# Supporting Information

## Ju et al. 10.1073/pnas.1214848109

### SI Materials and Methods

**Yeast Two-Hybrid Assay Constructs.** The sequence encoding the ETHYLENE-INSENSITIVE2 (EIN2) C-terminal domain (residues 516–1294) was PCR-amplified from an *EIN2* cDNA clone, pEIN2 (Arabidopsis Biological Resource Center), and cloned into prey vector pACTII (1) using *Xma*I and *Pst*I restriction sites added by the PCR primers shown in Table S3. The resulting clone was verified by DNA sequencing using primers shown in Table S4.

**Generation of Recombinant Virus Expressing $His_6$-CTR1-$KD^{WT}$ and $His_6$-CTR1-$KD^{D694E}$.** Recombinant baculoviruses expressing $His_6$-CTR1-$KD^{WT}$ and $His_6$-CTR1-$KD^{D694E}$ were generated according to the manufacturer's instructions using BaculoDirect Baculovirus Expression System (Invitrogen). In brief, the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) coding sequence for the kinase domain (residues 531–821) was amplified from *CTR1 WT* or *ctr1-1* (D694E) cDNA clones (2) and cloned into pENTR/D-TOPO (Invitrogen) using the primers described in Table S5 and sequence-verified. Using Gateway cloning, recombinant baculovirus DNA was generated by LR reaction with these entry clones and BaculoDirect linear DNA, which is designed to express target proteins with an N-terminal $His_6$ tag, as described by the manufacturer. The resulting recombinant DNA was used to transfect Sf9 insect cells, and recombinant virus was selected for by growth of the cells in media containing ganciclovir (Sigma). Expression of the both proteins was confirmed by Western analysis using P2 virus stock-infected Sf9 cells grown as an adherent culture in Grace's insect media at 28 °C.

**Mutagenesis of *EIN2* for Protein Expression.** Ser645 and Ser924 in EIN2 (residues 479–1294) in the pET21a_MCSpET28a_$EIN2^{479–1294}$ vector (3) were both mutated to Ala ($EIN2^{AA}$) using the QuikChange Site-Directed Mutagenesis Kit (Agilent) as recommended by the manufacturer using primers described in Table S6. Mutations were verified by DNA sequencing.

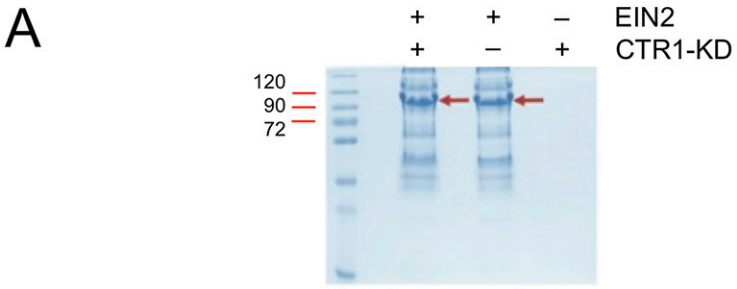
***EIN2* Constructs and Site-Directed in Vitro Mutagenesis for *Arabidopsis* Transformation.** All molecular cloning was carried out using the Gateway cloning system (Invitrogen) unless otherwise specified. Table S6 lists all of the primers used for cloning and mutagenesis. All clones were verified by DNA sequencing before and after mutagenesis using the primers listed in Table S4.

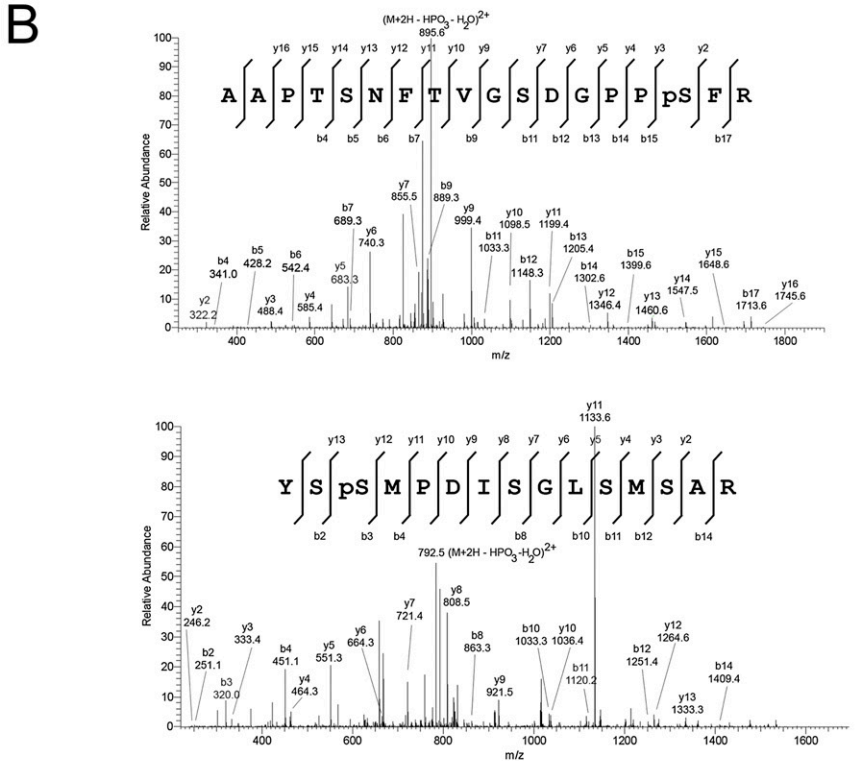
To create *EIN2p-EIN2*, we PCR-amplified two overlapping fragments of the full-length *EIN2* sequence (9.4 kb) using Col-0 genomic DNA as the template, and cloned the fragments into pDONR221 (Invitrogen). We next digested each clone with *Sgr*AI (introduced at the 5′ end of each clone by the PCR primers) and *Kpn*I (a unique site within the overlapping *EIN2* sequence of each clone) and used T4 DNA ligase to ligate the *Sgr*AI-*Kpn*I fragment of the *EIN2* N-terminal half into the vector containing the C-terminal half, thus creating the full-length clone in pDONR221. Mutations were introduced using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). The full-length fragment was then transferred into binary vector pBGW (4). The same method was used to construct *EIN2p-EIN2-GFP*, except that the C-terminal clone lacked the *EIN2* stop codon. Mutations were introduced as above. The full-length sequence was then transferred into binary vector pMDC107 (5). To construct *35S-EIN2*, we PCR-amplified *EIN2* from the start to stop codons using Col-0 genomic DNA as the template and cloned the fragment into pDONR221. Mutations were introduced as above. The sequence was then transferred into binary vector pEarleyGate100 (6). The same method was used to construct *35S-EIN2-YFP*, which encodes a C-terminal YFP tag, except the stop codon was omitted in the PCR amplification. Mutations were introduced as above. The sequence was then transferred into binary vector pEarleyGate101 (6).

**Bimolecular Fluorescence Complementation Constructs.** Full-length *CTR1* and *EIN2* coding sequences, without stop codons, were transferred from pDONR221 into the pSPYNE-35S and pSPYCE-35S vectors (7), respectively.

**EIN2 Constructs for Subcellular Localization in Onion Epidermal Cells.** To construct *35S-EIN2-GFP* for expression in onion epidermal cells, the full-length *EIN2* coding sequence was PCR-amplified using pEIN2 (Arabidopsis Biological Resource Center) as the template and cloned into pDONR221. The coding sequence was then transferred into the binary vector pEarleyGate103 (6). The primers used are shown in Table S7. To construct *35S-YFP-EIN2*, the *EIN2* genomic sequence (from start to stop codon) in pDONR221 (above) was transferred to pEarleyGate104 (6).


1. Clark KL, Larsen PB, Wang X, Chang C (1998) Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. *Proc Natl Acad Sci USA* 95(9): 5401–5406.
2. Huang Y, Li H, Hutchison CE, Laskey J, Kieber JJ (2003) Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. *Plant J* 33(2):221–233.
3. Bisson MMA, Bleckmann A, Allekotte S, Groth G (2009) EIN2, the central regulator of ethylene signalling, is localized at the ER membrane where it interacts with the ethylene receptor ETR1. *Biochem J* 424(1):1–6.
4. Karimi M, De Meyer B, Hilson P (2005) Modular cloning in plant cells. *Trends Plant Sci* 10(3):103–105.
5. Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* 133(2):462–469.
6. Earley KW, et al. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* 45(4):616–629.
7. Schütze K, Harter K, Chaban C (2009) Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in living plant cells. *Methods Mol Biol* 479: 189–202.

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451 CLILWLAATP LKSASNRAEA QIWNMDAQNA LSYPSVQEEE IERTETRRNE
501 DESIVRLESR VKDQLDTTSV TSSVYDLPEN ILMTDQEIRS SPPEERELDV
551 KYSTSQVSSL KEDSDVKEQS VLQSTVVNEV SDKDLIVETK MAKIEPMSPV
601 EKIVSMENNS KFIEKDVEGV SWETEEATKA APTSNFTVGS DGPPSFRSLS
651 GEGGSGTGSL SRLQGLGRAA RRHLSAILDE FWGHLYDFHG QLVAEARAKK
701 LDQLFGTDQK SASSMKADSF GKDISSGYCM SPTAKGMDSQ MTSSLYDSLK
751 QQRTPGSIDS LYGLQRGSSP SPLVNRMQML GAYGNTTNNN NAYELSERRY
801 SSLRAPSSSE GWEHQQPATV HGYQMKSYVD NLAKERLEAL QSRGEIPTSR
851 SMALGTLSYT QQLALALKQK SQNGLTPGPA PGFENFAGSR SISRQSERSY
901 YGVPSSGNTD TVGAAVANEK KYSSMPDISG LSMSARNMHL PNNKSGYWDP
951 SSGGGGYGAS YGRLSNESSL YSNLGSRVGV PSTYDDISQS RGGYRDAYSL
1001 PQSATTGTGS LWSRQPFEQF GVAERNGAVG EELRNRSNPI NIDNNASSNV
1051 DAEAKLLQSF RHCILKLIKL EGSEWLFGQS DGVDEELIDR VAAREKFIYE
1101 AEAREINQVG HMGEPLISSV PNCGDGCVWR ADLIVSFGVW CIHRVLDLSL
1151 MESRPELWGK YTYVLNRLQG VIDPAFSKLR TPMTPCFCLQ IPASHQRASP
1201 TSANGMLPPA AKPAKGKCTT AVTLLDLIKD VEMAISCRKG RTGTAAGDVA
1251 FPKGKENLAS VLKRYKRRLS NKPVGMNQDG PGSRKNVTAY GSLG
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**Fig. S1.** Analysis of EIN2 phosphorylation by collision-induced dissociation (CID) multi-stage mass spectrometry ($MS^2$ and $MS^3$). (*A*) Kinase assay input for mass spectrometry analysis consisting of $His_6$-$EIN2^{WT}$-$His_6$ (EIN2) reacted with $His_6$-$CTR1\text{-}KD^{WT}$ (CTR1-KD), and the control reactions containing EIN2 alone and CTR1-KD alone, examined on a Coomassie-stained acrylamide gel. Molecular weights are marked by the thin red lines on *Left*. The EIN2 protein bands are marked by the red arrows in the gel. (*B*) Linear ion trap CID-$MS^2$ product ion spectra for EIN2 phosphopeptides. Phosphate modification of S (pS) is denoted. The spectrum for AAPTSNFTVGSDGPPpSFR (*Upper*) is interpreted with phosphorylation of S645. The spectrum for YSpSMPDISGLSMSAR (*Lower*) is interpreted for phosphoric acid neutral loss from the parent ion and resulting dehydration of S924. Phosphosite positions were determined from the b and y ions, and $MS^3$ was performed on the $MS^2$ phosphoric acid neutral loss peaks to corroborate the $MS^2$ peptide-spectrum assignments. (*C*) EIN2 peptides found by mass spectrometry. Peptide coverage (bold red) for the C terminus of EIN2 in EIN2_CTR1 samples. Sixty-one peptides covering 85% of the C terminus of EIN2 were discovered in EIN2_CTR1 samples. Similar peptide coverage was obtained in the EIN2 control sample.

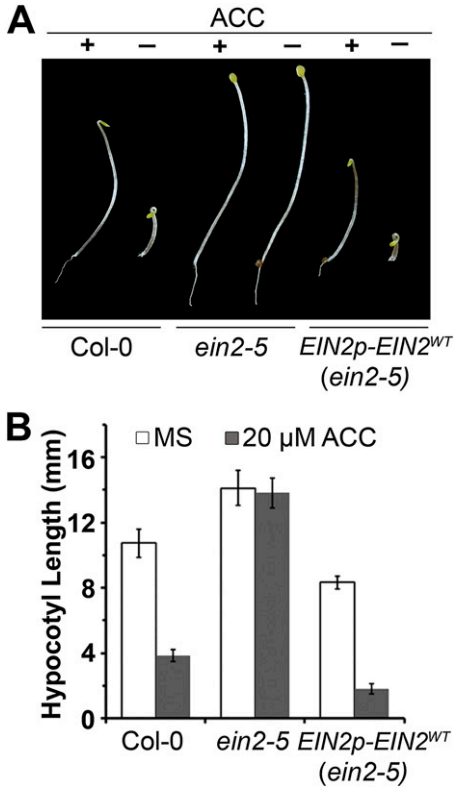


**Fig. S2.** Rescue of ethylene insensitivity in the *ein2-5* mutant by *EIN2p-EIN2*, the full-length *EIN2* genomic sequence with the native *EIN2* promoter region. (*A*) Four-d-old dark-grown seedlings germinated with or without 20 μM 1-aminocyclopropane carboxylic acid (ACC). Representative seedlings are shown in comparison with the WT (Col-0) and *ein2-5* mutant. (*B*) Hypocotyl length measurements of 4-d-old dark-grown seedlings. Each bar represents the average length ± SD of 20–30 seedlings per line.

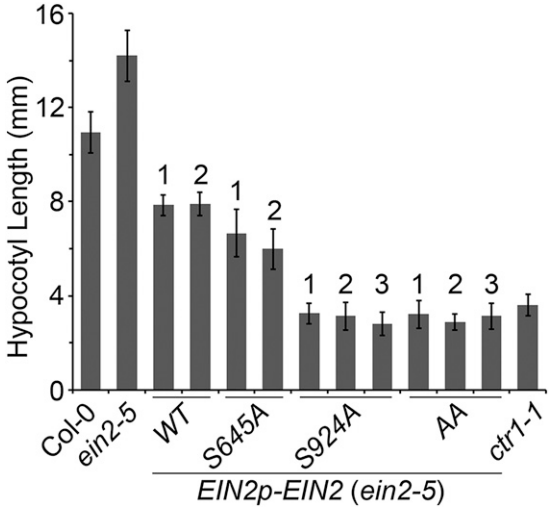


**Fig. S3.** Ser$^{645}$Ala and Ser$^{924}$Ala substitutions in EIN2 confer constitutive ethylene responses in *Arabidopsis*. For each transgene, independent homozygous lines of 4-d-old dark-grown seedlings, in the *ein2-5* mutant background, were grown in the absence of ethylene. Two or three independent lines per transgene (1–3) were measured. Hypocotyl length measurements represent the mean length ± SD of 20–30 seedlings per line.

**Table S1. Evidence for amino acid positioning of phosphorylation on EIN2 peptides**

| Phosphopeptide sequence matched for representative mass spectra | Mascot Ions score | Mascot Expect score | Evidence for peptide position determination, Π* | Confidence in position assignment* | Mascot delta score position assignment probability | EIN2 protein position for modification |
|---|---|---|---|---|---|---|
| AAPTSNFTVGSDGPP$^{\dagger}$SFR | | | | High | | S645 |
| $MS^2$: EIN2_CTR_B5.1477.1477.2.dta | 88 | 5.2e-8 | $MS^2$: +2 charge; y ions found for S16 [+80] | | $MS^2$: S16 [+80] 99.98% | |
| $MS^3$: EIN2_CTR_B5.1478.1478.2.dta | 83 | 7.4e-6 | $MS^3$: b and y ions found for S16 [−18] | | $MS^3$: S16 [−18] 99.07% | |
| LSNKPVGMNQDGPG$^{\dagger}$SR | | | | High | Not calculated | S1283 |
| $MS^2$: EIN2_CTR_B5.976.976.3 | 58 | 6.1e-5 | $MS^2$: +3 charge; b ion found for S2 and deduced for S15 [-18] | | | |
| $MS^3$: EIN2_CTR_B5.977.977.3 | 54 | 5.2e-3 | $MS^3$: deduced for S15 [−18] | | | |
| $^{\ddagger}$TPG$^{\dagger}$SID$^{\ddagger}$SLYGLQR | | | | High | | S757 |
| $MS^2$: EIN2_CTR_B5.1500.1500.2.dta | 59 | 3.8e-5 | $MS^2$: +2 charge; y ions found for S4 [+80] | | $MS^2$: S4 [+80] 96.69% | |
| $MS^3$: EIN2_CTR_B5.1501.1501.2.dta | 68 | 1.6e-4 | $MS^3$: y ions found for S4 [−18] in second ranking match | | $MS^3$: S4 [−18] 33.74% | |
| Y$^{\ddagger}$S$^{\dagger}$SMPDISGLSMSAR | | | | High | | S924 |
| $MS^2$: EIN2_CTR_B5.1556.1556.2 | 56 | 6.6e-5 | $MS^2$: +2 charge; b and y ions found for S3 [−18] | | $MS^2$: S3 [−18] 91.98% | |
| $MS^3$: EIN2_CTR_B5.1557.1557.2 | 83 | 6.7e-6 | $MS^3$: y ions found for S3 [−18] | | $MS^3$: S3 [−18] 94.85% | |
| APSSSEGWEHQQPA$^{\dagger}$TVHGYQMK | | | | High | Not calculated | T819 |
| $MS^2$: EIN2_CTR_B5.1242.1242.3.dta | 43 | 1.6e-3 | $MS^2$: +3 charge; b ions found for T15 [+80] | | | |
| $MS^3$: EIN2_CTR_B5.1243.1243.3.dta | 66 | 4.5e-4 | $MS^3$: b ions found for S3, S4, S5 and deduced T15 [−18] | | | |
| SLSGEGG$^{\ddagger}$SG$^{\ddagger}$TG$^{\dagger}$SL$^{\ddagger}$SR | | | | Moderate | | S659 |
| $MS^2$:EIN2_CTR_B5.971.971.2.dta | 80 | 2.7e-7 | $MS^2$: +2 charge; y and b ion deduced for S12 [+80] | | $MS^2$: S12 [+80] 97.99% | |
| $MS^3$: EIN2_CTR_B5.972.972.2.dta | 87 | 2.4e-6 | $MS^3$: y ion found for S12 [−18] | | $MS^3$: S12 [−18] 96.34% | |

*See Chen et al. (1) for confidence indicators; Π, for $MS^2$ spectra, if there is no neutral loss of phosphoric acid, then site-specific ions for S/T consider phosphorylation [+80], but if there is neutral loss of phosphoric acid, then site-specific ions for S/T consider the loss of water [+80−98 = −18] (3). For $MS^3$, dehydrated S/T is the likely result of neutral loss of phosphoric acid in the prior $MS^2$ event (3).

$^{\dagger}$The following amino acid is phosphorylated.

$^{\ddagger}$Alternative site of phosphorylation on following amino acid.

All but two EIN2 phosphopeptides previously reported by Chen et al. (1) were revalidated by the two approaches for assessing site positioning. Phosphorylation was confidently observed at Ser positions 645, 757, 924, and 1283. As a result of possible gas phase rearrangements (2), it is possible that S923 is phosphorylated instead of S924, and that T754 or S760 is phosphorylated instead of S757, although the Mascot Delta score method suggests S924 and S757 as being more likely. In addition, two other sites were identified at S659 and T819. Given the mass spectrometry evidence, Ser and Thr residues adjacent to S659 may be phosphorylated, but at lower rates. Peptides for GMDSQMTSSLYDSLKQQR and GMDSQMTSSLYDSLK were found, but evidence for phosphorylation was weak, as before (1).


1. Chen RQ, et al. (2011) Proteomic responses in *Arabidopsis thaliana* seedlings treated with ethylene. *Mol Biosyst* 7(9):2637–2650.
2. Palumbo AM, Reid GE (2008) Evaluation of gas-phase rearrangement and competing fragmentation reactions on protein phosphorylation site assignment using collision induced dissociation-MS/MS and MS3. *Anal Chem* 80(24):9735–9747.
3. Feng J, Garrett WM, Naiman DQ, Cooper B (2009) Correlation of multiple peptide mass spectra for phosphoprotein identification. *J Proteome Res* 8(11):5396–5405.


**Table S2. Primers for real-time quantitative PCR analysis**

| Primer name | Forward or reverse | Primer sequence (5′–3′) |
|---|---|---|
| *EIN2* F | F | TGAGACCTCAGCTAGGGTTTATC |
| *EIN2* R | R | TAAGTGCATGCGCAACTCCCAC |
| *ERF1* F | F | TCCCTTCAACGAGAACGACTC |
| *ERF1* R | R | ACGGATTTGATCGGAAGGTC |
| *GAPDH* F | F | CAAGGAGGAATCTGAAGGCAAAATGA |
| *GAPDH* R | R | CAACCACACACAAACTCTCGCCG |

**Table S3. Primers for *EIN2* yeast two-hybrid construct**

| Construct | Forward or reverse | Primer sequence (5′–3′) |
|---|---|---|
| EIN2 CEND pACTII | F | CCCGGGAGATACTACGTCTGTTAC |
| | R | CTGCAGTCAACCCAATGATCCG |

**Table S4. Primers for *EIN2* DNA sequencing**

| Primer name | Primer sequence (5′–3′) |
|---|---|
| 5′EIN2seq01 | TCGATCTAAAGTCCTGAGCC |
| 5′EIN2seq02 | TCGACAAGAAAGATTCTTTAAGC |
| 5′EIN2seq03 | CTATCCAATGTACATGTATATATACTTTCAC |
| 5′EIN2seq04 | AAAAGTAGAGTGTATTTGTTTTTTCAAT |
| 5′EIN2seq05 | CTGAAATGTATAATAGAACCTATTCATGTA |
| 5′EIN2seq06 | GCTGTTTGATGTATTATTATTACATGTAAT |
| 5′EIN2seq07 | ACTATTGTTGTTAGGTCAACTTGC |
| 5′EIN2seq08 | GCTGCTTCATACTAAGATCAATTATT |
| 5′EIN2seq09 | AATCCTACTCTGAGTAATTATATTATCAGATAG |
| 5′EIN2seq10 | TCGACCTTACCATGGTAGTTACT |
| 5′EIN2seq11 | CATTCTTATTTTGCTGGGGTA |
| 5′EIN2seq12 | GACTTCCTGAAGATAGAAATACCC |
| 5′EIN2seq13 | TCCATCTGTTCAAGAAGAGGA |
| 5′EIN2seq14 | AGTGGGACTGGAAGCCTT |
| 5′EIN2seq15 | ACAGTTCACGGATACCAGATG |
| 5′EIN2seq16 | ATGATGACATTTCTCAATCAAGAG |
| 5′EIN2seq17 | CCTGAGCTTTGGGGAAAG |
| 5′EIN2seq18 | GTACGGATCATTGGGTTGA |
| 5′EIN2seq19 | CGCTATCGCCTTTTATCTCA |

**Table S5. Primers for *CTR1* baculovirus construct**

| Construct | Forward or reverse | Primer sequence (5′–3′) |
|---|---|---|
| CTR1 kinase domain | F | CACCATGAACAGGGCAAATAGGGAACTTGGAC |
| | R | TTACAAATCCGAGCGGTTGGGC |

**Table S6. Primers for *EIN2* transgene constructs**

| Construct | Forward or reverse | Primer sequence (5′–3′) |
|---|---|---|
| EIN2p-EIN2 | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCGGTGCTTTCGATCTAAAGTCCTGAGCC |
| | R | GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGTGACAGTCCGCTGAAGACACCAAAGATGGC |
| | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCGGTGGAATGGAGTGTTAACTCGGTTAAATGG |
| | R | GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAAAGAGAGGACTTTTCTGCGTTGTG |
| EIN2p-EIN2-GFP and 35S-EIN2-YFP | R | GGGGACCACTTTGTACAAGAAAGCTGGGTAACCCAATGATCCGTACGCAGTCACGTTTTTTC |
| 35S-EIN2 and 35S-EIN2-YFP | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGAAGCTGAAATTGTGAATGTG |
| Primers for *EIN2* mutagenesis | | |
| S645A | F | GATCTGATGGTCCTCCTGCATTCCGCAGCTTAAGTGG |
| | R | CCACTTAAGCTGCGGAATGCAGGAGGACCATCAGATC |
| S924A | F | GCCAATGAGAAAAAATATAGTGCCATGCCAGATATCTCAGG |
| | R | CCTGAGATATCTGGCATGGCACTATATTTTTTCTCATTGGC |

**Table S7. Primers for *EIN2* transient expression constructs**

| Construct | Forward or reverse | Primer sequence (5′–3′) |
|---|---|---|
| 35S-EIN2-GFP | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGAAGCTGAAATTGTGAATGTG |
| | R | GGGGACCACTTTGTACAAGAAAGCTGGGTAACCCAATGATCCGTACGCAGTCACGTTTTTTC |
| 35S-YFP-EIN2 | F | GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGAAGCTGAAATTGTGAATGTG |
| | R | GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAACCCAATGATCCGTACGCAGT |