# Ethylene signaling: simple ligand, complex regulation

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The hormone ethylene, the simplest alkene ($C_2H_4$), plays numerous roles in the development and environmental responses of the plant. Seed germination, seedling growth, organ development and senescence, leaf and petal abscission, fruit ripening, stress and pathogen responses are among the many processes governed at least in part by ethylene [1,2]. The easy-to-score "triple response" phenotype of dark-grown *Arabidopsis* seedlings exposed to ethylene gas has enabled the identification of ethylene-insensitive and ethylene-constitutive-response mutants [3]. Subsequent cloning and characterization of these mutants has led to the generation of a primarily linear model of ethylene signal transduction that starts with hormone perception and ends in transcriptional regulation [4]. Recent discoveries, however, suggest existence of a much more complex pathway with both positive and negative regulatory feedback loops. This review focuses on the most relevant discoveries in the ethylene field of the past three years, with a special emphasis made on the studies that impacted the mechanistic understanding of how plants fine-tune the activity of the ethylene signaling cascade. The current model of ethylene signaling is shown in Figure 1. Intertwined with the linear pathway, by which the ethylene receptor-triggered signal is transduced via CTR1 and EIN2 to the nuclear-localized EIN3/EILs transcriptional regulators, are several regulatory modules: RTE1, EBFs and ETPs. Upon ethylene binding, the receptors transmit the signal to the CTR1 protein kinase inhibiting its ability to phosphorylate EIN2 and causing the C-terminal end of EIN2 to translocate to the nucleus, where the EIN2 C-end leads to the stabilization of EIN3/EILs and the initiation of transcriptional responses to ethylene.

## The ethylene-signaling pathway

The ethylene-signaling cascade starts with ethylene binding to its receptors. In all plants examined to date, including monocots, dicots and mosses, the ethylene receptors exist as a multimember family that in *Arabidopsis* is composed of ETR1, ERS1, ETR2, ERS2, and EIN4. These receptors work as negative regulators of the pathway, actively repressing the ethylene response in the absence of the hormone [5]. Although the receptors are largely redundant in the control of ethylene responses, some functional specificity among their different isoforms has recently been uncovered (shown in Table 1).

The receptors predominantly reside in the ER membrane, which is not a typical site for receptor-ligand binding. However, given that the ethylene gas can diffuse freely both in aqueous and lipid environments of the cell, this localization of the receptors might facilitate interactions with other cellular components and/or enable signal integration with other pathways [6].

Based on the phylogenetic analysis and shared structural features, the receptors have been divided into two subfamilies [reviewed in 3,7,8], but all of the ethylene receptors share a modular structure composed of an N-terminal

transmembrane domain responsible for ethylene binding, a GAF domain involved in protein-protein interactions between different receptor types, and a C-terminal domain required for the interaction with the downstream components of the pathway. Although the receptor C-termini show structural similarity to bacterial two-component histidine kinases, the autokinase activity of the receptors seems to play only a minor role in the ethylene response [reviewed in 6]. While a recent Arabidopsis study by Hall *et al*. suggested that the binding of ethylene to the receptors stimulates their autophosphorylation [9*], Kamiyoshihara et al. reported reduced receptor phosphorylation upon ethylene treatment in tomato [10*]. The latter study is in agreement with an older biochemical analysis of the Arabidopsis ETR1 that demonstrated that the binding of ethylene inhibits the receptor phosphorylation activity *in vitro* [11]. Thus, the relationship between ethylene binding and kinase activity remains currently unresolved. Interestingly, the kinase activity of ETR1 affects the interaction between ETR1 and EIN2 [12*], supporting a fine-tuning role of the receptor kinase domain.

The basic functional unit of the ethylene receptor is a homodimer capable of binding ethylene, although heterodimers can also form, at least in a yeast heterologous system [13]. Higher order associations can occur among homodimers interacting through the GAF domains, giving rise to clusters of receptors in the membrane. This allows for a differential composition of the ethylene receptor complexes in different plant tissues, with potential functional implications on the efficiency of hormone perception. This could explain the broad range of ethylene sensitivity (0.2nL/L to 1000µL/L) found in plants, signal attenuation and output, as well as the dominant nature of ethylene-insensitive receptor mutants [13, 14,15*].

Copper, supplied by the intracellular copper transporter RAN1, is required both for ethylene binding and for the receptor functionality [16]. Plants carrying loss-of-function (LOF) mutations in *ran1* lack ethylene-binding activity and display phenotypes similar to that of the LOF receptor mutants [17, 18]. Furthermore, weak alleles of *ran1* treated with copper chelators show phenotypes similar to that of ethylene-treated wild-type plants [17] and the addition of copper ions to these plants partially suppresses the *ran1* phenotype [16]. These results suggest that RAN1 plays an essential role in the biogenesis of ethylene receptors.

RTE1 is a negative regulator of ethylene responses [19] that co-localizes with the receptors at the ER and is also detected in the membrane of the Golgi apparatus [20]. RTE1 functions by specifically activating ETR1 by promoting its transition from the inactive (in the presence of ethylene) to the active (in the absence of ethylene) signaling state [21]. In tomato, the two different RTE1-like genes influence distinct but overlapping ethylene responses, suggesting the possibility of sub-functionalization [22*].

Although the exact output of the receptors is still obscure, genetic studies demonstrate that in the absence of ethylene the receptors activate CTR1, a negative regulator of the pathway [23]. CTR1 is a Ser/Thr protein kinase that homodimerizes when activated [24]. Unlike the ambiguous mode of action of the kinase domain in the receptors, the kinase activity of CTR1 is absolutely necessary for the downstream signaling to occur. While CTR1 lacks any predicted transmembrane domains, it also resides at the ER membrane due to its physical interaction with the receptors [25, 26]. This

physical association with the receptors is critical for the induction of the kinase activity of CTR1. The activated CTR1 kinase dimers engage in interactions that might enable crosstalk between ethylene receptor clusters [24].

Downstream of CTR1 is EIN2, a key player in the ethylene signaling cascade. The *ein2* mutant is defective in all examined ethylene responses [27]. Despite its importance, for over a decade *EIN2* remained the most elusive player in the ethylene-signaling pathway. The EIN2 protein consists of an N-terminal hydrophobic region made of 12 predicted transmembrane domains and a hydrophilic C-terminus [27] that harbors a conserved nuclear localization sequence [28**], but no other recognizable functionally defined structures. The hydrophobic domain has similarity to the NRAMP family of metal ion transporters, although no transport activity has been shown for EIN2 [29]. EIN2 resides in the ER membrane and physically interacts with the kinase domain of the ethylene receptors [12]. EIN2 accumulates upon ethylene treatment and is absolutely required for the stabilization of the downstream pathway component, EIN3 [30**]. Interestingly, overexpression of the C-terminal end of EIN2 constitutively activates ethylene responses in light-grown plants, although it is not sufficient to trigger full-scale ethylene response nor to restore ethylene sensitivity to null *ein2* mutants [27].

Even though EIN2 functions as a critical component in ethylene signaling, it took more than 13 years to determine how this intriguing molecule transduces the ethylene signal from the receptors in the ER to the transcription factors EIN3/EIL1 in the nucleus that regulate downstream gene expression. It was the work done by three different groups [28**,31**,32**] and published in the last year that finally shed some light on this part of the pathway. In these three independent studies, the authors were able to show that there is a physical movement of the C-terminal end of EIN2 from the membrane of the ER to the nucleus, allowing the ethylene signal to reach the downstream components EIN3 and EILs. Importantly, the regulatory mechanism linking the ethylene signal with the movement of the C-terminus of EIN2 to the nucleus was also uncovered. Chen *et al.* (2011) showed that in the presence of ethylene EIN2 lacks phosphorylation at multiple Ser and Thr residues [33*]. Shortly after, Ju *et al*. (2012) demonstrated that there is a physical interaction between EIN2 and CTR1, and that CTR1 is the protein kinase that in the absence of ethylene directly phosphorylates the C-terminal end of EIN2, thus preventing it from signaling to the downstream components EIN3 and EILs [31**]. It is not yet clear, however, whether or not the dephosphorylation directly promotes EIN2 cleavage or enhances the stability of this part of the protein [34]. Due to the structural similarities of CTR1 with MAPKKKs, the controversial involvement of a MAP kinase cascade in ethylene signaling has long been hypothesized [reviewed in 34 and 29]. The results presented by Ju *et al*. [31**] imply that there is no need for a MAPKK or MAPK activity for the signal transduction between CTR1 and EIN2. Once in the nucleus, the C-terminal end of EIN2 leads to the stabilization of EIN3 and the activation of the EIN3/EILs-dependent transcriptional cascade [28**,31**,32**].

EIN3 and its homologs (EILs, EIL1 in *Arabidopsis*) are short-lived proteins that act as positive regulators of the ethylene-signaling pathway. EIN3 and EIL1 are the two master transcription factors that generate the primary output of ethylene responses and are both necessary and sufficient

for the regulation of the ethylene-responsive genes' expression [35]. EIN3/EILs function as dimers and, at least in the case of the tomato EIL1, a mutation at a conserved phosphorylation site disrupts fluorescence signal in a tobacco BiFC system, as well as abolishes the activity of the respective transgene in tomato plants [36]. Upon transcriptional activation by EIN3/EILs, the ethylene target genes mediate a wide array of the plant responses to ethylene [4]. Using ChIP-seq, Chan *et al*. [37] have found that EIN3 regulates the downstream genes' transcription in a four-wave manner, with each of the waves encompassing a unique subset of the EIN3 targets that cumulatively modulate a multitude of downstream transcriptional cascades. Importantly, some of the downstream targets of EIN3 correspond to key components of other hormone-signaling pathways, reinforcing the idea of the existence of a complex net of interactions among the different plant hormones.

**Turnover of the signaling components and feedback regulation**

With the recent discoveries, the largely linear signaling pathway described above is gradually transforming into a more complex route that includes feedback-regulated transcriptional networks, as well as protein and mRNA turnover regulatory modules [4]. Proteasome-mediated protein degradation plays a major role in the regulation of the ethylene-signaling cascade. At the receptor level, ethylene induces ETR2 degradation through the 26S proteasome [38]. At the same time, this hormone transcriptionally activates *ETR2*, *ERS1,* and *ERS2* [39]. The newly synthesized receptors, and therefore not yet occupied by ethylene, would allow for the inhibition of the downstream pathway as soon as the levels of the hormone decrease. This up-regulation in the levels of mRNA and reduction in that of the proteins in response to ethylene have also been described for the tomato ethylene receptors NR, LeETR4 and LeETR6 [40]. Thus, both transcriptional regulation and proteasome-mediated degradation of the receptors may form part of a sophisticated desensitizing mechanism to this stress-related hormone.

The protein levels of EIN2 and EIN3/EIL1 are also tightly regulated; in this case, by specific F-box proteins that trigger their proteasome-mediated degradation in the absence of ethylene [41,42,43,44]. ETP1 and ETP2 control EIN2 levels [41], whereas EBF1 and especially EBF2 regulate the levels of EIN3 in response to the ethylene signal [42,43,44]. To add further complexity to this regulatory module, the EBF1/2 and ETP1/2 protein levels are down-regulated by ethylene and, at least in the case of EBF1/2, this process is mediated by the proteasome [41, 30**]. Although the mechanistic details of this are still to be determined, functional EIN2 is clearly required for the degradation of EBF1 and EBF2 [30**]. The different roles of each EBF in the ethylene response [45,46] can be explained by the fact that *EBF2* is itself a target of EIN3, being transcriptionally induced by ethylene [45], thus creating an intricate regulatory feedback mechanism. As the final output of these regulatory loops, the protein levels of EIN3/EIL1 in the nucleus are finely tuned to orchestrate the activation of the proper set of ethylene responses. In other words, the balance between the ethylene-dependent increase in *EBF2* transcription and decrease in EBF1 and EBF2 protein stability is thought to modulate the EIN3/EIL1 turnover, providing a dynamic mechanism of adjusting the plant responsiveness to this hormone.

An additional layer of regulation is provided by the 5'-3' exoribonuclease XRN4/EIN5 that is believed to down-regulate the levels of *EBF1* and *EBF2* mRNA by an unknown mechanism. Due to the molecular nature of EIN5, an RNA degradation module in the control of the ethylene response has been suggested [4]. In contrast with other regulatory loops described above, neither *EIN2* nor *ETP*s are transcriptionally regulated by ethylene [41].

**Future perspectives**

The greater understanding of the ethylene-signaling pathway reached as a result of the work of multiple research groups has also brought to light new and intriguing questions that are yet to be answered. Thus, the finding that the C-terminus of EIN2 translocates to the nucleus in response to ethylene has opened up the search for the molecular elements and mechanisms that a) trigger and execute the C-terminal cleavage allowing for the EIN2 C-end translocation to the nucleus, and b) activate the transcriptional signaling cascade. Similarly, lack of full activation of ethylene responses upon expression of the nuclear-localized C-terminus of EIN2 suggests that other parts of this intriguing protein may carry out additional (and so far uncharacterized) functions. In that sense, it is important to point out the current lack of functional information on the highly conserved N-terminus of EIN2 that shares clear sequence similarity with the NRAMP family of metal-ion transporters.

Other important challenges lying ahead relate to the findings that implicate additional regulatory modules in the ethylene pathway or reveal alternative signaling routes that deviate from the core linear cascade described above. Thus, for example, the mechanisms by which the 5'-3' exoribonuclease EIN5 participates in the ethylene response is not yet fully understood [44,45], although the accumulation of the full-length and 3'UTR region of the *EBF1* and *EBF2* mRNA has been suggested as the likely culprit. Perhaps, related to this are the findings that the long 3'UTR of *EBF2* has a negative regulatory effect on the activity of *EBF2,* as indicated by the hyper-activation of *EBF2*-mediated responses when the native 3'UTR is eliminated [45]. Since the nature of the 3'UTR in part determines the stability and/or translatability of the mRNA, the aforementioned results suggest that an EIN5-dependent regulatory module may control the stability of the *EBF1* and *EBF2* mRNAs. No direct evidence, however, has been found for such a mechanism [47,48], leaving the mode of EIN5 action and the role of the *EBF1/EBF2* 3'UTRs unknown at the moment and open for future studies.

Finally, several different lines of research have suggested the existence of alternative signaling pathways in which one or several of the classical core components are bypassed in triggering a specific set of ethylene responses. In this regard, the recent work by Qiu *et al*. [49] explored the possibility of RTE1 and the N-terminal domain of ETR1 working together to mediate ethylene signaling through a CTR1-independent pathway. Conversely, the detailed morphometric analysis of the growth inhibition mediated by ethylene had also suggested the existence of an alternative fast-response signaling pathway that does not require the activity of the key transcriptional regulators EIN3 and EIL1 [50]. Thus, although it is clear that the majority of well-characterized responses to this hormone are mediated by

the canonical ethylene signaling pathway described above, the possible existence of alternative cascades that skip one or several of the classical ethylene signaling components needs to be further investigated.

**Table 1. Examples of distinct functions played by the ethylene receptors**

| Biological process | Species | Receptor isoforms involved |
|---|---|---|
| Ethylene-induced nutational bending of the apical hook | Arabidopsis | Activated by ETR1 [51] Inhibited by ETR2, ERS1, ERS2 and EIN4 [51] |
| Inhibition of ethylene signaling by silver ions | Arabidopsis | Mainly ETR1 [52] |
| Functional dependence on RTE1 | Arabidopsis | ETR1 [53] |
| Recovery of growth after ethylene treatment | Arabidopsis | ETR1, ETR2 and EIN4 [54] |
| Development of light-grown seedlings | Arabidopsis | ETR1 and ERS1 [55] |
| Ethylene response in an ETR1-dependent manner | Arabidopsis | ERS1 [56] |
| Trichome branching | Arabidopsis | Induced by ETR2 [57] |
| Response to fumonisin treatment | Arabidopsis | ETR1 inhibits the response and EIN4 activates it [58] |
| Starch accumulation | Rice | ETR2 [59] |
| Control of flowering time | Rice | ETR2 [59] |
| Control of fruit ripening | Tomato | LeETR4 and LeETR6 [60] |
| Responses to salt stress | Tobacco | NTHK1 [61] |

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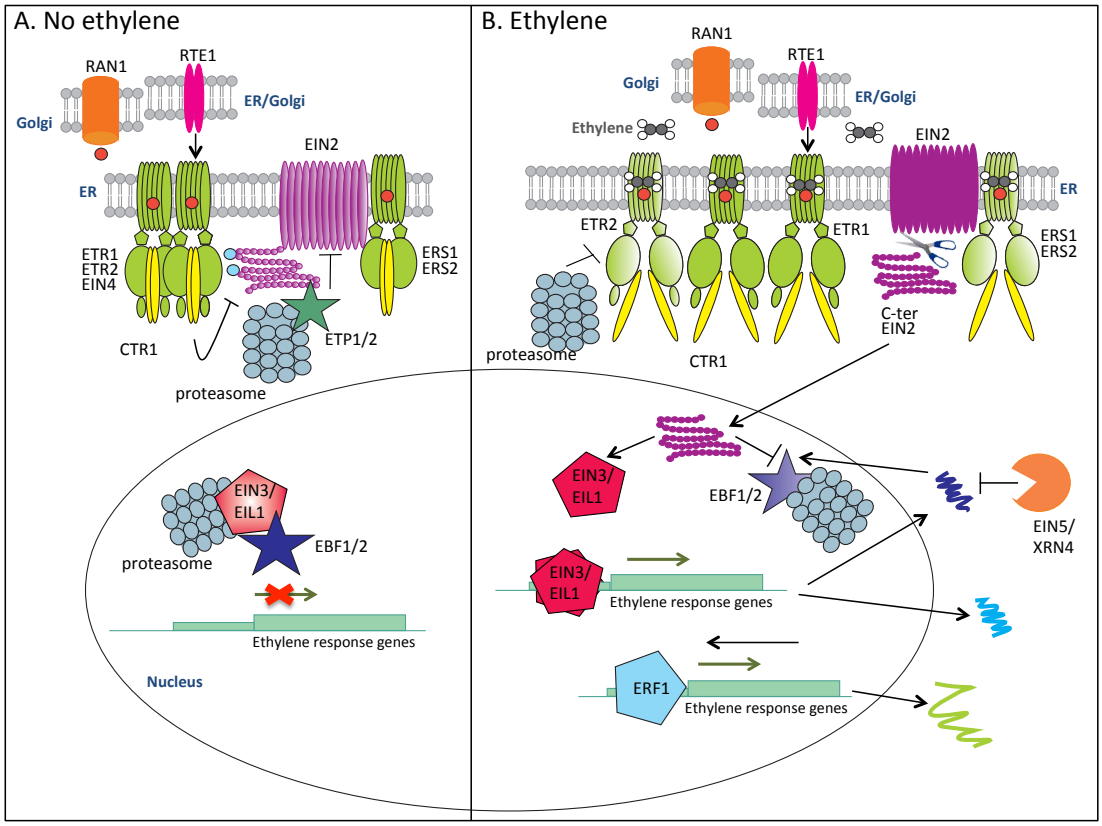


**Figure1**. **Current model of the ethylene signaling pathway in *Arabidopsis***. Ethylene is perceived by the receptor proteins ETR1, ERS1, ETR2, ERS2 and EIN4 (represented in green), all of which bind ethylene with high affinity. In the figure the receptors are grouped into two classes based on the presence (ETR1, ETR2 and EIN4) or absence (ERS1 and ERS2) of the receiver domain. The receptors work as homodimers but form higher order complexes in the ER membrane by interacting with other receptors through their GAF domains (represented as pentagons in the receptors´ cytosolic domain). Copper (red circles) serves as a cofactor for ethylene binding and is delivered to the receptors by the copper transporter RAN1 (represented in orange). RTE1 (in pink) is associated with ETR1 and mediates the receptor signal output. The receptors are negative regulators of ethylene signaling. **A.** In the absence of the hormone, the receptors activate CTR1 (in yellow), a Ser/Thr kinase that dimerizes when active and suppresses the ethylene response. CTR1 inactivates EIN2 (in purple) by directly phosphorylating (blue circles) its C-terminal end. EIN2 can directly interact with the kinase domain of the receptors (represented as the larger ovals under the pentagons in the cytosolic domain of the receptors). The levels of EIN2 are negatively regulated by the F-box proteins ETP1 and ETP2 (green star) via the 26S proteasome (grey). In the nucleus, the transcription factors EIN3/EIL1 (in red) are being degraded by two other F-box proteins, EBF1/2 (blue star), through the proteasome. In the absence of EIN3/EIL1, transcription of the ethylene response genes is shut off. **B**. In the presence of ethylene, the receptors bind the hormone and become inactivated, which in turn, switches off CTR1. This inactivation prevents the phosphorylation of the positive regulator EIN2. The C-terminal end of EIN2 is cleaved off by an unknown mechanism and moves to the nucleus where it stabilizes EIN3/EIL1 and induces degradation of EBF1/2. The transcription factors EIN3/EIL1

dimerize and activate the expression of ethylene target genes, including the F-box gene *EBF2* (dark blue curly line) [which generates a negative feedback loop dampening the activity of the ethylene pathway] or the transcription factor gene *ERF1* (light blue line) [which, in turn, initiates a transcriptional cascade resulting in the activation and repression of hundreds of ethylene-regulated genes]. Among the ethylene responsive genes is the receptor gene *ETR2* (green line), whose mRNA is upregulated by ethylene and is translated into the new batch of ethylene-free receptor molecules which then activate the negative regulator CTR1, thus providing the means of tuning down ethylene signaling in the absence of additional ethylene. Other regulatory nodes in the pathway are the exoribonuclease EIN5 (light orange), which controls the levels of *EBF2* mRNA, and the F-box proteins ETP1 and ETP2 (green star) that are degraded in the presence of ethylene leading to the stabilization of EIN2. All of the aforementioned ethylene signaling components identified in *Arabidopsis* are conserved in evolutionary distant plant species, suggesting that the mechanism of ethylene signaling in plants is universal. Positive and negative arrows (-> and -|) represent activation and downregulation processes, respectively. Molecules shown in fading colors (EIN3/EIL1 in “no ethylene”, or ETP1/2 and EBF1/2 in “ethylene”) correspond to unstable proteins targeted to proteasome-mediated degradation. Curly lines indicate specific mRNAs, with their colors matching that of the corresponding proteins.