Cell Research (2012) 22:1613-1616.
© 2012 IBCB, SIBS, CAS All rights reserved 1001-0602/12 $ 32.00
www.nature.com/cr

LETTER TO THE EDITOR

Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus

Cell Research (2012) **22**:1613-1616. doi:10.1038/cr.2012.145; published online 16 October 2012

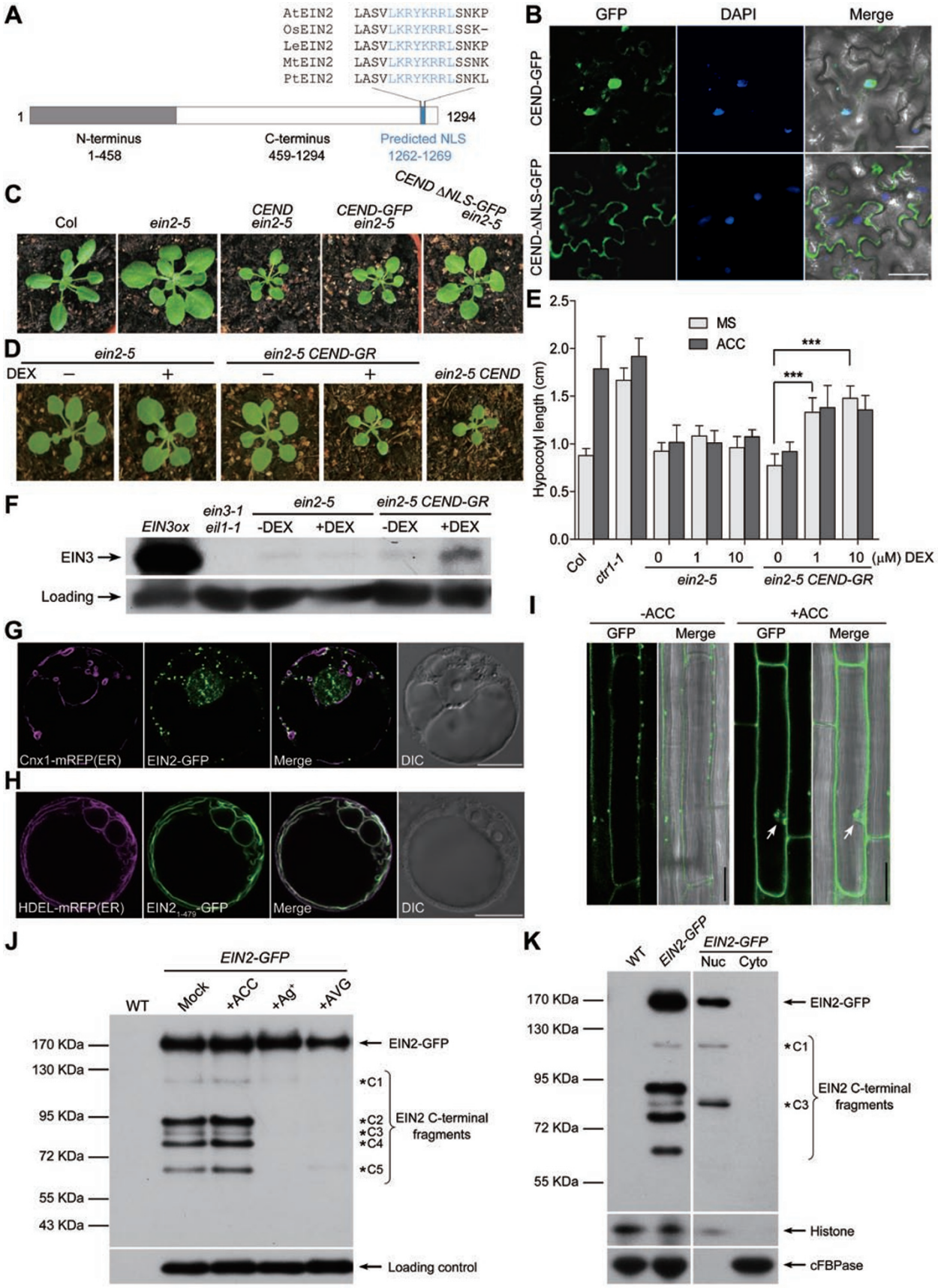
Dear Editor,

Ethylene gas is a classic phytohormone regulating many aspects of plant development and defense. Molecular and genetic studies have revealed a highly conserved signaling pathway starting from the ER membrane-associated receptors to transcription factors in the nucleus [1]. EIN2 (ETHYLENE INSENSITIVE2) is an essential positive regulator of ethylene signaling in *Arabidopsis thaliana*, as loss-of-function *ein2* mutants are completely insensitive to ethylene [2]. EIN2 encodes a 1 294 amino acid protein that comprises a membrane-spanning amino terminus and a functionally unknown carboxyl terminus [3]. EIN2 was reported to localize at the ER membrane when transiently expressed in tobacco leaf cells [4]. EIN3 (ETHYLENE INSENSITIVE3) and its homolog EIL1 (EIN3-LIKE1) are two nuclear-localized transcription factors genetically acting downstream of EIN2 [5, 6]. However, it remains a long mystery on how the ethylene signal is transmitted from ER-located EIN2 into the nucleus to modulate EIN3/EIL1-directed transcription. Here we report that the carboxyl end of EIN2 (CEND) is a trafficking signal translocating from ER membrane to the nucleus. Ethylene signal promotes the cleavage of CEND from ER-located EIN2, and facilitates its nuclear localization to stabilize EIN3 protein.

It was reported that constitutive expression of EIN2 carboxyl terminal end (CEND, amino acids 459-1 294) leads to partial activation of ethylene responses in light-grown plants [3], implying that CEND might function in signal transmission. The CEND fragment contains a putative nuclear localization signal (NLS, amino acids 1 262-1 269), which is highly conserved among numerous EIN2 orthologs of angiosperms (Figure 1A) [7]. To determine whether CEND is nuclear-localized, we expressed CEND-GFP in tobacco leaf cells and found that GFP fluorescence was detected in both nucleus and cytoplasm (Figure 1B). By contrast, deletion of the NLS retained the CEND-GFP fusion protein exclusively in the cytoplasm (Figure 1B), suggesting that the NLS sequence is responsible for CEND's nuclear localization. Constitutive expression of CEND has been shown to induce ethylene response phenotypes, including elongated hypocotyl of light-grown seedlings, compacted rosette, reduced fertility, abnormal flower with protruded gynoecium, and activation of downstream gene expression [3]. Similarly, constitutive expression of CEND-GFP was also able to induce these ethylene responses (Figure 1C, Supplementary information, Figure S1A and S1B). In contrast, removal of the NLS eliminated the ability of CEND to activate ethylene signaling (Figure 1C, Supplementary information, Figure S1A and S1B), indicating that the nuclear localization of EIN2 CEND is required for its action.

To address whether the nuclear localization of CEND is sufficient to activate ethylene signaling, we employed a glucocorticoid receptor (GR) system, which has been widely used to induce nuclear translocation of transcriptional regulators upon dexamethasone (DEX) treatment in animals and plants [8, 9]. We found that transgenic plants constitutively expressing CEND-GR in the *ein2-5* mutant were indistinguishable from *ein2-5* plants without DEX application (Figure 1D and 1E, Supplementary information, Figure S1C-S1E). Upon DEX treatments, *CEND-GR/ein2-5* plants exhibited elongated hypocotyls, abnormal flowers with protruded gynoecia, reduced fertility, and activation of ethylene-responsive gene expression, all reminiscent of CEND-expressing plants (Figure 1D and 1E, Supplementary information, Figure S1C-S1E). Consistent with these constitutive ethylene response phenotypes, we found that promoting the nuclear transport of CEND-GR by DEX application notably increased EIN3 protein level (Figure 1F). Therefore, we conclude that the nuclear localization of CEND is both required and sufficient for the activation of ethylene signaling.

A previous study demonstrated that EIN2 transiently expressed in tobacco leaf cells was localized at the ER membrane [4]. Our above results indicate that CEND is partly located in the nucleus, which is essential for its function. To reconcile this seemingly discrepancy, we re-examined the subcellular localization of EIN2. We


A
AtEIN2 LASVLKRYKRRLSNKP
OsEIN2 LASVLKRYKRRLSSK-
LeEIN2 LASVLKRYKRRLSNKP
MtEIN2 LASVLKRYKRRLSSNK
PtEIN2 LASVLKRYKRRLSNKL
1
1294
N-terminus 1-458
C-terminus 459-1294
Predicted NLS 1262-1269
B
GFP
DAPI
Merge
CEND-GFP
CEND-ΔNLS-GFP
C
Col
ein2-5
CEND ein2-5
CEND-GFP ein2-5
CEND ΔNLS-GFP ein2-5
D
ein2-5
ein2-5 CEND-GR
DEX
ein2-5 CEND
E
MS
ACC
Hypocotyl length (cm)
Col
ctr1-1
ein2-5
ein2-5 CEND-GR
(μM) DEX
F
EIN3ox
ein3-1 eil1-1
ein2-5
ein2-5 CEND-GR
-DEX
+DEX
EIN3
Loading
G
Cnx1-mRFP(ER)
EIN2-GFP
Merge
DIC
H
HDEL-mRFP(ER)
EIN2 1-479-GFP
I
-ACC
+ACC
GFP
Merge
J
EIN2-GFP
WT
Mock
+ACC
+Ag+
+AVG
170 KDa
130 KDa
95 KDa
72 KDa
55 KDa
43 KDa
EIN2-GFP
*C1
*C2
*C3
*C4
*C5
EIN2 C-terminal fragments
Loading control
K
WT
EIN2-GFP
EIN2-GFP
Nuc
Cyto
170 KDa
130 KDa
95 KDa
72 KDa
55 KDa
EIN2-GFP
*C1
*C3
EIN2 C-terminal fragments
Histone
cFBPase

verified the functionality of the EIN2-GFP fusion protein based on its ability to complement the *ein2-5* mutant (Supplementary information, Figure S2A). Consistent with previous report, EIN2-GFP was co-localized with the ER marker protein when transiently expressed in tobacco leaf cells (Supplementary information, Figure S2B). However, when treated with ACC, EIN2-GFP was also observed in the nucleus (Supplementary information, Figure S2C and S2D). We further expressed EIN2-GFP in protoplasts of *Arabidopsis* PSB-D suspension cells and detected the GFP fluorescence in both ER and nucleus (Figure 1G). By comparison, a fusion protein of GFP and EIN2 N-terminal membrane-spanning domain (amino acids 1-479) was exclusively detected in ER membrane (Figure 1H), implying that the N-terminal end is responsible for EIN2's location on ER. We also detected weak fluorescence in the nucleus of transgenic *Arabidopsis* plants expressing EIN2-GFP upon ACC treatment, but not in untreated plants (Figure 1I). Taken together, these results indicate that ethylene treatment facilitates the translocation of EIN2-GFP from ER membrane into the nucleus.

Given the above results, we hypothesized that CEND could be a trafficking molecule transported into the nucleus when ethylene signal is on. To test this hypothesis, we generated an *Arabidopsis* suspension cell line stably expressing EIN2-GFP. Immunoblot assays with anti-GFP antibody showed that, in addition to full-length EIN2-GFP, at least five smaller fragments (designated as C1-C5) were detected in the transformed cell line (Figure 1J). Interestingly, ACC treatment hardly altered the pattern or intensity of these fragments, whereas treatment with silver ion or aminoethoxyvinylglycine (AVG), inhibitor of ethylene perception or biosynthesis, respectively, led to the disappearance of those fragments (Figure 1J). This result indicates that EIN2 C-terminal end could be cleaved and ethylene signal induces the cleavage(s). The lack of an ACC effect implied that *Arabidopsis* suspension cells are already saturated with high ethylene production, likely induced by mechanical stresses during cell preparation and propagation. It also provides an explanation for the observed nuclear localization of EIN2-GFP in *Arabidopsis* protoplasts even without ACC treatment (Figure 1G).

To further confirm that EIN2 C-terminal end could be cleaved and translocated into the nucleus, we isolated nuclear fraction (Nuc) and soluble cytosolic fraction (Cyto) from the EIN2-GFP-expressing *Arabidopsis* suspension cells. Immunoblot assay with anti-GFP antibody showed that full-length EIN2-GFP as well as C1 and C3 fragments were detected in the nuclear fraction, whereas no fragments were present in the soluble cytoplasm (Figure 1K). It is likely that fragments C2, C4, C5 might associate with other non-nuclear organelles or insoluble particles. Collectively, these findings demonstrate that, in the presence of ethylene signal, EIN2-GFP is cleaved and the resulting C-terminal ends are selectively translocated from ER to the nucleus to activate downstream ethylene signaling.

Previous studies revealed that EIN2 is required for EIN3 and EIL1 protein accumulation [6, 10]. We found that while EIN3 protein was undetectable in the *ein2* mutant, expression of CEND in *ein2* led to the re-accumulation of EIN3 protein (Supplementary information, Figure S3A). Genetic analysis further indicated that EIN3 and EIL1 are required for the action of CEND, as loss of EIN3/EIL1 function completely suppressed CEND activity in all ethylene response phenotypes examined, as well

Figure 1 EIN2 is subjected to ethylene-induced processing and nuclear translocation that is necessary and sufficient for the activation of ethylene-regulated responses. **(A)** Schematic representation of EIN2 and alignment of a predicted nuclear localization sequence (NLS). **(B)** Transient expression of CEND-GFP and CEND-GFP with a deletion of the putative NLS (CEND-ΔNLS-GFP) in tobacco leaves. DAPI was used as the nuclei marker. **(C)** Phenotypes of 4-week-old adult plants of the indicated genotypes. **(D)** Comparison of phenotypes of 4-week-old adult plants with or without 60 μM DEX application. **(E)** The hypocotyl lengths of 5-day-old light-grown seedlings on 1/2 MS plate containing DEX of the indicated concentrations. Mean ± SD, $n > 10$. *** indicates $P < 0.001$ in two-tailed Student's *t*-test. **(F)** Immunoblot assay indicates the levels of endogenous EIN3 protein in 5-day-old light-grown seedlings with or without 100 μM DEX treatment for 4 h. **(G**, **H)** Subcellular localization of EIN2 **(G)** and EIN2 N-terminus **(H)** in *Arabidopsis* suspension cells. EIN2-GFP and $EIN2_{1-479}$-GFP were transiently expressed in *Arabidopsis* suspension cells. Cnx1-mRFP and HDEL-mRFP were co-transformed as ER markers, respectively. DIC, differential interference contrast. Scale bars, 20 μm. **(I)** GFP fluorescence in the roots of 3-day-old etiolated seedlings of *35S:EIN2-GFP/ein2-5* transgenic plants with or without ACC treatment. Arrows indicate the nuclei. Scale bars, 20 μm. **(J)** Ethylene signal promotes the cleavage of EIN2 C-terminus. *Arabidopsis* suspension cells stably expressing EIN2-GFP were immunoprecipitated with the GFP-Trap_A beads and immunoblotted with anti-GFP antibody. Five C-terminal fragments (C1-C5) are indicated by asterisk (*). **(K)** Subcellular fraction and immunoblot analysis of EIN2 fragments. Proteins were fractionated from *Arabidopsis* suspension cells stably expressing EIN2-GFP. Nuc, nucleus; Cyto, cytoplasm. Histone H3 and cFBPase were used as controls of nuclear and cytosolic fractions, respectively.

as ethylene-responsive gene expression (Supplementary information, Figure S3B-S3F). Therefore, CEND acts to activate EIN3-mediated transcription and ethylene responses. We are currently investigating how CEND stabilizes EIN3 protein in the nucleus.

In this study, we have demonstrated that EIN2 harboring a functional NLS is subjected to a hormone-induced cleavage event, followed by transporting its carboxyl end (CEND) into the nucleus, where CEND acts to stabilize EIN3 and activate ethylene responses (Supplementary information, Figure S4). This model uncovers a molecular mechanism to bridge two separate compartments (i.e., ER and nucleus) involved in ethylene signal transduction, wherein CEND serves as a trafficking molecule conveying ethylene signal from ER membrane to the nucleus. A recent study by Qiao *et al*. [11] reported similar findings of EIN2 processing and subcellular translocation. In comparison with their results, we observed at least 5 ethylene-induced C-terminal cleavage fragments, and only 2 fragments are preferentially associated with nuclear fraction, including the one reported by Qiao *et al*. Meanwhile, we also observed that EIN2-GFP formed speckles in cytoplasm apart from nuclear localization (Figure 1G, Supplementary information, Figure S2B-S2D), implying that EIN2 might play additional roles in ethylene signaling other than activating EIN3-mediated transcription. Detailed methods are described in the Supplementary information, Data S1.

Acknowledgments

We thank Drs D Guo and Y Wang for critical reading of the manuscript. This work was supported by the National Natural Science Foundation of China (91017010), Ministry of Science and Technology (2009CB119101), and Ministry of Agriculture of China (2010ZX08010-002), and the 111 Project of Peking University to H G.

Xing Wen[1, *], Cunli Zhang[1, *], Yusi Ji[1, *], Qiong Zhao[1, 2], Wenrong He[1], Fengying An[1], Liwen Jiang[2], Hongwei Guo[1]

[1]*State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China;* [2]*School of Life Sciences, the Chinese University of Hong Kong, Hong Kong, China*

*These three authors contributed equally to this work.
Correspondence: Hongwei Guo
E-mail: hongweig@pku.edu.cn

References

1 Guo H, Ecker JR. The ethylene signaling pathway: new insights. *Curr Opin Plant Biol* 2004; **7**:40-49.
2 Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR. Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. *Genetics* 1995; **139**:1393-1409.
3 Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* 1999; **284**:2148-2152.
4 Bisson MM, Bleckmann A, Allekotte S, Groth G. EIN2, the central regulator of ethylene signalling, is localized at the ER membrane where it interacts with the ethylene receptor ETR1. *Biochem J* 2009; **424**:1-6.
5 Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR. Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 1997; **89**:1133-1144.
6 An F, Zhao Q, Ji Y, *et al.* Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in *Arabidopsis*. *Plant Cell* 2010; **22**:2384-2401.
7 Cokol M, Nair R, Rost B. Finding nuclear localization signals. *EMBO Rep* 2000; **1**:411-415.
8 Huang W, Perez-Garcia P, Pokhilko A, *et al.* Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. *Science* 2012; **336**:75-79.
9 Lloyd AM, Schena M, Walbot V, Davis RW. Epidermal cell fate determination in *Arabidopsis*: patterns defined by a steroid-inducible regulator. *Science* 1994; **266**:436-439.
10 Guo H, Ecker JR. Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* 2003; **115**:667-677.
11 Qiao H, Shen Z, Huang SS, *et al.* Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. *Science* 2012 Sep 25; doi:10.1126/science.1225974

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)