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Ethylene signaling: new levels of complexity and regulation

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Summary

The gaseous plant hormone ethylene plays important roles in plant growth and development. Recent discoveries have expanded our linear view of ethylene signaling by revealing an elaborate signaling network with multiple regulatory circuits. At the membrane, the ethylene receptors form heteromeric and higher order complexes providing enhanced sensitivity and fine-tuning of signaling. Ethylene sensitivity is further enhanced by the rapid degradation of ethylene receptors upon ethylene binding and by dependence on a novel protein REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1)/GREEN-RIPE (GR). In the nucleus, EIN3-BINDING F-BOX1 and 2 (EBF1/2) coordinately control 26S proteasome degradation of the critical transcription factors EIN3 and EIL1. EBF1/2 expression is repressed by *ETHYLENE-INSENSITIVE5* (*EIN5*), which encodes the exoribonuclease XRN4. Additionally, EIN3 possesses two mitogen-activated protein kinase (MAPK) phosphorylation sites that have opposing effects on EIN3 stability.

Introduction

Ethylene is a gaseous plant hormone that plays a key role in many processes, including seed germination, leaf senescence, fruit ripening, abscission and responses to abiotic and biotic stresses [1]. The molecular dissection of ethylene signal transduction began with genetic screens based on the well-documented triple response phenotype of ethylene-treated etiolated *Arabidopsis* seedlings [2]. Initial studies uncovered a linear framework for the ethylene-signaling pathway, leading from ethylene perception at the membrane to transcriptional activation in the nucleus.

Briefly, ethylene is perceived by a family of membrane-bound receptors [2,3] having similarity to two-component histidine protein kinase receptors and derived from a cyanobacterial origin [4**,5]. Each receptor has an N-terminal membrane-spanning domain that binds ethylene with a copper cofactor [6] provided by the RAN1 copper transporter [7]. Although the receptors display protein kinase activity in vitro, their biochemical signaling mechanism is unknown [2,3]. Genetically, the receptors are negative regulators of ethylene signaling [8,9*]; in the absence of ethylene, the receptors repress downstream ethylene responses through the Raf-like protein kinase CTR1 [10] and, when ethylene is bound, the receptors no longer repress ethylene responses [2,3]. CTR1 negatively regulates ethylene responses by repressing the positive regulator EIN2 [11], which relays the ethylene signal by an unknown mechanism to the transcription factors EIN3 and EIL, which in turn activate the ERF1 transcription factor [12]. ERF1 activates transcription of ethylene responsive genes such as PDF1.2 [12]. EIN3 and EIL1

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are constitutively expressed and controlled by protein degradation through a 26S proteasome-dependent pathway [13–15]. The components and overall mechanisms of ethylene signaling are conserved among dicots and monocots [2,16–18].

Recent advances have expanded our linear view of the ethylene-signaling pathway into an increasingly complex signaling system that includes multiple pathways of regulation and feedback (Figure 1). In this review, we focus on discoveries in ethylene signaling reported in the last two years. These latest findings have revealed new levels of regulation, particularly with respect to the ethylene receptors and the EIN3/EIL1 transcription factors. Due to space limitations, we do not discuss ethylene crosstalk with other pathways or descriptions of ethylene responses, which can be found in recent reviews on ethylene signaling [2], ethylene biosynthesis [19] and crosstalk [20–23].

Multiple modes of ethylene receptor regulation

Subfamily I versus Subfamily II receptors

The ethylene receptors fall into two subfamilies as shown in Figure 1. Subfamily I receptors have three amino-terminal transmembrane domains, while subfamily II receptors have four [3]. Subfamily I receptors possess histidine kinase activity, whereas subfamily II receptors possess serine/threonine kinase activity. *Arabidopsis* has five ethylene receptors, two of which (ETR1 and ERS1) are members of subfamily I. Tomato has six ethylene receptors, three of which belong to subfamily I [24].

Individual receptor isoforms contribute differentially to overall signaling. In *Arabidopsis*, subfamily I plays a larger role than subfamily II and cannot be replaced by subfamily II members [2,3]. In tomato, subfamily II receptors (*LeETR4* and *LeETR6*) play the largest role in ethylene responses [24,25**]. To clarify the role of *Arabidopsis* subfamily I receptors, Qu et al. [9*] analyzed new T-DNA insertion alleles of *ETR1* and *ERS1*. The new *ers1-3* mutant exhibits hypersensitivity to ethylene not seen previously for *ers1-2*, and correspondingly, the subfamily I double null mutant carrying *ers1-3* has a stronger phenotype. In addition to having a more severe constitutive triple response, the double mutant is dwarfed with reduced fertility, premature leaf senescence, and novel filamentous structures at the base of the flower. The double mutant still shows a response to ethylene, representing signaling from subfamily II.

Repression of ETR1 signaling by the ETR1 N-terminal domain

The first mutations isolated in the ethylene receptor genes were dominant gain-of-function mutations conferring ethylene insensitivity. Single receptor loss-of-function mutants are essentially indistinguishable from the wild type due to a high degree of functional redundancy among the receptors [3], but null mutant combinations, such as the subfamily I mutant described above, exhibit varying degrees of constitutive ethylene responses, demonstrating that the ethylene receptors are negative regulators of ethylene responses. From this, it was deduced that ethylene perception shuts off, rather than activates ethylene receptor signaling [8]. Interestingly, all of the known dominant mutations reside within or immediately after the N-terminal ethylene-binding domain.

Greater insight into the relationship between the N-terminal domain and signaling domain was recently obtained from a structure/function analysis of ETR1 [4**]. Wang et al. [4**] introduced amino acid substitutions for 37 residues of the ETR1 N-terminal domain and measured the effects on both ethylene binding and signal output. Interestingly, only two of these mutations cause a loss of function with respect to ETR1 signaling. Most of the remaining mutations result in gain-of-function (constitutive) ETR1 signaling. A subset of these constitutive signaling mutations disrupt ethylene binding (and are primarily located in the mid-

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regions of transmembrane helices I and II), but the others (located near the cytoplasmic ends of transmembrane helices I and III) do not. The latter hold the receptor in an intermediate state, in which ethylene is bound to the receptor but receptor signaling remains on. These results suggest that the predominant function of the N-terminal domain is to inhibit the signaling domain.

Ethylene receptor degradation: enhancing sensitivity

Protein degradation plays a key role both in ethylene biosynthesis through the regulation of ACC synthase [26] and in ethylene signaling through the regulation of EIN3 [13–15]. Recently, it was found that one or more ethylene receptors are subject to regulated degradation as well. Upon ethylene binding, the *Arabidopsis* ETR2 receptor is targeted for endoplasmic reticulum (ER)-associated degradation by a proteasome-dependent pathway, triggered perhaps by a conformational change in the receptor [27**]. Similarly, tomato LeETR4 and LeETR6 receptors are degraded rapidly in the presence of ethylene, most likely by the 26S proteasome [25**]. The degradation of either LeETR4 or LeETR6 results in early fruit ripening, providing a nice demonstration of how receptor levels can control the sensitization of plant tissues to ethylene [25**]. This degradation helps to explain why the dramatic increase in ethylene receptor gene transcript levels during ripening does not result in the inhibition of ripening.

Whether all ethylene receptors are regulated in this manner remains to be seen. As explained by Chen *et al.* [27**], the receptors exhibit slow ethylene dissociation kinetics when expressed in yeast cells, but in plants, both rapid (< 30 minute half-life) and slow (> 12 hour half life) release kinetics have been observed. The degradation of certain ethylene receptors might account for this rapid dissociation component.

Interactions among the ethylene receptors

New data indicate that the *Arabidopsis* ethylene receptors are capable of forming heteromeric complexes, which has implications for signal amplification and fine-tuning of ethylene responses. It was shown previously that ETR1 and ERS1 exist as disulfide-linked homodimers [3], although the cysteine residues required for the disulfide bonds do not appear to be essential for receptor signaling [28]. Recently, protein-protein interactions for all possible ethylene receptor combinations have been observed, both in a membrane recruitment assay in tobacco cells and in the yeast split ubiquitin assay [29*]. Similar interactions, as well as higher order complexes, have been detected by co-purification of epitope-tagged ethylene receptors in *Arabidopsis* [Gao *et al.*, submitted]. Such interactions may explain why a truncated form of ETR1 (residues 1–349 comprising the ethylene-binding domain, GAF domain and coiled coil domain) is capable of signaling in the presence of other ethylene receptors [3,28]. In several studies, the GAF domain has been indicated as a site of interaction between the receptors [28,29*; Gao *et al.*, submitted]. Because the ethylene receptor genes have overlapping but distinct expression patterns, the composition of the receptor complex is likely to vary among different plant tissues, resulting in a broad range of differential responses to ethylene [29*].

Membrane localization and topology of the ethylene receptors

The ethylene receptors might be present at one or more membrane systems. *Arabidopsis* ETR1 and ETR2 were previously localized to the ER by sucrose density gradient fractionation [3], and recently, all five *Arabidopsis* ethylene receptors were localized to the ER when expressed in tobacco leaf epidermal cells [29*]. In contrast, immunohistochemistry showed ETR1 to be primarily at the Golgi apparatus in *Arabidopsis* roots [30*], whereas tobacco NTHK1 (subfamily II) was reported to be at the plasma membrane in protoplasts [3].

The predicted membrane topology of the receptors was confirmed using CmERS1, a melon subfamily I ethylene receptor that localizes to the ER [31]. The N-terminal domain of CmERS1

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spans the membrane three times with the N-terminus on the lumenal side and the C-terminus in the cytosolic side of the ER membrane.

RTE1, a membrane protein that represses ethylene responses through ETR1

Arabidopsis REVERSION-TO-ETHYLENE SENSITIVITY1 (*RTE1*) encodes a novel integral membrane protein involved in regulating ETR1 function [32**]. *RTE1* is highly conserved in plants, animals and some protists, but its molecular function is unknown. The RTE1 protein co-localizes with ETR1 in the Golgi and ER [30*]. *RTE1* was identified on the basis of loss-of-function *rte1* mutants that suppress the ethylene-insensitive receptor mutation *etr1-2* [32**]. *rte1* mutants have hypersensitivity to ethylene, similar to the *etr1* null mutant [32**, 33*]. *GREEN-RIPE* (*GR*) is a tomato homolog of *RTE1*. The dominant *Green-ripe* (*Gr*) mutant exhibits inhibition of fruit ripening and other ethylene-insensitive phenotypes [34**]. The *Gr* mutant carries a deletion in the *GR* promoter region that causes ectopic over-expression of *GR* [34**]. In *Arabidopsis*, ethylene insensitivity from *RTE1* over-expression is largely dependent on *ETR1* but not on the other ethylene receptors, suggesting that *RTE1* has specificity for *ETR1* [32**,33*]. In addition, *rte1* does not suppress ethylene insensitivity conferred by the *etr1-1* allele nor ethylene-insensitive mutations in the other ethylene receptor genes [32**]. *RTE1* expression is ethylene-inducible, suggesting that *RTE1* is involved in negative feedback on ethylene signaling [32**].

Downstream regulation of ethylene signaling

Regulation of the Raf-like kinase CTR1

Acting downstream of the ethylene receptors is a putative MAPK kinase kinase CTR1, a negative regulator of ethylene responses with similarity to Raf protein kinases [10]. CTR1 has a non-catalytic N-terminal region that physically interacts with subfamily I receptors, resulting in association of CTR1 with the ethylene receptor complex [35,36]. *Arabidopsis* has one copy of *CTR1*, while tomato has three to four copies. The tomato subfamily I receptor *NEVER-RIPE* (*NR*) interacts *in vivo* with *LeCTR1, LeCTR3, and LeCTR4* [37*]. Apart from interaction with the receptors, little is understood about CTR1 regulation. Most models of CTR1 regulation are based on what is known about Raf kinases. The mammalian Raf-1 kinase can bind phosphatidic acid (PA), leading to translocation of Raf-1 from the cytosol to the plasma membrane for activation [38]. In plants, PA levels increase rapidly in response to biotic and abiotic stresses [39]. Recently, it was found that PA is capable of binding to the CTR1 kinase domain and blocking kinase activity *in vitro* [40], raising the possibility that PA negatively regulates CTR1.

Post-transcriptional regulation of the EIN3 transcription factor

The deactivation/inhibition of CTR1 kinase activity leads to the downstream activation of two transcription factors, EIN3 and EIL1, which are positive regulators of ethylene signaling [41]. In the absence of ethylene perception, two F-box proteins, EBF1 and EBF2, in a Skp-Cullin-F-box (SCF) E3 ligase complex, target EIN3 and EIL1 for degradation via the 26S proteasome [13-15,42]. While *ebf1* and *ebf2* single mutants are only slightly hypersensitive to ethylene, the *ebf1 ebf2* double mutant displays severe growth arrest [15]. Binder *et al*. [43**] showed that by introducing *ein3* and *eil1* mutations into the *ebf1 ebf2* background, the severity of the *ebf1 ebf2* double mutant is not only alleviated, but the resulting quadruple mutant is ethylene insensitive. Thus, EIN3 and EIL1 are the predominant targets of EBF1/2 in ethylene signaling. Kinetic analyses of ethylene response in *ebf1* and *ebf2* suggest that EBF1 degrades EIN3/EIL1 prior to ethylene signaling, whereas EBF2 plays a larger role in degrading EIN3/EIL1 after ethylene responses have been activated [43**]. This role of EBF2 is consistent with previous suggestions that an ethylene-induced negative feedback loop is involved in EIN3 regulation by the $SCF^{EBF1/2}$ complex [13–15,43**]. The distinct but overlapping roles of EBF1

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and EBF1 provide fine-tuned post-transcriptional regulation of EIN3 and EIL1, permitting, for example, the rapid induction of ethylene responses during biotic stresses such as pathogen attack.

Another significant finding is that the stability of EIN3 appears to be controlled through two MAPK phosphorylation sites, one required for stabilization of EIN3 and the other involved in its degradation [44**]. Yoo et al. obtained *in vivo* evidence that two MAPKs, MPK3 and MPK6, phosphorylate EIN3 on Threonine174 to stabilize EIN3. They also showed that MPK3/6 can be activated by MKK9, forming a potential MAPK cascade in ethylene signaling. In contrast, a constitutively active form of CTR1 results in EIN3 degradation, but loses this effect when the second EIN3 phosphorylation site (Threonine592) is mutated [44**]. This suggests that CTR1 can activate a separate MAPK, but whether CTR1 has direct control of a MAP kinase cascade remains an open question.

EIN3 seems to be a point of convergence of the ethylene, glucose and possibly light signaling pathways [41,42,45]. Therefore, multiple protein kinases serving multiple functions in different pathways may be involved in regulating EIN3. For example, MPK6 plays a role in ethylene biosynthesis [46] and possibly EIN3 stabilization [44**].

EIN5, an exoribonuclease that affects EBF1/EBF2 levels

EBF1 and *EBF2* are regulated by *ETHYLENE-INSENSITIVE5* (*EIN5*), which acts downstream of *CTR1* and encodes the 5′→ 3′ exoribonuclease XRN4 [47**,48**]. *EIN5/XRN4* is homologous to yeast exoribonucleases Xrn1p and Rat1p, which function in mRNA and rRNA degradation, respectively. Similar to Xrn1p, EIN5 localizes to the cytoplasm [48] and can rescue yeast *xrn1* but not *rat1* [47**]. Interestingly, *EBF1/2* transcripts, which are ethylene-inducible, accumulate in the *ein5/xrn4* background [47**,48**]. Consequently, EIN3 protein does not accumulate in the *ein5*/*xrn4* background in the presence of ethylene and, similarly, many other ethylene-inducible transcripts are expressed at much lower levels relative to wild type [47**,48**]. EIN5/XRN4 does not appear to directly degrade *EBF1/2* transcripts, because the half-life of *EBF1/2* transcripts in the *ein5* mutant background is the same as in the wild type [48**]. Therefore, the accumulation of *EBF1/2* mRNAs may be due to increased transcription with *EIN5*/*XRN4* promoting a repressor of *EBF1/2* transcription.

EER3 and EER4 may be involved in transcription

Arabidopsis ENHANCED ETHYLENE RESPONSE3 (*EER3*) and *4* (*EER4*) were identified through mutants exhibiting enhanced ethylene response in seedlings [50*,51]. *EER3* encodes a previously uncharacterized prohibitin, AtPHB3 [50*]. In mammals, some prohibitins are involved in the formation of transcriptional complexes. *EER4* encodes a transcription factor containing a putative TATA-binding factor (TFIID)-interacting domain [51]. In yeast, TFIID binds to the TATA box and initiates formation of the RNA polymerase II complex. An *eer3* mutant completely suppresses *ein3-1* ethylene-insensitivity, whereas an *eer4* mutant partially suppresses *ein3-1*. If EER3 and EER4 are functionally conserved with their mammalian and yeast homologs, respectively, then these two proteins may be involved in transcription leading to particular ethylene responses.

Conclusions

In recent years, our understanding of ethylene signal transduction has advanced significantly, making ethylene signaling one of the best-characterized pathways in plants. The latest discoveries have begun to address the complexities surrounding the interplay of the ethylene receptors and how they function to provide exquisite sensitivity and signaling efficiency, and have revealed multiple mechanisms of feedback and regulation. Interactions between the receptors, receptor turnover, differing signaling strengths and differential expression patterns

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all contribute to the fine tuning of ethylene perception and signaling. Transcription factor EIN3 has emerged as a key player that is regulated by rapid protein turnover. Intriguing data suggests that two distinct MAPK cascades have opposing effects on EIN3 stability. EIN3 (and EIL1) turnover is blocked by down-regulation of *EBF1/2* F-box gene expression involving an exoribonuclease. *EBF1/2* have distinct but overlapping functions that provide added fine-tuning of this pivotal signaling step.

Remaining key questions include the biochemical mechanism of ethylene receptor signaling, the biochemical function of RTE1/GR, the mechanism of CTR1 regulation and downstream targets of CTR1, the identification of additional MAPK cascade components, the biochemical function, targets and regulation of EIN2, the targets of XRN4, how ethylene perception regulates EBF1/2 and the precise roles of *EER3* and *EER4*. The continued integration of molecular, genetic, cell biological, kinetic and biochemical approaches will help to elucidate the answers to these and many other questions.

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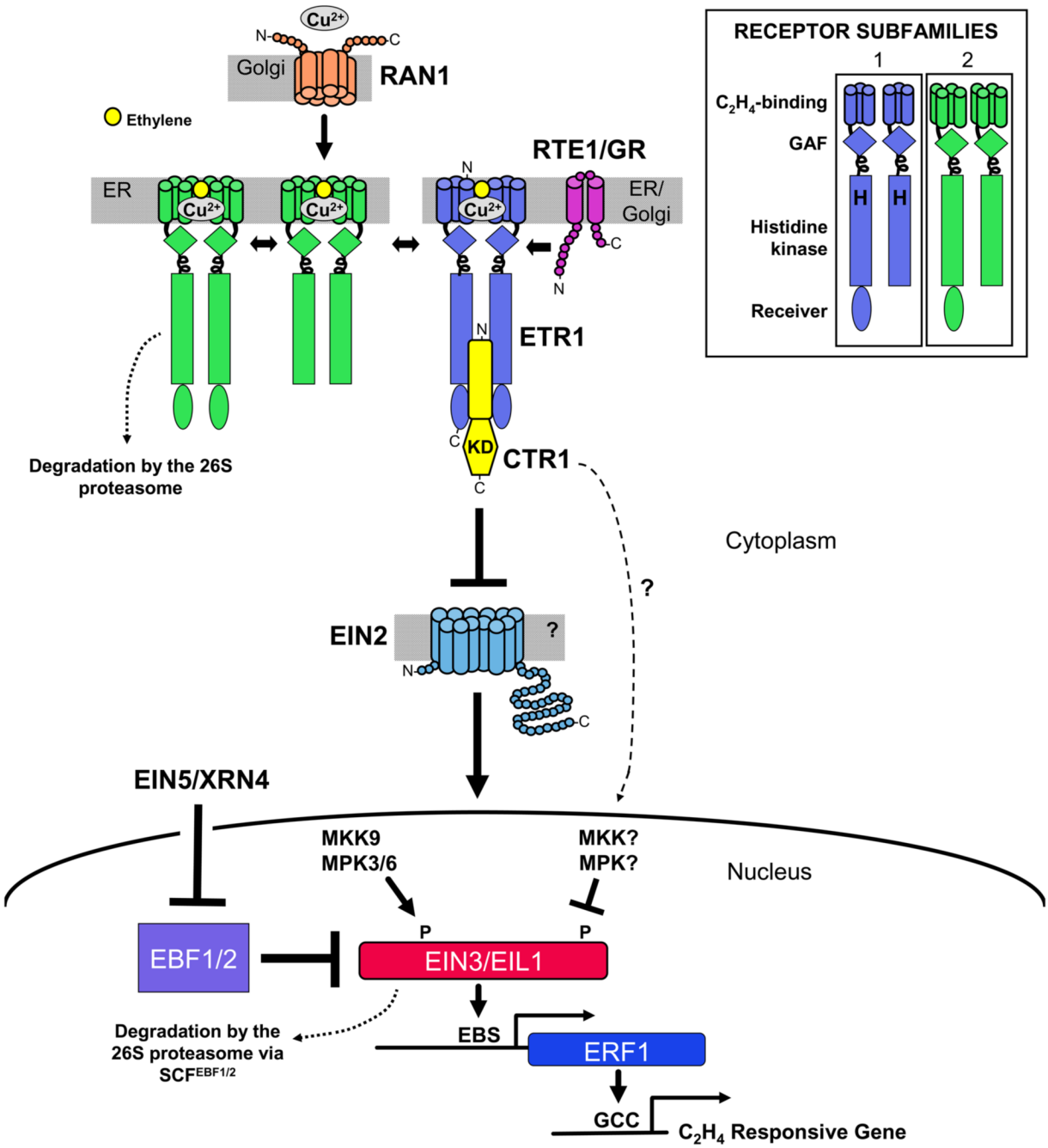

Figure 1. Current model of the ethylene-signaling pathway

Ethylene is perceived at the endomembranes by a family of receptors (see inset) that share similarity to prokaryotic two-component histidine kinase receptors [3]. The receptors form higher order complexes of homodimers and heterodimers [29*; Gao *et al.*, unpublished]. RAN1 is a P-Type ATPase copper transporter homolog [7] in the Golgi membrane [52] that provides the copper cofactor for ethylene binding. RTE1/GR is a novel protein [32**,34**] in the Golgi and ER [30*] that positively regulates ETR1 receptor signaling in *Arabidopsis* [32**,33*]. In the absence of ethylene binding, the receptors repress ethylene responses by signaling through CTR1, a Raf-like MAPKK kinase that negatively regulates responses [10]. When ethylene binds to the receptors, receptor signaling is inactivated, causing the CTR1 kinase domain (KD)

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to be inactivated, allowing downstream signaling to proceed through EIN2, which has similarity to theNramp family of metal ion transporters [11]. *EIN2* is a positive regulator of ethylene responses, and loss of *EIN2* renders the plant completely insensitive to ethylene [11]. *EIN2* regulates a transcriptional cascade initiated by *EIN3* and *EIL1*, two members of a small family of DNA-binding proteins [41]. EIN3 activates ethylene responses by binding to the EIN3-binding site (EBS) in the promoter of *ERF1* [12]. ERF1 encodes a transcriptional activator that binds to the GCC-box in the promoters of several ethylene-responsive genes. A key regulatory step in the pathway is the degradation of EIN3 and EIL1 by the 26S proteasome-dependent pathway, mediated by an $SCF^{EBF1/2}$ E3 ligase complex containing F-Box proteins EBF1 and EBF2 [13–15,42,43**]. Stability of EIN3 is promoted by phosphorylation of T174 through a MAP kinase cascade consisting of MKK9 signaling to MPK3/6, whereas degradation of EIN3 is promoted by phosphorylation on T592, possibly through a separate MAP kinase cascade involving CTR1[44**]. Repression of *EBF1* and *EBF2* transcription is mediated by an exoribonuclease encoded by *EIN5/XRN4* [47**,48**].

Inset: Each receptor has a transmembrane (TM) N-terminal domain, which binds ethylene with a copper cofactor and localizes the receptor to the endomembranes [3]. Subfamily I receptors have three TM domains. Subfamily II receptors have a fourth TM domain, which might serve as a signal sequence. In the cytosol, the receptor contains a GAF domain adjacent to a coiled coil region followed by a histidine kinase (HK)-like domain. In some receptors, the HK domain is fused to a receiver domain, which appears to have a subtle role in signaling [3]. The GAF domain may play a role in transmitting the signal between the receptors [28,29*; Gao *et al.*, unpublished], as well as from the N-terminal domain to the HK and receiver. Although the HK domain is required for proper signaling, protein kinase activity does not play a major role [3]. Subfamily I receptors have autokinase activity on the histidine (H) [3]. Subfamily II receptors have degenerate HK domains and possess serine/threonine protein kinase activity [3]. (Subfamily I receptor ERS1 in *Arabidopsis* is capable of both activities [3].)