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Separation anxiety

An analysis of ethylene-induced cleavage of EIN2

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Since the discovery of the CTR1 protein kinase and the endoplasmic reticulum (ER)-localized EIN2 protein nearly 20 y ago, plant biologists have wondered how these proteins respectively serve as negative and positive regulators of ethylene-mediated signal transduction in plants.[1,2] Now with the publication of four studies, it can be concluded that in the absence of ethylene (ET) in *Arabidopsis thaliana*, CTR1 phosphorylates EIN2 thereby inactivating ET signal transduction, while in the presence of ET, CTR1 no longer phosphorylates EIN2 and the cytosolic C-terminus of EIN2 is released from the ER to translocate to the nucleus to promote gene transcription.[3-6] Chen et al. (2011) showed that EIN2 is differentially phosphorylated at amino acids (a.a.) S^{645} and S^{924} after ET treatment.[6] Ju et al. (2012) then proved that CTR1 phosphorylates EIN2 at those positions and that the lack of phosphorylation at S^{645} and S^{924} leads to the translocation of an EIN2 C-terminus peptide.[5] Wen et al. (2012) and Qiao et al. (2012) also demonstrated ET-induced translocation of an EIN2 C-terminus peptide, while Qiao et al. (2012) proved that EIN2 has a nuclear localization signal sequence required for translocation, confirmed phosphorylation at S^{645} and said that proteolytic cleavage occurs at S^{645} in absence of phosphorylation there.[3,4] Despite the revelation of this elegant switch, there are contradictory indications for specific cleavage at EIN2 S^{645}. This article investigates the data and concludes that EIN2 may be cleaved at alternative positions.

Keywords: ethylene signaling, triple response, mass spectrometry, semi-tryptic peptide, phosphorylation

Abbreviations: ER, endoplasmic reticulum; ET, ethylene; a.a., amino acid; pMRM, pseudo-multiple reaction monitoring

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The first sign of inconsistency has to do with the theoretical molecular weight of the EIN2 C-terminus from S^{645} to the stop codon which is approximately 70 kDa. A 70 kDa fragment could migrate to the nucleus if cleavage occurred at S^{645}, but the Qiao et al. immunoblots (see Fig. 3A and B in ref. 3) showed an ~80 kDa EIN2 C-terminus peptide. The EIN2 C-terminus-YFP fusion peptide (Fig. 4G and H in ref. 3) was also larger than expected from an S^{645} cleavage site. Although protein sizes can be difficult to estimate by SDS-PAGE, a cleavage site upstream of S^{645} could reasonably explain the ~10 kDa size excess. Wen et al. also revealed a product larger than predicted by cleavage at S^{645} and several smaller fragments. Hence, the immunoblots do not appear to be consistent with a single cleavage position at S^{645}.

Qiao et al. reasoned that S^{645} is the cleavage site because EIN2 is differentially phosphorylated at S^{645} as originally shown by Chen et al. Subsequently, Qiao et al. used pseudo-multiple reaction monitoring (pMRM) mass spectrometry to detect changes in abundance between EIN2 tryptic peptides and their phosphorylated analogs before and after ET treatment. According to the Qiao et al. model (rendered in **Fig. 1**), if EIN2 is cleaved at S^{645} after ET treatment, then the C-terminus moves from the ER to the nucleus. This means that the abundance of peptides downstream of S^{645} should decrease in ER membrane fractions after ET treatment and concomitantly increase in the nucleus. Immunoblots showing a preponderance of EIN2 antigen in nuclear preparations after ET treatment were consistent with

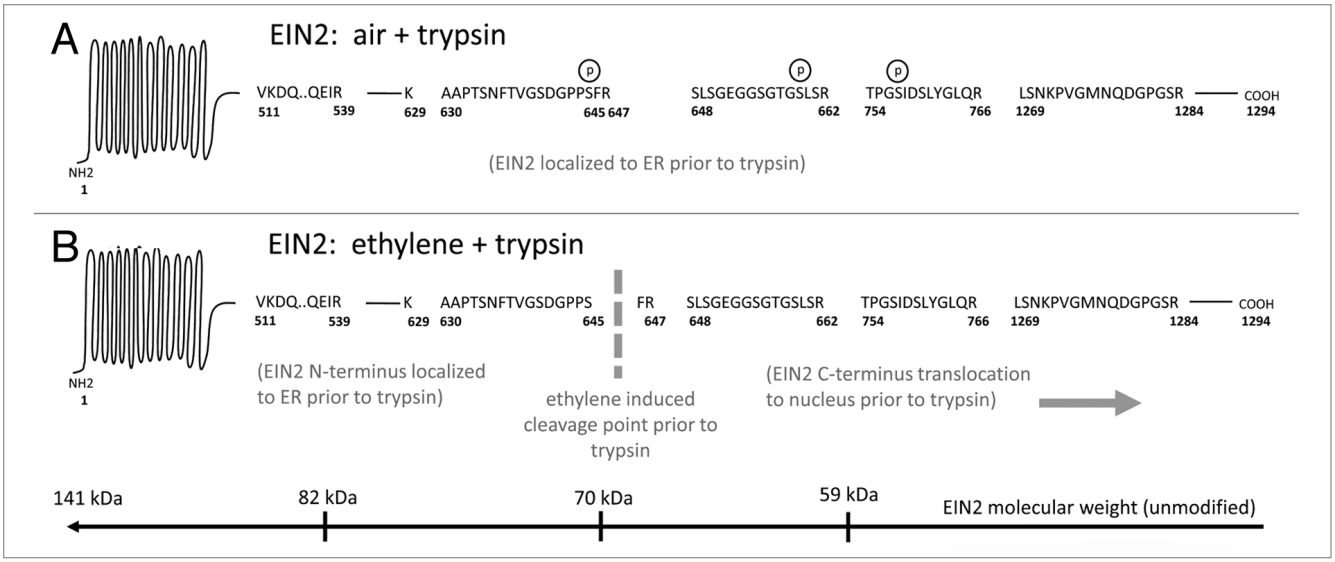

Figure 1. EIN2 phosphorylation and cleavage products based on Qiao et al.[3] (**A**) EIN2 in absence of ET (ambient air exposure) and digested with trypsin. EIN2 is phosphorylated (circled p) and remains anchored at the ER. (**B**) EIN2 in the presence of ET. EIN2 is not phosphorylated and is cleaved in vivo at S^{645} and the EIN2 C-terminus translocates to the nucleus (prior to trypsin digestion). The bottom bar shows the approximate molecular weight of native EIN2 as measured from the C-terminus. The author contends that the ET-induced cleavage site in model in **B** is not sufficiently proven by published results.

the model (Fig. 3B in ref. 3; Fig. 3K in ref. 4), but the abundances of downstream peptides measured by pMRM were inconsistent (Table S1A in ref. 3): Assuming that pMRM precisely measured a 10-fold change for phosphopeptide a.a. 648–662 in ER membranes, then the data unexpectedly showed no decrease in abundance of the analogous nonphosphorylated peptide a.a. 648–662 after ET treatment.

Interestingly, Qiao et al. observed a 19-fold decrease for nonphosphorylated peptide a.a. 630–647 after ET treatment, but they attributed this to cleavage at S^{645} even though upstream cleavage would also explain the observation. It may be unintuitive why Qiao et al. reached that conclusion, so **Figure 1** is provided for clarity. The amino acids K^{629} and R^{647} are trypsin digestion sites that flank S^{645} and after ET treatment and tryptic digestion the semi-tryptic peptide a.a. 630–645 should become more prevalent with increasing amounts of nonphosphorylated EIN2. Thus, Qiao et al. concluded that tryptic peptide a.a. 630–647 decreased because of prior, ET-mediated proteolytic cleavage at S^{645}. Thus, they expected this would lead to an increased semi-tryptic variant after ET treatment and trypsin digestion and they found evidence of this by pMRM, which they cited as proof of cleavage at S^{645}. Since the model dictates that the semi-tryptic peptide is prevalent after ET treatment, I re-examined the mass spectrometry data from Chen et al. which were sufficient to reveal differential phosphorylation of EIN2 at S^{645}.[6] Mascot searches for semi-tryptic termini and error-tolerant searches for hundreds of mass deviations reconfirmed the phosphorylated tryptic peptide a.a. 630–647 in ambient air control seedlings and the nonphosphorylated form in ET-treated seedlings (**Table 1**). There was, however, no other prevalent mass modification in EIN2 peptides and the semi-tryptic peptide a.a. 630–645 was not apparent (**Table 1**). Of course, not finding a peptide by shotgun mass spectrometry rarely invalidates its existence,[7] but it is suspect that an essential nonphosphorylated semi-tryptic peptide predicted by Qiao et al. was more difficult to observe than its inherently-difficult-to-detect phosphorylated precursor. Thus, it can be argued that the available pMRM and shotgun proteomics data do not conclusively support EIN2 cleavage at S^{645}.

Qiao et al. and Ju et al. genetically assessed EIN2 phosphosites and showed that these are crucial for regulating EIN2 nuclear translocation, but these experiments may not have provided conclusive insight on positions of cleavage. For example, Qiao et al. substituted S^{645} with alanine ($S^{645}A$), expressed $EIN2^{S645A}$-YFP in transgenic plants, observed the translocation of YFP to the nucleus, and found an ET phenotype in the absence of ET treatment. Their results implied that the loss of phosphorylation is a regulatory signal that sends EIN2 to the nucleus. But while the $S^{645}A$ mutation will indeed inhibit phosphorylation at that position, it seems plausible that the mutation could also change the recognition site for the unknown protease that Qiao et al. concluded catalyzed hydrolysis there. Qiao et al. did not test by pMRM for the abundance of peptides with $S^{645}A$ termini, so it remains unknown whether the existence of such peptides were adversely affected. Notwithstanding, it is reasonable to suspect that the $S^{645}A$ mutation may have not inhibited potential upstream cleavage positions (evidenced by the larger-than-predicted size of the EIN2 fragment in Fig. 4G in ref. 3). Thus, these genetic experiments supported a functional role of phosphorylation, but did not validate cleavage at S^{645}.

In fact, there are other sites of phosphorylation on EIN2 shown by Chen et al. and Ju et al. that were not fully investigated by Qiao et al. Independent mutations on two different CTR1-regulated phosphosites revealed that ambient-air grown *A.*

Table 1. Peptide-tandem mass spectrum matches from air (control) and ET-treated *A. thaliana* etiolated seedlings

Air treated (control)									
Spectrum query	EIN2 amino acid start	EIN2 amino acid end	Observed m/z	Mr (expt)	Mr (calc)	Parent ion ppm error	Mascot Ions score	Mascot expect value	EIN2 peptide sequence match
329	502	506	603.345	602.3378	602.3388	−1.67	25	0.063	D.ESIVR.L
1221474	511	539	1104.5542	3310.6408	3309.6286	306	51	0.00039	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
1221420	511	539	1104.2208	3309.6406	3309.6286	3.63	41	0.0035	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
1221418	511	539	1104.2197	3309.6374	3309.6286	2.64	36	0.0091	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
811749	552	567	886.933	1771.8515	1771.8476	2.23	44	0.00092	K.YSTSQVSSLKEDSDVK.E
812432	552	567	887.4352	1772.8558	1771.8476	569	45	0.00098	K.YSTSQVSSLKEDSDVK.E
811742	552	567	886.9304	1771.8463	1771.8476	−0.73	34	0.006	K.YSTSQVSSLKEDSDVK.E
812434	552	567	887.4361	1772.8576	1771.8476	570	33	0.0073	K.YSTSQVSSLKEDSDVK.E
812425	552	567	887.4332	1772.8519	1771.8476	567	25	0.091	K.YSTSQVSSLKEDSDVK.E
878122	630	647	944.4185	1886.8224	1886.82	1.24	92	0.00000033	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
878123	630	647	944.4192	1886.8238	1886.82	2.01	78	0.0000028	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
878470	630	647	944.9172	1887.8199	1886.82	530	64	0.000017	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
878121	630	647	944.4182	1886.8219	1886.82	0.98	56	0.000034	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
878126	630	647	944.4207	1886.8269	1886.82	3.63	62	0.00005	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
878117	630	647	944.4091	1886.8036	1886.82	−8.72	58	0.000087	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
878472	630	647	630.2815	1887.8227	1886.82	531	26	0.096	K.AAPTSNFTVGSDGPPSFR.S + Phospho S (S)
936929	648	666	668.6009	2002.7807	2001.7847	498	33	0.06	R.SLSGEGGSGTGSLSRLQGL.G + 2 Phospho S (S); Phospho T (T)
423230	700	710	646.8458	1291.6771	1291.6772	−0.094	44	0.0026	K.KLDQLFGTDQK.S
423193	700	710	646.8409	1291.6673	1291.6772	−7.65	30	0.075	K.KLDQLFGTDQK.S
542771	754	766	703.8643	1405.714	1405.7201	−4.39	69	0.000033	R.TPGSIDSLYGLQR.G
542775	754	766	703.8674	1405.7203	1405.7201	0.12	59	0.000064	R.TPGSIDSLYGLQR.G
542786	754	766	703.8703	1405.726	1405.7201	4.2	50	0.00038	R.TPGSIDSLYGLQR.G
542779	754	766	703.8681	1405.7217	1405.7201	1.13	44	0.0011	R.TPGSIDSLYGLQR.G
608871	754	766	743.8513	1485.6881	1485.6865	1.09	31	0.04	R.TPGSIDSLYGLQR.G + Phospho S (S)
608879	754	766	743.8526	1485.6907	1485.6865	2.87	35	0.068	R.TPGSIDSLYGLQR.G + Phospho S (S)
274965	835	843	551.3035	1100.5925	1100.5938	−1.16	45	0.0099	K.ERLEALQSR.G
754564	922	936	841.355	1680.6954	1680.6888	3.91	31	0.0072	K.YSSMPDISGLSMSAR.N + Phospho S (S)
52751	1211	1217	699.45	698.4427	698.4439	−1.65	20	0.062	A.AKPAKGK.C
790762	1269	1284	868.8923	1735.77	1735.7713	−0.74	31	0.062	R.LSNKPVGMNQDGPGSR.K + Phospho S (S)

Spectra from Chen et al.[6] were searched as described in Chen et al.,[6] but for semi-tryptic termini. Only the peptide-spectrum matches with expect values < 0.1 are considered.

Table 1. Peptide-tandem mass spectrum matches from air (control) and ET-treated *A. thaliana* etiolated seedlings (continued)

			Ethylene treated						
Spectrum query	EIN2 amino acid start	EIN2 amino acid end	Observed m/z	Mr (expt)	Mr (calc)	Parent ion ppm error	Mascot Ions score	Mascot expect value	EIN2 peptide sequence match
902435	511	539	1104.2163	3309.6271	3309.6286	−0.46	44	0.0012	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
902516	511	539	1104.5494	3310.6265	3309.6286	302	34	0.016	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
902436	511	539	1104.2182	3309.6327	3309.6286	1.24	29	0.056	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
902520	511	539	1104.5546	3310.6419	3309.6286	306	31	0.087	R.VKDQLDTTSVTSSVYDLPENILMTDQEIR.S
518666	552	567	591.6218	1771.8437	1771.8476	−2.22	26	0.06	K.YSTSQVSSLKEDSDVK.E
536908	630	647	904.4321	1806.8497	1806.8537	−2.2	55	0.00013	K.AAPTSNFTVGSDGPPSFR.S
241596	700	710	646.8446	1291.6747	1291.6772	−1.98	59	0.00026	K.KLDQLFGTDQK.S
242470	700	710	647.3521	1292.6897	1291.6772	784	35	0.013	K.KLDQLFGTDQK.S
241600	700	710	646.8446	1291.6747	1291.6772	−1.93	32	0.04	K.KLDQLFGTDQK.S
778132	805	826	819.3732	2455.0977	2454.1022	406	65	0.000017	R.APSSSEGWEHQQPATVHGYQMK.S
778133	805	826	819.3735	2455.0987	2454.1022	406	71	0.000056	R.APSSSEGWEHQQPATVHGYQMK.S
777982	805	826	819.0399	2454.0977	2454.1022	−1.84	39	0.0054	R.APSSSEGWEHQQPATVHGYQMK.S
142268	835	843	551.3033	1100.592	1100.5938	−1.61	37	0.065	K.ERLEALQSR.G
309469	839	850	698.3436	1394.6727	1393.6715	718	23	0.096	E.ALQSRGEIPTSR.S + Phospho S (S)
461623	1269	1284	828.9051	1655.7956	1655.8049	−5.63	58	0.000044	R.LSNKPVGMNQDGPGSR.K
461625	1269	1284	828.9077	1655.8009	1655.8049	−2.46	38	0.0029	R.LSNKPVGMNQDGPGSR.K
461621	1269	1284	828.9038	1655.7931	1655.8049	−7.18	37	0.0054	R.LSNKPVGMNQDGPGSR.K
461619	1269	1284	552.9373	1655.7901	1655.8049	−8.95	31	0.051	R.LSNKPVGMNQDGPGSR.K

Spectra from Chen et al.[6] were searched as described in Chen et al.,[6] but for semi-tryptic termini. Only the peptide-spectrum matches with expect values < 0.1 are considered.

thaliana seedlings transgenic for EIN2^{S645A} expressed by the native *EIN2* promoter exhibited little ET-response phenotype, whereas EIN2^{S942A} transgenic seedlings exhibited a much stronger phenotype. Since the S^{645} site retained the potential to be phosphorylated in the EIN2^{S924A} seedlings in ambient air, it is likely that specific cleavage was blocked at S^{645} under the Qiao et al. model. But because EIN2^{S924A} produced a strong phenotype whereas EIN2^{S645A} did not, cleavage likely occurred elsewhere.

So what explains the strong phenotype for S^{645}A observed by Qiao et al. when the same mutation conferred a weak phenotype for Ju et al.? Transgenic expression and protein accumulation may be the difference. Ju et al. revealed that transgenic seedlings overexpressing wild-type EIN2 from the constitutive CaMV *35S* promoter exhibited an unexpected, abnormal, strong ET-response phenotype in ambient air. Consequently, Ju et al. switched to using the native *EIN2* promoter. When they did, their EIN2 transgenics more closely resembled nontransgenic wild-type plants. Hence, expression and accumulation differences also likely explain why Ju et al. observed a slight ET phenotype for EIN2^{S645A} transgenic seedlings with the native *EIN2* promoter but a stronger ET phenotype for the EIN2^{S645A} transgenic seedlings with a *35S* promoter. Therefore, it is possible that the same strong phenotype for the same S^{645}A mutation observed by Qiao et al. may have been due to their use of the *35S* promoter as well. In that case, excessive and constant accumulation of EIN2^{S645A} may have short-circuited CTR1 control, preventing phosphorylation at the unexamined S^{924} site (mimicking S^{924}A in Ju et al.) and leading to an inadvertent but stronger phenotype that masked the weaker effect of S^{645}A.

The results from four papers reveal that differential phosphorylation of EIN2 controls EIN2-mediated activation of transcription at the nucleus and leads to ET-regulated proteomic changes.[3-6] Nevertheless, on the basis of the conflicting evidence, specific cleavage at S^{645} is controversial.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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