylated EIN2 Variants Does Not Confer Constitutive Ethylene Signaling

The unphosphorylated EIN25645A protein is considered active independent of ethylene, and its overexpression leads to a constitutive ethylene response phenotype in the *ein2*-5 loss-of-function mutant. Expression of the transgene encoding the phosphomimetic EIN25645E protein does not complement the *ein2*-5 mutation (Qiao et al., 2012). Our data suggested that without the negative regulation by CTR1-mediated phosphorylation, EIN2 still responds the ethylene signal and triggers ethylene signaling. EIN2 Ser645/Ser924 phosphorylation status and ethylene signaling appeared loosely associated, which prompted us to examine ethylene responsiveness by the nonphosphorylated EIN25645A (designated EIN2*) and EIN25645A/S924A (designated EIN2*2A*) variants.

The 3SS:*EIN2*2A*-YFP* seedling (in wild-type Col-0, from Qiao et al., 2012) did not show the reported typical constitutive ethylene response phenotype, and the etiolated seedlings were slightly shorter than the wild type by 1.82 to 0.86 mm (Figure 2A, CI0.95, of mean difference, power = 1). Consistent with the seedling growth inhibition phenotype, expression of *ETHYLENE RESPONSE FACTOR1* (*ERF1*), which marks degrees of the ethylene response, was slightly higher in 3SS:*EIN2*2A*-YFP* seedlings than in wild-type seedlings (Figure 2B). The *EIN2*2A*-YFP* transgene expression was determined by quantitative RT-PCR (qRT–PCR). In this study, the qRT–PCR primer pair matched the *YFP* sequence, and the *EIN2*--*YFP* transcript copy

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(E) qRT–PCR measures of *ERF1* level. Data are mean and SE, and symbols indicate independent measurements (*n* = 3, for three independent biological replicates).

(F and G) Ethylene dose–response curves for *EIN2p:EIN2*2A*-GFP* *ein2*-1 (*F*) and *EIN2p:EIN2-GFP* *ein2*-1 (*G*) seedlings; each genotype involves three independent transformations lines and *n* > 30 for each measurement.

(H) Silver treatment prevents ethylene-induced hypocotyl growth inhibition of *EIN2p:EIN2*2A*-GFP* *ein2*-1 seedlings. Data are mean and SE, and dots represent individual values, *n* > 30.

(I) Hypocotyl measurement of the indicated genotypes and transgenic lines respectively expressing *EIN2p:EIN2*, *EIN2p:EIN2*2A*, *EIN2p:EIN2*D0*, and *EIN2p:EIN2*2A* in *ein2*-5.

(J and K) qRT–PCR measures of transgene expression (*EIN2* copy number) (*J*) and *ERF1* levels for degrees of the constitutive ethylene responsiveness (*K*). For qRT–PCR, symbols indicate individual measurements, and error bars are SE. Each measurement involves at least 50 pooled seedlings as one biological replicate.

(L) Ethylene-induced seedling hypocotyl growth inhibition is prevented by 1-methycyclopropene (1-MCP). For the seedling hypocotyl measurement, dots are individual values and error bars are SEs, *n* > 30 for each genotype.
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number was determined by calibrating against the corresponding YFP CDNA. Similarly, the EIN2 transcript copy number in the wild type was measured by an EIN2-specific primer pair, calibrated against the EIN2 cDNA. qRT–PCR showed a similar copy number for EIN2 and EIN2Δ-YFP transcripts (Student’s t-test, power = 0.1), which indicates a similar transcript level for EIN2Δ-YFP and the endogenous EIN2 (Figure 2C).

The slightly increased growth inhibition phenotype and ERF1 level of 35S:EIN2Δ-YFP (Col-0) seedlings likely resulted from elevated total EIN2 transcripts from the endogenous EIN2 and the ectopically expressed transgene. Alternatively, the YFP tag, the Ser645Ala replacement, or both may marginally but not greatly elevate EIN2Δ-YFP activity. The effect of ethylene signaling activation by EIN2Δ-YFP appeared very minor, and whether the protein can be activated by ethylene remains to be determined. We next expressed the 35S:EIN2A-YFP transgene in ein2-1. As reported, EIN2 transgenes are highly prone to transgene silencing (Ju et al., 2012). In this study, unless specified, transgenic lines in the ein2-1 mutant background were analyzed at the homozygous T4 generation, and transgenics in some transgenic lines were partly or heterogeneously silenced/underexpressed at T4 and completely silenced at T5 and higher generations. The transgenic plants showed elevated degrees of seedling growth inhibition and were fully responsive to ethylene treatment (Figure 2D, one-tailed Student’s t-test, power = 1; Supplemental Figure 2A). Consistently, ERF1 levels were also elevated in the two lines in the absence of ethylene treatment, as compared with the untransformed parent genotype ein2-1 (Figure 2E; mean differences = 0.33 and 0.31, respectively, by Dunnett’s multiple comparisons test). The EIN2 transcript copy number of the wild type (Col-0) from the two independent measurements (Figure 2C and Supplemental Figure 2B) was not statistically significant, determined by Welch’s t-test (P = 0.119), whereas EIN2Δ-YFP transcript copy numbers were greater in lines L2 and L8 (Supplemental Figure 2B, in ein2-1 background) than in 35S:EIN2Δ-YFP (in Col-0; Figure 2G), determined by Dunnett’s multiple comparisons test (see Supplemental Figure 2C for the effect size). The stronger seedling growth inhibition of 35S:EIN2A-YFP ein2-1 lines, as compared with 35S:EIN2Δ-YFP (Col-0) seedlings, was likely associated with levels of the transgene expression.

Considering that CTR1 phosphorylates EIN2 at Ser645 and Ser924 (Ju et al., 2012), the weak constitutive ethylene responsiveness by EIN2Δ-YFP expression may result from incomplete prevention of phosphorylation. We next examined whether EIN2A-activated ethylene can be independent of ethylene. The EIN2p:EIN2AA-GFP transgene was expressed in ein2-1, and the ethylene dose–response curves were similar for the transgenic lines and wild type (Col-0) (Figure 2F), with various degrees of differences. As a control, we expressed EIN2p:EIN2-GFP in ein2-1, and the ethylene dose–response curves were similar for the lines expressing EIN2p:EIN2AA-GFP and EIN2p:EIN2-GFP (Figure 2F and 2G). The difference was not associated with the type of transgenes, indicative of a similar function for EIN2AA-GFP and EIN2-GFP in ethylene-triggered growth inhibition.

The relevance of transgene expression levels and degrees of the constitutive ethylene response was investigated. Of note, degrees of growth inhibition varied greatly for seedlings of the transgenic lines L1 and L2 at higher generations (T4 and higher) (Figure 2H and Supplemental Figure 2D). The large variability may be caused by heterogeneous underexpression of the transgene of individual seedlings, and transcription levels of the transgene in seedlings with elevated growth inhibitions may be underestimated by the variability. Regardless, the average EIN2 transcript copy by qRT–PCR revealed an association between EIN2 levels and degrees of the seedling growth inhibition (Figure 2H and Supplemental Figure 2D–2F). The ethylene-induced seedling hypocotyl growth inhibition was also associated with ERF1 induction, except for line L2 (Supplemental Figure 2G), for which the transgene may be heterogeneously silenced to various degrees, and qRT–PCR for pooled seedlings of great variability may underestimate ERF1 levels of individual seedlings with an elevated growth inhibition phenotype.

Silver may compete with the cuprous ion for ethylene binding to the ethylene receptor ETR1 and convert ETR1 to an ethylene-insensitive state to prevent ethylene signaling (Rodríguez et al., 1999; McDaniel and Binder, 2012). Ethylene-treated seedlings grown on medium supplemented with silver nitrate prevented the ethylene-induced growth inhibition and ERF1 induction (Figure 2H; Supplemental Figure 2D and 2G). EIN2AA-activated ethylene signaling involves the inactivation of ETR1 and possibly the receptor family members upon ethylene treatment. The present results may support the argument that EIN2AA can be activated by ethylene and that EIN2 and EIN2AA may behave similarly in response to ethylene. The elevated degrees of constitutive ethylene responsiveness are likely associated with expression levels of the EIN2AA-GFP transgene.

Expression of EIN2AA, EIN2DD, and EIN2EE Reverses ein2-5 to Ethylene Responsiveness

Our results suggest that ethylene may activate the underphosphorylated EIN2AA to mediate ethylene signaling, which prompted us to examine whether the phosphomimic EIN2AA-activated ethylene-induced growth inhibition and ERF1 induction (designated EIN2AA) and EIN2DD-activated ethylene signaling involves the inactivation of ETR1 and possibly the receptor family members upon ethylene treatment. The present results may support the argument that EIN2AA can be activated by ethylene and that EIN2 and EIN2AA may behave similarly in response to ethylene. The elevated degrees of constitutive ethylene responsiveness are likely associated with expression levels of the EIN2AA-GFP transgene (Ju et al., 2012).

Stable transgenic lines in the ein2-5 mutant background expressing EIN2 and its variants were studied at the homozygous T4 generation. All the non-treated (air) transgenic lines had shorter hypocotyl lengths than the ethylene-treated wild type (Figure 2I; Supplemental Figures 3A and 4). qRT–PCR measurement revealed higher levels of transgene expression for the transgenic lines than wild type (Figure 2J and Supplemental Figure 3B). Unlike the large variation in the seedling hypocotyl length of ethylene-treated EIN2p:EIN2AA-YFP ein2-1 transgenic lines that were studied at the T4 generation, the variation for ethylene-treated transgenic lines were similar to that of the wild type (Figure 2I and Supplemental Figure 4); likely the transgenes were stably expressed at the T4 generation. In line with the slightly increased seedling growth inhibition, qRT–PCR results revealed slightly elevated ERF1 levels for the transgenic
lines (except for EIN2AA of L5, which was slightly shorter than wild-type seedlings) than for the wild type (Col-0) (Figure 2K and Supplemental Figure 3C) in the absence of ethylene treatment.

We showed a reversion of ethylene-induced seedling growth inhibition by silver treatment for EIN2p:EIN2AA-GFP ein2-1 transgenic lines, which implies involvement of the ethylene receptors in the negative regulation of EIN2AA activation. As silver might exert side effects on seedling growth, we investigated the involvement of the ethylene receptors in EIN2 inactivation with treatment of 1-methylcyclopropene (1-MCP), which competes with the ethylene binding site to prevent ethylene signaling (Hall et al., 2000). In line with results of silver treatment, 1-MCP prevented ethylene-induced seedling hypocotyl growth inhibition in all transformation lines (Figure 2L).

Variability in the Subcellular Distribution of EIN2-YFP

The present model proposes a coupling of a decrease of ER-bound EIN2 and an increase of nuclear EIN2-C as a result of proteolytic cleavage of the underphosphorylated EIN2 at the ER membrane trafficking to the nucleus (Ju et al., 2012; Qiao et al., 2012). Our results suggested an irrelevance of EIN2Ser645/Ser924 phosphorylation status and EIN2-activated ethylene signaling, raising a question as to whether subcellular EIN2 redistribution is associated with ethylene signaling.

Transgenic lines requested from reported studies (Ju et al., 2012; Qiao et al., 2012) appeared to be greatly silenced for the 35S:EIN2-YFP transgene, and the fluorescence signal was not detectable. Among these, one unstable line (line 7 from C. Chang) showed a wide range of constitutive ethylene responsiveness at the seedling stage, and ethylene treatment increased degrees of seedling growth inhibition (Figure 3A and 3B). The constitutive ethylene response phenotype heterogeneously disappeared soon at later stages, possibly due to heterogeneous transgene silencing of individual seedlings.

Among these seedlings, the YFP fluorescence was detectable only by laser scanning confocal microscopy (LSCM) from seedlings with the typical ethylene growth inhibition phenotype (4 mm and shorter in seedling hypocotyl length). The LSCM setting detected an autofluorescence background for ethylene-treated wild-type seedlings at the apical hook and root tip regions but not in hypocotyls (Figure 3C–3E). To avoid false positives, we did not examine the fluorescence for seedlings with a weaker phenotype with the use of unreasonably strong laser settings and a larger pinhole, and only fluorescence in hypocotyl cells was examined.

Complying with image data integrity that presents all aspects of image information from an observation (https://blogs.plos.org/plos/2019/08/redefining-standards-in-image-data-reporting-a-new-policy-at-plos-one-and-plos-biology-requires-raw-blot-and-gel-image-data/) (Not picture-perfect, 2006), the LSCM images shown were not cropped or touched. Consistent with most of the LSCM images presented in previous studies in Arabidopsis cells, two types of the EIN2-YFP fluorescence pattern were observed for the non-treated seedling cells: fluorescence particles and aggregated patches in the cytosol and speckles in the nucleus (Ju et al., 2012; Qiao et al., 2012) (Figure 3F and Supplemental Figure 5). Of note, cell-to-cell variability in the fluorescence distribution was wide over the z axis (focal planes); nevertheless, cytosolic fluorescence particles/aggregates and nuclear EIN2-YFP fluorescence speckles were consistently detectable in different cells (Supplemental Figure 5).

Details of the nuclear speckles, as well as a few nearby cytosol EIN2-YFP fluorescence particles, observed by the Airyscan technique (Zeiss LSM 880), are shown in Figure 3G and 3H. Independent cytosolic fluorescence particles and nuclear speckles, but not the ER-characteristic network, were observed at a high magnitude over the z axis (63× objective and 10× ocular, Supplemental Figure 6). However, the ER-characteristic network structure shown in previously published LSCM images from tobacco cells was not observed under our LSCM setting in Arabidopsis cells.

The full-field LSCM image revealed extensive variability in subcellular fluorescence over the z axis in independent cells (Supplemental Figure 5). The cell-to-cell variability may lead to distinct or biased interpretations about the EIN2-YFP distributions when certain focal planes and image regions are cropped and sampled, and inference for cell biology observations requires appropriate statistical evidence (Vaux, 2012). Paired samples (before and after treatment, at the same focal plane) instead of independent samples (unpaired; treated versus non-treated) are studied for EIN2-YFP relocation in response to ethylene treatment (Zhang and Wen, 2019). Practically, observing paired samples is not achievable by ethylene treatment, and ethylene treatment was replaced by 1-aminocyclopropane-1-carboxylic acid (ACC) as a proxy. A 10-min ACC treatment is sufficient to observe changes in EIN2-YFP levels and distribution (Qiao et al., 2012). In this case a 15-min ACC treatment was applied by replacing water with ACC without disturbing the samples. A prolonged ACC treatment is not considered because ACC conversion to ethylene is oxygen dependent, and oxygen may be depleted with a prolonged treatment in aqueous solution.

The fluorescence of the same cells at the same focal plane, before and after the ACC treatment, was quantified and compared (Supplemental Figure 7A–7C for large, untouched images) by paired t-test instead of a standard Student’s t-test (Lu and Wen, 2019; Zhang and Wen, 2019). This allowed us to directly evaluate the effects of ACC treatment on paired cells, avoiding the sample-to-sample variability. Our results revealed statistical significance (one-tailed t-test, $z = 0.05$) for the difference in total (power = 0.999; the Pearson correlation coefficient $r = 0.97$ suggested an effective pairing), cytosol (power = 0.998, $r = 0.966$), and nuclear fluorescence intensity (power = 0.965, $r = 0.978$) after the ACC treatment (Figure 3I). The before-and-after treatment fluorescence difference for cells of paired samples suggested an increase in EIN2-YFP levels in the cytosol and nucleus (Figure 3J). Because of sample-to-sample variability as well as various degrees of transgene silencing, the amount of difference for different paired samples varied. The increase in the fluorescence intensity was much greater in the cytosol than in the nucleus, and the increase in nuclear fluorescence cannot simply be due to reduced cytosolic EIN2-YFP by its cleavage and trafficking to the nucleus, as previously inferred (Qiao et al., 2012).
In conclusion, the EIN2-YFP fluorescence was predominantly observed in both the cytosol and nucleus, regardless of ethylene treatment. The cytosol EIN2-YFP fluorescence was dispersed particles, as revealed at a high magnification and by the Airyscan technique, distinct from the characteristic ER network structure. Ethylene may elevate EIN2-YFP levels but have little effect on EIN2 redistribution.

Subcellular Localization of EIN2 and EIN2 Variants in Arabidopsis

EIN2, underphosphorylated EIN2AA, and phosphomimic EIN2DD and EIN2EE exhibited similar behaviors in ethylene signaling. This prompted us to determine the subcellular localization of these variants, which may help address whether they may function similarly.

In their homozygous transgenic lines, degrees of the constitutive ethylene response were mild, and the expressed YFP fusion proteins were not detectable by LSCM or immune assays. Nevertheless, a few individual transformation seedlings at the T1 or T2 generation showing a constitutive ethylene response phenotype were studied for the subcellular distribution of those EIN2 variants (Supplemental Figure 8A for the T1 seedling phenotype). Consistently, the fluorescence was observed as particles and aggregates in cytosol and speckles in the nucleus for seedlings.
expressing EIN2AA-GFP, EIN2DD-GFP, and EIN2EE-GFP (Figure 4A–4C; see Supplemental Figure 8B for independent large images). Subcellular distribution of these proteins was also observed in Arabidopsis protoplasts by transient expression. YFP-fused EIN2AA, EIN2DD, and EIN2EE were each co-expressed with EIN2-mRFP, which served as an internal reference, and fluorescence of the EIN2 variants and the wild-type EIN2-mRFP merged completely (Figure 4D–4F).

EIN2 may bind the 3’ untranslated region (UTR) of EBF1 and EBF2 transcripts to P-bodies for translational inhibition, which prompted us to determine whether the cytosolic EIN2-YFP fluorescence was associated with P-bodies. DCP1 and EIN5 are considered two P-body components (Xu and Chua, 2009; Li et al., 2015), and the fluorescence particles of EIN5-YFP and mRFP-DCP1 can be fully superimposed in Arabidopsis protoplasts regardless of their relative levels (Figure 4G and 4H). The fluorescence pattern for mRFP-DCP1 and EIN5-YFP was the same as the previously reported DCP1-YFP fluorescence pattern in tobacco epidermis cells and Arabidopsis seedling cells (Xu and Chua, 2009). In contrast, the fluorescence patterns were distinct for EIN5-YFP and EIN2-mRFP and barely overlapped (Figure 4I).

The fluorescence merge of transiently expressed mRFP-EIN2 and YFP-tagged EIN2 variants suggested the same subcellular distribution and perhaps the same functionality in subcellular compartments. EIN2-mRFP and EIN5-YFP did not co-localize, whereas mRFP-DCP1 and EIN5-YFP merged completely in Arabidopsis protoplasts. In this regard, most of the cytosol EIN2 was not associated with P-bodies under our experimental setting. Alternatively, the amount of EIN2-mRFP greatly exceeded levels of the endogenous EBF1/EBF2 transcripts and thus was not observed at P-bodies.

**Complexity of EIN2 Protein**

EIN2-C, cleaved from the full-length underphosphorylated EIN2 upon ethylene treatment, is suggested to traffic to the nucleus and activate ethylene signaling. However, western blot analysis detected six proteins from immunologically purified EIN2-GFP, and the full-length EIN2-GFP is detectable from the nuclear fraction (Wen et al., 2012). Among the many EIN2 fragments, the exact EIN2-C(s) that activates ethylene signaling in the nucleus and at P-bodies remains to be determined.

The complexity of the EIN2 protein was reported in only one study by immune assays, which confirms that EIN2 cleavage products will be important for shedding light on the association of EIN2 functionality and its subcellular localization. With the nature of various degrees of heterogeneous transgene silencing (Figure 3A and 3B), 35S:EIN2-YFP seedlings with a typical constitutive ethylene growth inhibition phenotype were sampled to examine EIN2-YFP protein and its cleavage. To comply with image data integrity (Not picture-perfect, 2006), we presented results from independent, full western blots. Besides a relatively

**Figure 4. Subcellular Localization of EIN2 Variants.**

(A–F) Fluorescence of transformation seedlings, with a typical triple-response phenotype, expressing EIN2p:EIN2AA-GFP (A), EIN2p:EIN2DD-GFP (B), and EIN2p:EIN2EE-GFP (C) in the indicated genotypes. Co-localization of EIN2-mRFP with EIN2AA-YFP (D), EIN2DD-YFP (E), and EIN2EE-YFP (F) in Arabidopsis protoplasts. (G and H) The P-body components EIN5-YFP and mRFP-DCP1 are co-localized. (I) EIN2-mRFP and the P-body component EIN5-YFP have distinct fluorescence patterns.
faint immune signal that was barely detected near the expected molecular weight position (170 kDa) for the full-length EIN2-YFP, complex immune signals at other molecular weight positions were detected (Figure 5A). The strength of the immune signals (Figure 5B) was associated with degrees of seedling growth inhibition (Figure 5C) and ERF1 level (Figure 5D). Except for a few background signals, these signals were not detected for proteins from the wild-type seedlings. Silver treatment that prevents ethylene signaling had little effect on the immune pattern of EIN2-YFP (Figure 5E), which is indicative of proteolytic cleavage of EIN2-YFP independent of ethylene. Notably, independent western blots revealed that EIN2-YFP may form high-molecular-weight aggregates of various degrees above the 245-kDa marker position (Figure 5A, 5B, and 5E).

Figure 5. Analyses of EIN2 Protein.
(A–D) Immune signals of proteins from light-grown, 3-day-old 3SS:EIN2-YFP seedlings with a typical constitutive ethylene response phenotype (A). EIN2-YFP level (B) is associated with degrees of growth inhibition (C) and ERF1 level (D).
(E) Immune assays for EIN2-YFP for seedlings grown on media with the indicated silver dosages.
(F) A graphical presentation of EIN2 peptides detected from two independent mass spectrums analyses for proteins from the indicated positions in (A), from two independent biological samples.
(G) EIN2 sequence and the putative trypsin cleavage sites (highlighted in green and yellow). The shaded area indicates the putative N-terminal transmembrane and inter-transmembrane regions.
(H) Red and yellow fluorescence by the mRFP-EIN2-YFP fusion protein expressed in Arabidopsis protoplasts.
(I and J) Full field of LSCM images shows the fluorescence of GFP-EIN2 from EIN2p:GFP-EIN2 ein2-5 seedlings with a typical constitutive ethylene response phenotype at the indicated magnitudes. For qRT–PCR, symbols indicate individual measurements, and error bars are SE. Each measurement involves at least 50 pooled seedlings as one biological replicate.
Results from our western assay were greatly in line with the previously reported study that was conducted with suspension culture cells expressing EIN2-GFP. One exception is that the transient (4-h) silver treatment to the suspension culture pre-culture cells expressing EIN2-GFP cleavage (Wen et al., 2012), whereas our experiments involved living seedlings grown on medium supplemented with silver. Whether the difference is caused by experimental materials and settings needs further determination.

To examine whether the immune signals were from specific interactions of the antibodies and the YFP tag, we individually analyzed proteins from SDS–PAGE gel slices at the indicated molecular weight positions (Figure 5A; E1: 170 kDa; E2: 100 kDa; E3: 80 kDa; E4: >245 kDa) by the mass spectrum analysis technique. Two independent SDS–PAGE and mass spectrum analyses from independent plant samples were conducted (Figure 5F). Except for most of the N-terminal transmembrane regions (the predicted residues 12–458, including the inter-transmembrane regions) (Alonso et al., 1999), EIN2 peptides were detected from proteins at the 170-kDa position (E1). Peptides of the transmembrane region were not detected from proteins from the other two gel slices (E2 and E3; Figure 5F). Only a few peptides of the EIN2 transmembrane region were detected in this analysis, likely due to rare trypsin sites at the region; thus, the large fragment was not detectable by the mass spectrum technique (Figure 5G).

The present data indicate that, regardless of ethylene treatment, overexpressed EIN2 protein may already exist as various proteolytic forms. The complexity of EIN2 agrees with a previous independent study that detected multiple immune signals (Wen et al., 2012). The association of EIN2-GFP with the nuclear fraction unveiled by Wen et al. (2012) is distinct from the previous inferred at the ER membrane. We tagged mRFP and YFP at each terminus of EIN2, mRFP-EIN2-YFP, to examine whether the fluorescence distribution may differ due to the inferred proteolytic cleavage. Expressed in Arabidopsis protoplasts, the fluorescence by mRFP and YFP of mRFP-EIN2-YFP completely merged (Figure 5H). The fluorescence in protoplasts could not clearly reveal its distribution in detail. GFP-EIN2 subcellular localizations were next investigated in the ein2-5 mutant, with GFP tagged at the amino (N) terminus of EIN2. Although stable EIN2p:GFP-EIN2 ein2-5 transgenic lines were unavailable, likely due to transgene silencing, individual T1 seedlings with a typical constitutive triple-response phenotype were examined by the LSCM technique. If the N terminus is associated with the ER membrane, the fluorescence is expected to be characteristic of the ER network and outside the nucleus. Of note, fluorescence by the N-terminal-tagged GFP-EIN2 was similar to that by the C-terminal-tagged EIN2-YFP; the fluorescence was particles in the cytosol and speckles in the nucleus (Figure 5I and 5J; see Supplemental Figure 9 for independent large images over the z axis). This result may support the nuclear localization of the full-length EIN2 by western blots, and EIN2 distribution is not likely to be associated with the ER. However, the subcellular distribution of the many proteolytically cleaved EIN2 products remains to be determined.

The present data revealed the complexity of EIN2 protein products and their subcellular distribution, with their functionality remaining to be determined. In addition, how the >245-kDa aggregate may form and why it was partly resistant to SDS/β-mercaptoethanol treatment remains to be investigated. One explanation is that an excess amount of membrane-bound protein may aggregate and misfold to high degrees; however, this explanation is not supported by the mass spectrum data in which peptides at the transmembrane region were not detected.

### EIN2 C-Terminal Subcellular Distribution and Its Activation by Ethylene

The EIN2 C terminus (EIN2-C) is considered the cleaved product of EIN2, translocating to the nucleus to activate ethylene signaling (Qiao et al., 2012; Zhang et al., 2017). However, the putative, endogenous EIN2-C fragment has yet to be identified among the many cleavage products. In contrast to the report of constitutive activation of ethylene signaling by EIN2-C (Qiao et al., 2012), an independent study already reported that EIN2-C overexpression complemented the ein2-1 mutation and that the plants are ethylene responsive (Bisson and Groth, 2010). Previous immune assays revealed the complexity of the EIN2 protein, regardless of ethylene treatment (Wen et al., 2012), and our LSCM studies revealed a similar subcellular fluorescence distribution pattern for GFP-EIN2 and EIN2-YFP. Functionality and subcellular localization of the artificially designed EIN2-C may need further confirmation to address its role in ethylene signaling activation.

Expression of the EIN2p:EIN2-C transgene, encoding EIN2<sup>1259–1294</sup>, as described (Wen et al., 2012) in ein2-1 (homozygous T<sub>3</sub> generation), resulted in elevated degrees of constitutive ethylene responses, and the plants were ethylene responsive as determined by degrees of the ethylene seedling growth inhibition (Figure 6A and Supplemental Figure 10) and ERF1 expression (Figure 6B). Degrees of the constitutive ethylene response of the transgenic lines were associated with levels of the transgene expression (Figure 6C).

Stable transgenic lines overexpressing fluorescence protein-tagged EIN2-C and EIN2-C variants with the Ser<sup>645</sup>/Ser<sup>924</sup> modification for the LSCM observation at a detection level were unavailable. A few independent T<sub>1</sub> or T<sub>2</sub> transformation seedlings with a typical or mild constitutive ethylene response phenotype were studied for EIN2-C subcellular distribution, and the fluorescence was prevalently distributed in the cytosol, without a specific pattern (Figure 6D). The subcellular EIN2-C-GFP was carefully examined over the z axis, and part of the fluorescence was outlined but not localized within the nucleus (Supplemental Figure 11). Images of the bright field and fluorescence were contrasted, and the fluorescence was not observed for many of the nuclei regardless of the Ser<sup>645</sup>/Ser<sup>924</sup> modifications (Supplemental Figure 12). EIN2-C-GFP fluorescence that surrounded or was at the nucleus was distinct from the nuclear fluorescence speckles shown by the full-length EIN2-YFP and EIN2-GFP.

The transgenic lines were ethylene responsive, which prompted us to investigate whether the ethylene-induced seedling growth inhibition was associated with EIN2-C translocation to the nucleus. To prevent false negatives caused by the removal of ethylene treatment for prolonged LSCM observation, we treated the seedlings with ACC as a proxy of the ethylene gas. LSCM revealed that ACC treatment did not result in fluorescence...
Figure 6. Activation of EIN2-C by Ethylene and Its Subcellular Localization.
(A–C) Hypocotyl length (A), ERF1 expression level (B), and EIN2 transcript level of EIN2:pEIN2-C ein2-1 transgenic lines (C). Data are means and SE, and numbers in (A) are CI.95 of mean differences as the effect size of ethylene treatment. Dots (A) and symbols (B and C) indicate individual values. More than 30 seedlings (n > 30) were measured for hypocotyl length. For gene expression measurement, at least 50 seedlings were pooled as one independent sample, and three independent samples were measured.
(D) Subcellular fluorescence distribution of the indicated transgenic lines.
(E) EIN2-C-YFP is not co-localized with the nuclear protein SR33-mRFP.
(F) EIN2-C-YFP fluorescence in tobacco epidermis.
(G) Western blot for EIN2-C-YFP and the indicated variants expressed in Arabidopsis protoplasts; proteins from wild type (Col-0) are a control. Numbers indicate the corresponding molecular weights (kDa).
localization to the nucleus, which indicates that EIN2-C and its variants did not translocate into the nucleus to activate ethylene signaling (see Supplemental Figure 13A–13C for independent large images).

Subcellular localization of EIN2-C was also determined by transient expression of 35S:EIN2-C-YFP in the wild-type Arabidopsis protoplast. Observed by LSCM, EIN2-C-YFP fluorescence was predominantly cytosolic. With SR33-mRFP as a nuclear marker protein (Xu et al., 2015), EIN2-C-YFP and SR33-mRFP were largely distinct in their fluorescence patterns and did not overlap; these data were insufficient to support a nuclear localization for EIN2-C-YFP (Figure 6E). Moreover, the YFP fluorescence was primarily detected in the cytosol in tobacco epidermis with transient expression of EIN2-C-YFP (Figure 6F; driven by the UBIQUITIN10 promoter), in line with a previous study (Bisson and Groth, 2015).

The complexity of EIN2 by immune assays and mass spectrum analyses indicates the presence of multiple EIN2 fragments. To determine whether the expressed EIN2-C (EIN2459–1294) may directly mediate ethylene signaling without post-translational proteolytic modifications, we expressed EIN2-C-YFP and its variants, with the 5′ and 3′ modifications, in Arabidopsis protoplasts and analyzed them by immune assays. Our western blots revealed the presence of multiple immune signals, and some of the immune signals for the full-length EIN2-YFP (Figure 5). EIN2-C-YFP, and EIN2-C-YFP variants (Figure 6G) were highly similar, supporting the presence of multiple cleavage products of overexpressed EIN2-C.

Our results showed an association of elevated EIN2-C expression with degrees of the constitutive ethylene response. Degrees of the response were enhanced by ethylene, hence EIN2-C activation requires ethylene. Unlike the subcellular localization patterns of EIN2 and the P-body components EIN5 and DCP1 (Figure 4), EIN2-C is prevalently localized to the cytosol, without a specific pattern. The characteristic nuclear speckles by EIN2-YFP were not observed in cells expressing EIN2-C-YFP and its variants. The phosphorylation status of EIN2-C-YFP were not observed in cells expressing EIN2-C-YFP and its variants. The phosphorylation status of Ser645/Ser924 had little effect on ethylene signaling activated by EIN2-C. The expressed EIN2-C may undergo post-translational cleavage that produces multiple fragments with a complexity similar to that of the full-length EIN2. The constitutive ethylene response phenotype by EIN2-C overexpression is likely a consequence of gain-of-function effects, and the exact EIN2-C species that responds to ethylene and activates the signaling remains to be determined.

**DISCUSSION**

The Ser645 and Ser924 residues of EIN2 are considered the major phosphorylation sites by CTR1, and overexpression of EIN2, EIN2S645A, EIN2S924A, and EIN2AA confers elevated degrees of constitutive ethylene signaling activation, whereas expression of the phosphominic EIN2S645E does not. Therefore, EIN2S645S924 phosphorylation status is suggested to be associated with its activity (Chen et al., 2011; Ju et al., 2012; Qiao et al., 2012). In contrast to previous reports, our findings suggested a similar behavior for the wild-type EIN2 and its variants in response to ethylene, regardless of their phosphorylation-site modifications. Evidence from our study suggested an association of degrees of the constitutive ethylene signaling with levels of the transgene expression. The tight association of fluorescence or protein levels by the fluorescence protein-tagged EIN2/EIN2 variants and degrees of the constitutive triple-response phenotype and ERF1 expression also supports activation of ethylene signaling by an excess amount of EIN2 and its variants. Transgenic lines were ethylene responsive, which indicates activation of the EIN2 variants requiring ethylene, regardless of their phosphorylation status. Taken together, the elevated constitutive ethylene response phenotype by overexpression of those transgenes was likely a result of gain-of-function effects and less likely a result of constitutive EIN2 activation by the phosphorylation modifications. This raises the question of whether the phosphorylated EIN2 and phosphominic EIN2 variants are still active and not subject to ubiquitination modification by ETP1 and ETP2 to the 26S proteasome-mediated degradation (Qiao et al., 2009). We do not exclude that the D and E residues cannot convert EIN2 to a phosphominic conformation and thus the replaced residues prevent phosphorylation by CTR1. This scenario may suggest that the EIN2S645A, EIN2S924A, and EIN2DKD variants are underphosphorylated and cannot confer constitutive ethylene signaling independent of ethylene.

In addition to the transformation study, genetic evidence does not support the association of EIN2 phosphorylation status and constitutive ethylene signaling activation. EIN2 is conceivably underphosphorylated in the ctr1 ein2/EIN2 sesquimutant, which exhibits a mild constitutive ethylene response phenotype and is ethylene responsive. The ethylene responsiveness of the sesquimutant suggests activation of the non-phosphorylated EIN2 requiring ethylene and presence of a cryptic mechanism that prevents ethylene signaling activated by the non-phosphorylated EIN2 in the absence of CTR1. Presence of such a mechanism is previously proposed based on the study that ethylene signaling can be largely prevented in ctr1 mutants expressing the truncated ETR11–349 (Xie et al., 2006, 2012). REVERSION-TO-ETHYLENE SENSITIVITY 1 (RTE1) is required for the repression of constitutive ethylene signaling of ctr1 mutants by the ETR11–349 fragment, and RTE1 overexpression confers ethylene insensitivity in ETR11–349 ctr1 plants (Qiu et al., 2012). RTE1 may be a component of the proposed mechanism, namely ETR1 N-terminal signaling. CTR1 may also be a component of the mechanism, and the loss of CTR1 leads to elevated, instead of complete, ethylene responses in ein2/EIN2 plants. Alternatively, CTR1 and the unidentified mechanism may act synergistically (Figure 7). The mechanism of repression of ethylene signaling by the non-phosphorylated EIN2 in ctr1 mutants remains to be unveiled. Four phosphorylation sites of EIN2 were revealed (Chen et al., 2011; Ju et al., 2012), and the Ser645/Ser924 phosphorylation status appears to have little effect on EIN2 activity. We do not exclude that phosphorylation of the other residues by other proteins may determine EIN2 activity. ETR1 and EIN2 may interact (Bisson and Groth, 2010, 2015), and EIN2-activated ethylene signaling may be prevented via its phosphorylation by ETR1. Alternatively, the proposed ethylene signaling via a phosphorelay involving the ethylene receptors, AHPs (ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINS) and ARRs (ARABIDOPSIS RESPONSE REGULATORS), may serve as a CTR1-independent mechanism (Shakeel et al., 2013; Street et al., 2015; Zdarska et al., 2019).
EIN2-C is inferred as the proteolytic product of underphosphorylated EIN2 and activates ethylene signaling in the nucleus and at P-bodies (Qiao et al., 2012; Li et al., 2015; Merchante et al., 2015; Zhang et al., 2016, 2018), and EIN2-C overexpression leads to constitutive ethylene signaling of various degrees (Alonso et al., 1999; Qiao et al., 2012; Wen et al., 2012). In our study, degrees of the constitutive ethylene response by EIN2-C were associated with levels of the transgene expression. The phenotype of those EIN2-C overexpression lines likely results from gain of function due to excess amount of the protein instead of constitutive activation independent of ethylene. Of note, our observations are not novel, and EIN2-C transgene expression can complement the ein2-1 mutation without conferring constitutive ethylene signaling; the transformation plants are fully responsive to ethylene (Bisson and Groth, 2010, 2015). Although the artificially created EIN2-C were associated with the nucleus by LSCM observations (Qiao et al., 2012), our LSCM observations over different focal planes and sample cells revealed a predominant cytosolic localization. Moreover, the characteristic cytosolic particles and nuclear speckles of the EIN2-YFP fluorescence were distinct to features of the EIN2-C-GFP fluorescence. These data suggest that EIN2-C and EIN2 may localize to distinct compartments. The observed cytosolic EIN2-C localization is in agreement with the report by Bisson and Groth (2010). The expressed EIN2-C is artificially created, and the presence of the inferred EIN2-C remains to be experimentally confirmed (Cooper, 2013; Qiao et al., 2013). Our western blots revealed high complexity of the EIN2-C protein, and whether it is the inferred EIN2-C or its proteolytic...
products that may mediate ethylene signaling awaits further investigation, as does the mechanism by which the cytosolic EIN2-C responds to ethylene.

Cell biology studies of subcellular localizations of EIN2 and its products may help determine the process of ethylene signaling by EIN2. Although EIN2 is suggested to be localized at the ER membrane, published LSCM images as well as our observations show a bipartite fluorescence pattern in the cytosol predominantly as particles or aggregates and in the nucleus as speckles in cells of Arabidopsis plants, regardless of ethylene treatment. The fluorescence characteristics of the ER network were observed primarily from studies involving transient expression in tobacco cells. Of note, the fluorescence pattern by GFP tagged at the N terminus of EIN2 was similar to that by YFP tagged at the EIN2 C terminus. These image data may suggest that a fraction of the full-length EIN2 protein is already transported to the nucleus independent of ethylene, which agrees with the immunological detection of EIN2-GFP from the nuclear fraction (Wen et al., 2012). The result that EIN2-GFP is detected only from the microsome fraction of non-treated plants may reflect the localization of EIN2 that is not activated by ethylene (Ju et al., 2012). Also, Qiao et al. (2012) showed a predominant fluorescence nuclear localization of the underphosphorylated EIN2Δ-YFP-HA by LSCM and a large fraction of EIN2 to be the uncleaved, full-length form by western blots. These results may support a tight association of the full-length EIN2Δ-YFP-HA protein and its nuclear localization. We consider that the full-length EIN2 may traffic to the nucleus, where it is processed to produce cleaved fragments while some of the products are exported to the cytosol. This process may support the hypothesis that EIN2 can specifically target the EBF1 and EBF2 transcripts in the nucleus as part of ribonucleoproteins that export the protein–transcript complex to P-bodies (Zhang and Wen, 2015) (Figure 7). The molecular weight of the fluorescence protein-tagged EIN2 is large, and the protein, with 12 predicted transmembrane domains, likely does not leak into the nucleus. How the full-length GFP-EIN2/EIN2-YFP may traffic to the nucleus remains to be investigated. EIN2 may associate with other types of membrane, such as transport vesicles, that can be delivered to the nucleus.

Comparing EIN2 subcellular distributions and inferring its trafficking involving independent cells (unpaired samples) may lead to various interpretations due to the great variability of EIN2 localization and level. Our studies involving paired samples from independent samplings avoided the variability and did not support the process of cytosol EIN2 trafficking to the nucleus. Caution should be exercised in inferring the process of EIN2 trafficking from results of overexpression studies. The tight association of subcellular EIN2 localization and activation of ethylene signaling may suggest the subcellular sites of the functionally active EIN2. In contrast, the subcellular sites where EIN2 is not activated, or its activity is prevented, may or may not be the same as the sites of activated EIN2. The present LSM data may more likely infer the possible subcellular sites where EIN2 activates ethylene signaling, but the sites where EIN2 localizes before its activation need to be determined. This may in part explain why the EIN2-YFP fluorescence was not observed at the ER membrane by LSM observations whereas EIN2-YFP can be detected from microsomal fraction (Ju et al., 2012). The complexity of EIN2 and EIN2-C as well as their subcellular distribution adds uncertainty to inferring functionality of the protein fragments in ethylene signaling at different subcellular compartments.

A model that is proposed to explain results from independent experiments may not necessarily describe the scientific truth of a biological process, and a model is subject to changes (Editors, 2005). The present molecular studies well explain the genetic model inferred from previous genetic analyses, while framed in the genetic framework, and do not externally explain ethylene signaling in the context of other genetic evidence, such as ETR1 receptor N-terminal signaling (Qiu et al., 2012) and the ethylene responsiveness of ctrl1-1 ein2W308/EIN2 (Zhang et al., 2014). Beyond the present model, there may be an alternative mechanism repressing EIN2-activated ethylene signaling independent of CTRL1 (Zhang et al., 2014). The present genetic framework is constructed based on epistatic effects in the context of certain alleles, and effects of the involved components are inferred. Of note, the effect of a gene could be larger in the context of specific genetic backgrounds but minor in another (Gibson and van Helden, 1997; de Visser et al., 2003). It is not unusual that effects of the CTRL1-independent mechanism appear minor in ctrl1 mutants whereas the mechanism becomes evident in other genetic backgrounds. The epistatic effects that differ in different genetic backgrounds do not necessarily reflect their biological significance, and epistasis does not conclude an upstream or downstream relationship between two proteins.

We propose an alternative model to explain ethylene signaling in the context of genetic analyses from the present genetic framework and our studies. In the scenario of the alternative model, the ethylene receptors and EIN2 are the two key components that respectively prevent and activate ethylene signaling. CTRL1 may act as an activator or co-factor of the ethylene receptors, via protein–protein interaction (Clark et al., 1998; Huang et al., 2003), and the receptors may repress EIN2 activation via an unidentified mechanism (Figure 7). Ethylene prevents the receptor activity and thus inactivates the mechanism that prevents EIN2 activity. In ctrl1 mutants defective in CTRL1 kinase activity, the mutant CTRL1 proteins cannot activate the receptors and EIN2 activity is not repressed. The ethylene responsiveness of ctrl1-1 and ctrl1-3 mutants may reveal a basal-level receptor activity that inhibits EIN2 activity, and the basal-level activity is further prevented by ethylene treatment. This model may explain the extreme constitutive ethylene response phenotype of receptor quintuple mutants, complete ethylene insensitivity of ein2 mutants, and the ethylene responsiveness of ctrl1 alleles and the ctrl1 ein2/EIN2 sesquimutant. The truncated ETR11-349 protein may fold into a conformation with elevated activity, lacking the CTRL1 binding domain, and greatly prevent constitutive ethylene signaling of the ctrl1-1 and ctrl1-2 mutants (Qiu et al., 2012). We do not rule out that repression of ethylene signaling by the ETR1 N terminus could be a gain-of-function effect, and naturally there is not such a truncated protein. However, if there were not the hypothesized cryptic mechanism, the ETR11-349 fragment would not be able to exert the gain-of-function effect and repress EIN2-activated ethylene signaling independent of CTRL1.
Plant Communications

METHODS

Plant Materials

The wild type is Col-0, and the loss-of-function ein2 mutant was described. 35S:EIN2-YFP (line 1, in Col-0) was from C. Chang (Ju et al., 2012). Arabidopsis plants were grown and seedling hypocotyl lengths were measured by a standardized procedure (Xie et al., 2006; Lu and Wen, 2019); in brief, seeds were stratified at 4°C for 72 h, germinated on half-strength Murashige and Skoog medium at 22°C for 80 h, and the hypocotyl length was measured involving the use of Video testT (Moscow). The 35S:EIN2-YFP plant (in Col-0) was from H. Qiao (Qiao et al., 2012). 35S:EIN2-YFP, EIN2p::EIN2-GFP, EIN2p::EIN2A-YFP, EIN2p::EIN2D-GFP, EIN2p::EIN2E-GFP, EIN2p::EIN2A-YFP, EIN2p::EIN2D-GFP, EIN2p::EIN2E-GFP, and EIN2p::EIN2C lines (in ein2-1 and ein2-5 backgrounds) were obtained by Agrobacterium transformation. Transgenic lines generated in this study in ein2-1 were analyzed at the T3 homozygous generation and in ein2-5 analyzed at the T4 homozygous generation. Unless specified, ethylene (10 μl l–1) was involved for ethylene treatment.

Clones and Transgenes

The 35S:EIN2-YFP clone was from H. Qiao and EIN2p::EIN2-GFP and EIN2p::EIN2A-YFP clones were from C. Chang (Ju et al., 2012; Qiao et al., 2012). The EIN2-35S, EIN2E-35S genomic DNA fragments were cloned by PCR, with the primer pairs EIN2-645D-F: 5'-ATT TCC GCA GCT TAA GTG GGC AA-3' and EIN2-645D-R: 5'-CAG GAG CAT CAG ATC CGA CAG T-3'. EIN2-924D-F: 5'-ACA TGG CAT ATA TGT GAT TGT CT-3' and EIN2-924D-R: 5'-CAG TAT ATT TTT TCT CAT GGA CT-3', EIN2-2942D-F: 5'-AGA TCG GAG CAT TCT CAG TGT CT-3' and EIN2-2942D-R: 5'-CAG TAT ATT TTT TCT CAT GGA CT-3', EIN2p::EIN2-F: 5'-TCA GAT AGA TTT AGG GGT ACC ATG AGC CGG CTG GTA CCC AAT GAT CCG TAC GCA GTC A-3'.

Protein Isolation and Immune Assays

Approximately 100 mg of light-grown seedlings (about 100–300 seedlings, depending on the genotype), quickly frozen by liquid nitrogen, were ground into fine powder in a homogenizer (Precelly 24, Berin) with stainless steel beads and 200 μl of extraction buffer (50 mM Tris–HCl [pH 7.5]), 150 mM NaCl, 5 mM EDTA, 2% (w/v) Nonidet P-40, 1 mM PMSF, 1% (v/v) cocktail [EDTA-free protease inhibitor cocktail tablets, Roche (Mannheim)], and 5 mM DTT). Once homogenized, the debris was spun down with a microfuge and the supernatant mixed with 0.2 volume of 6× SDS sample buffer (360 mM Tris–HCl [pH 6.8], 12% SDS, 0.06% bromophenol blue, 50% glycerol, 30% 2-mercaptoethanol) to bring the buffer to 1×.

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EYFP or mRFP and the 35S promoter and NOS terminator cassette, respectively at the SaI–BarHl and SalI–Spel sites. The nuclear protein marker SR33-mRFP is as described by Xu et al. (2015).

Transient Transgene Expression in Arabidopsis Protoplasts

Arabidopsis protoplast preparation and protoplast transient expression were followed as described previously (Wu et al., 2009; Pitzschke and Persak, 2012). The fluorescence and images were acquired involving Leica TSC SPE STED 3X involving the objective HCX PL APO CS 10×/0.40 DRY (objective: 20×; scanner: HyD; main gain: HyD 815, triple dichroic (TD) 192; pinhole: 90 μm; laser: 2.0×). Emission wavelengths between 542 and 578 nm were acquired for EYFP fluorescence, and between 623 and 650 nm for mRFP fluorescence.

Mass Spectrum Analyses

Gel slices containing proteins at the indicated molecular weight position of interest were minced into 1 × 1-mm pieces, destained, washed, and dehydrated with acetonitrile. The in-gel proteins were reduced with dithiothreitol at 50°C, washed, reacted with iodoacetamide in darkness, and dehydrated with acetonitrile. Trypsin solution (10 ng μl–1) was added to the dehydrated gel, and in-gel digestion was performed at 37°C for 16 h. The trypsin-digested peptides were isolated twice, dehydrated with acetonitrile, and lyophilized. The resulting samples were desalted with C18 tip and lyophilized. Mass spectrometry involved Thermo Fisher Easy-nLC 1000 for chromatography and Thermo Fisher LTQ Orbitrap ETD for the mass spectrum. The resulting data in RAW format were analyzed with Proteome Discoverer 1.4 (Thermo) with the database uniprot-Arabidopsis thaliana.fasta. The analyses were a custom service by AIMS Scientific (Shanghai, China).

Laser Scanning Confocal Microscopy

LSCM and the Aryscan technique were performed with Zeiss LSM880 Airyscan. The LSCM setting is as follows. For subcellular localization studies of transgenic lines: objective: 20× M27 and 63× M27; scanner: photomultipier tube (PMT); main gain: PMT 815, TD 192; pinhole: 90 μm; laser: 514 nm (2.0×). For Arysan: objective: 63× M27; scanner: PMT; main gain: PMT 800; pinhole: 178 μm; laser: 514 nm (4.5×); Arysan mode:

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superresorlution; and Airyscan parameter: 6.6 (2D). For fluorescence quantification studies: objective: 20x M27; scanner: GaAsP; main gain: GaAsP 667, TD 313; pinhole: 81 μm; laser: 514 nm (2.6%). Data were analyzed by ImageJ 1.52a. For ACC treatment, water was carefully withdrawn from one side of the slide by tissue and ACC solution was added to the opposite side of the slide that carried the seedling sample; the images before and after (15-min) ACC treatment were acquired at the same focal plane.

Quantitative RT–PCR

Gene expression was determined by qRT–PCR involving the use of StepOne Plus (Applied Biosystems) and TaKaRa SYBR Premix Ex Taq. To determine the endogenous EIN2 and ectopically expressed EIN2-GFP (YFP) transcript copy number, their corresponding cDNA templates were each serially diluted to plot a standard curve (only a curve with R² > 0.99 was used for the calibration). EIN2 and GFP (or YFP) cDNAs were each reversely transcribed with oligo(dT) as the primer, with the use of 200 ng of RNA, and 8 ng of cDNA as template in each qPCR. The primer sequences for EIN2 and GFP (or YFP) were GFP-F: 5'-ACG TAA ACG GCC ACA AGT TC-3' and GFP-R: 5'-AAG TCG TGG TGC TTC ATG TG-3'; EIN2endo-F: 5'-TCG CTT TTG ACT GCT CT-3' and EIN2endo-R: 5'-ACC GGA TTC GTG TTG TAG C-3', qRT–PCR primers measuring expression levels of EIN2AA, EIN2OD, and EIN2EE involved the common reverse primer EIN2q-645Ser-R: 5'-GTG CCA AAC AGC TGA TCT-3' and the respective forward primers EIN2q-645AA-F: 5'-TCT GAT GGT CCT CCT GAA-3', EIN2q-645DD-F: 5'-TCT GAT GGT CCT CCT GAT-3', and EIN2q-645EE-F: 5'-TCT GAT GGT CCT CCT GAA-3'. ERF1 expression was determined with the primer pairs ERF1-F: 5'-TTT CTC GAT GAG AGG GTC-3' and ERF1-R: 5'-AAG CTC CTC AAG GTA CTG-3', with UBQ10 as the internal calibrator (UBQ-F: 5'-ATG GAA AAT CCC ACC TAC TAA ATT-3' and UBQ-R: 5'-GTG GAG AGG GTC-3').

Data Acquisition and Statistics

Comparisons between two independent sample means were conducted by standard one- or two-tailed Student’s t-test. Comparisons involving multiple sample means (≥3) were each serially diluted to plot a standard curve (only a curve with R² > 0.99 was used for the calibration). EIN2 and GFP (or YFP) cDNAs were each reversely transcribed with oligo(dT) as the primer, with the use of 200 ng of RNA, and 8 ng of cDNA as template in each qPCR. The primer sequences for EIN2 and GFP (or YFP) were GFP-F: 5'-ACG TAA ACG GCC ACA AGT TC-3' and GFP-R: 5'-AAG TCG TGG TGC TTC ATG TG-3'; EIN2endo-F: 5'-TCG CTT TTG ACT GCT CT-3' and EIN2endo-R: 5'-ACC GGA TTC GTG TTG TAG C-3', qRT–PCR primers measuring expression levels of EIN2AA, EIN2OD, and EIN2EE involved the common reverse primer EIN2q-645Ser-R: 5'-GTG CCA AAC AGC TGA TCT-3' and the respective forward primers EIN2q-645AA-F: 5'-TCT GAT GGT CCT CCT GAA-3', EIN2q-645DD-F: 5'-TCT GAT GGT CCT CCT GAT-3', and EIN2q-645EE-F: 5'-TCT GAT GGT CCT CCT GAA-3'. ERF1 expression was determined with the primer pairs ERF1-F: 5'-TTT CTC GAT GAG AGG GTC-3' and ERF1-R: 5'-AAG CTC CTC AAG GTA CTG-3', with UBQ10 as the internal calibrator (UBQ-F: 5'-ATG GAA AAT CCC ACC TAC TAA ATT-3' and UBQ-R: 5'-GTG GAG AGG GTC-3').

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SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

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