Review

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Emerging connections in the ethylene signaling network

Sang-Dong Yoo[1,2], Younghee Cho[1] and Jen Sheen[1]

[1] Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, MA 02114, USA
[2] Department of Biological Science, Sungkyunkwan University, Suwon, Korea

The gaseous plant hormone ethylene acts as a pivotal mediator to respond to and coordinate internal and external cues in modulating plant growth dynamics and developmental programs. Genetic analysis of *Arabidopsis thaliana* has been used to identify key components and to build a linear ethylene-signaling pathway from the receptors through to the nuclear transcription factors. Studies applying integrative approaches have revealed new regulators, molecular connections and mechanisms in ethylene signaling and unexpected links to other plant hormones. Here, we review and discuss recent discoveries about the functional mode of ethylene receptor complexes, dual mitogen-activated protein kinase cascade signaling, stability control of the master nuclear transcription activator ETHYLENE INSENSITIVE 3 (EIN3), and the contextual relationships between ethylene and other plant hormones, such as auxin and gibberellins, in organ-specific growth regulation.

Ethylene: chemically simple but functionally complex

A simple hydrocarbon gas, ethylene (C_2H_4) is produced in most plant tissues and cell types [1–4] and modulates growth dynamics and developmental events, including seed germination, seedling growth, leaf, root, stem and flower development, fruit ripening, and organ senescence and abscission [1–3]. Ethylene synthesis is regulated by developmental cues and other hormones, such as auxin, gibberellin (GA), cytokinin and brassinoride, and is greatly enhanced by diverse stresses, such as wounding, salt, drought, cold, ozone, flooding, and pathogen and insect attack. Thus, ethylene has a pivotal role in the coordination of internal growth, defense and survival in response to environmental challenges (Figure 1). However, it is still not fully understood how this chemically simple hormone is involved in such diverse functions. Emerging evidence has suggested that ethylene sensitivity differs in various tissues and/or distinct developmental stages as a result of signaling interactions with other plant hormones, metabolites and environmental signals [2–7].

The key signaling components initially identified by molecular genetics and bioinformatics in *Arabidopsis* (*A. thaliana*) are multiple membrane receptors, an intracellular signaling protein kinase (PK), a membrane transporter-like regulator, nuclear transcription factor families and F-box proteins [2,7–12]. These key regulators have also been identified in many plant species, implicating a high level of conservation in ethylene signaling mechanisms that have evolved to function in diverse lifestyles and developmental programs [3,8–10]. Currently, a main challenge in understanding ethylene signaling and functions is to integrate genetically identified regulatory components into mechanistic actions at the biochemical, molecular and cellular levels. There is also the need to distinguish direct and specific signaling components from indirect and general regulators or shared signaling mediators to clarify ethylene functions in the signaling network that modulates pleiotropic phenotypes.

Here, we focus on recent advances in understanding ethylene signaling, mainly the molecular action and modulation of the membrane-associated ethylene receptor complexes, the inducible nuclear events and the mitogen-activated protein kinase (MAPK) cascades that connect and specify the hormonal signaling inputs and outputs. We also highlight recent studies uncovering the molecular links between ethylene actions and other plant hormones, GAs and auxin. Such signaling integration would provide novel mechanisms that could underlie diverse ethylene-driven responses in coordinating endogenous developmental programs with environmental cues to modulate organ-specific growth and plant adaptation and survival.

The ethylene signal transduction pathway

Several key components of ethylene signaling, from membrane receptors to nuclear activators, have been identified in *Arabidopsis* (Figure 2). Multistep regulation of the ethylene signaling components via feedback mechanisms adds to the dynamics and complexity of hormone signaling [1–3,7,9,11,12]. Five membrane receptors in *Arabidopsis* (ETHYLENE RESPONSE1 [ETR1], ETR2, ETHYLENE SENSOR1 [ERS1], ERS2 and ETHYLENE INSENSITIVE4 [EIN4]) are related to bacterial two-component histidine kinase (HK) sensors [13–23] and bind to ethylene through their N-terminal domain, which is localized within the endoplasmic reticulum (ER) [20,21]. Analyses of ethylene receptor null mutations have led to the proposal of an inverse-agonist model for ethylene receptor signaling [18–23]. For instance, in the absence of ethylene, the receptor is constitutively signaling, whereas when ethylene is bound, the receptor is switched off. These receptors act as negative regulators through another genetically identified negative regulator, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), which encodes a putative Raf-like MAPK kinase kinase (MKKK) [24]. CTR1 is mainly

Corresponding author: Sheen, J. (sheen@molbio.mgh.harvard.edu).

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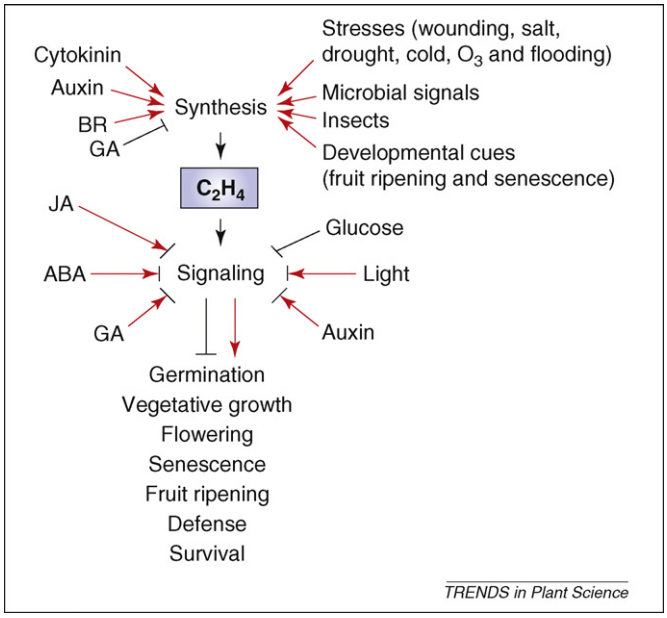

Figure 1. The ethylene regulatory network. Ethylene biosynthesis and signaling are regulated by multiple plant hormones, as well as by developmental and environmental signals, to modulate important plant processes from germination through to senescence. Both activation and inhibition can be observed in the relationships between ethylene and other signals in different tissues and cell types under diverse physiological, developmental and environmental conditions [2–8,69,70,73–90]. Abbreviations: ABA, abscisic acid; BR, brassinosteroid; JA, jasmonic acid.

associated with the receptor protein complexes through ETR1 and ERS1 [24–26]. A membrane metal transporter-like EIN2 has a pivotal role in ethylene signaling downstream of CTR1 [27]. It seems to regulate the availability of the key transcription factor EIN3 through an as yet unknown mechanism [28,29]. Based on yeast two-hybrid screens and mutant phenotype analyses, EBF1 and EBF2, two EIN3-binding F-box proteins with overlapping functions, have been identified to target EIN3 constantly for degradation through the 26S proteasome [29–32]. In response to ethylene, EIN3 is stabilized and accumulated in the nucleus to activate hormone-inducible primary transcription [29–32]. EIN3 and EIN3-LIKE1 (EIL1) transcription factors activate *ETHYLENE RESPONSE FACTOR1* (*ERF1*), *EBF2* and other primary responsive genes containing EIN3-binding sites (EBSs) in their promoter regions [7,11,29,31,33,34]. ERF1 and related transcription factors conferring GCC element binding activities induce the expression of the secondary response genes in ethylene-dependent transcription cascades. These gene products are eventually involved in modulating plant survival, defense and growth [2–12]. Further studies have shown that signaling components of the linear pathway are highly conserved in tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tobacum*), rice (*Oryza sativa*) and maize (*Zea mays*) [3,8,10].

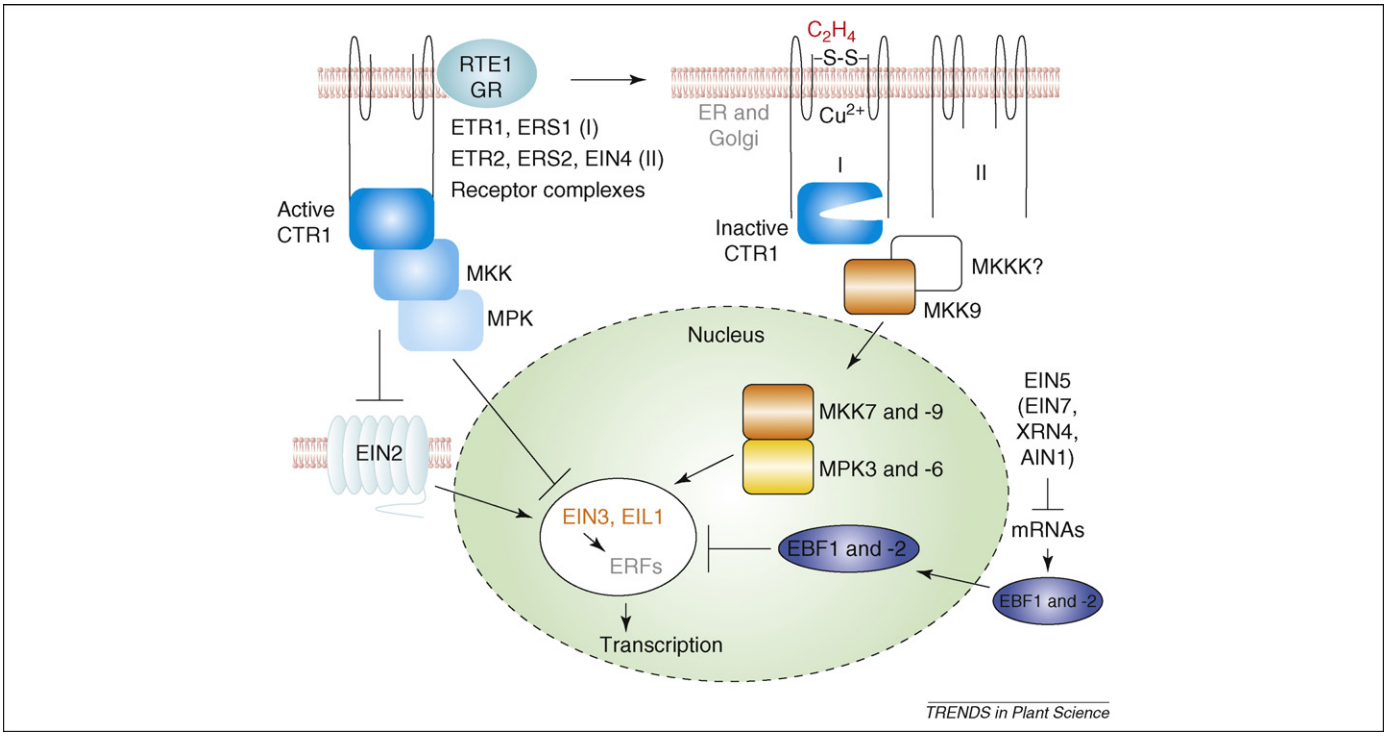

Figure 2. Current model for the ethylene signal transduction pathway. The ethylene receptor complexes comprising subfamily I and II members act synergistically but differentially to activate the CTR1 PK activity in the ER without ethylene binding [12,21,46]. *Arabidopsis* RTE1 and tomato GR are orthologous negative regulators of ethylene signaling and are localized in the ER and Golgi [38,52–55]. CTR1 is probably an MKKK that activates multiple MKKs and MPKs as cascades, and outcomes of the activated cascades then phosphorylate the Thr^{592} and Thr^{546} residues of the transcription factors EIN3 and EIL1, respectively, in the nucleus [24,28,67]. The CTR1-dependent phosphorylation of EIN3 and EIL1 might enhance their interaction with the F-box proteins EBF1 and EBF2 to promote protein degradation through the 26S proteasome and suppress ethylene signaling [24,28–32,67]. Upon ethylene binding to the receptors, CTR1 is inactivated, resulting in the simultaneous suppression of the CTR1–MAPK pathway and the activation of the MKK9–MPK3 and -6 cascade, which phosphorylates the Thr^{174} and Thr^{176} of EIN3 and EIL1, respectively, in the nucleus [67]. MKK9–MPK3 and -6-dependent phosphorylation of EIN3 and EIL1 probably increases their stability by reducing their interaction with EBF1 and -2. It is unclear whether MKK9–MPK3 and -6 is activated by an MKKK that is different from CTR1. The expression of *MKK7* is activated by ethylene and might act in long-term responses [67]. EIN2 is a membrane protein that is crucial for the accumulation of EIN3 [27,29]. EIN5 (EIN7, XRN4, AIN1) indirectly affects the transcript levels of *EBF1* and *-2* [39–41].

Many key components in the ethylene signal transduction pathway are also controlled at the transcript levels by ethylene, thus providing layers of negative- or positive-feedback loops for the fine tuning of response dynamics and patterns. Notably, *ERS1*, *ERS2* and *ETR2* transcripts increase as primary responses to ethylene [15,17,35]. The newly synthesized receptors that have not yet perceived ethylene might suppress downstream ethylene signaling and diminish or reset the hormone response as a negative feedback mechanism. By contrast, ethylene binding can initiate the proteasome-dependent degradation of *Arabidopsis* ETR2 within the ER at higher ethylene levels, and might increase plant sensitivity to ethylene [36]. Likewise, tomato NEVER-RIPE (NR), LeETR4 and LeETR6 receptor proteins decrease significantly at the onset of fruit ripening. This could serve as a major determinant of fruit-ripening stage-dependent hormone sensitivity by lowering negatively regulating components in ethylene signaling [37]. As a recently discovered negative regulator of ethylene signaling, the expression of *Arabidopsis REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*) is also enhanced by ethylene [38].

The expression of *EBF1* and *EBF2* encoding the EIN3-targeting F-box proteins of the E3 ubiquitin ligase is induced by ethylene and could provide another negative-feedback regulation by enhancing the degradation of EIN3 in the nucleus to reset the pathway [29,31,34]. Extensive molecular and transgenic analyses have identified the crucial *cis*-element for the direct control of the *EBF2* promoter by EIN3. Unexpectedly, the sequence downstream of the *EBF2* coding region also contributes to the modulation of *EBF2* expression and sensitivity to ethylene [29,31,34].

The genetically defined positive regulator EIN5 (EIN7, EXORIBONUCLEASE4 [XRN4], ACC INSENSITIVE1 [AIN1]) is a 5′-3′ exoribonuclease that might indirectly modulate *EBF1,2* mRNA metabolism (Figure 2). Recent investigations indicate that EIN5 (EIN7, XRN4, AIN1) has a novel role in RNA metabolism and small RNA biogenesis and might also influence other signaling pathways indirectly [39–41].

Novel modulators of ethylene signaling

To identify peripheral factors that might modulate ethylene signaling, a new genetic screen for *enhanced ethylene response* (*eer*) mutants has been carried out in *Arabidopsis*. These *eer* mutants typically enhance *ctr1* phenotypes and are assumed to have negative roles in ethylene signaling [42–45]. Epistasis analysis shows that EER3 acts downstream of EIN2, whereas EER4 and EER5 are likely to act upstream. Interestingly, there is a lack of molecular correlation between the enhanced ethylene inhibition of growth and the partial loss of ethylene-responsive gene regulation in *eer3*, *eer4* and *eer5* mutants [43–45]. EER proteins seem to act simultaneously as negative regulators in ethylene-related growth repression and as positive regulators in ethylene-responsive gene activation.

The identification of four *EER* genes suggests that there are several ways to modulate the ethylene-mediated growth responses. For example, *eer1* contains a point mutation in *ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1* (*RCN1*), one of three protein phosphatase 2A (PP2A) regulatory subunits. *RCN1* has been shown to associate with the kinase domain of CTR1 and is required for the catalytic activity of PP2A [42]. It remains to be seen whether and how EER1 modulates CTR1 kinase activity, given that EER1 and CTR1 are both negative regulators in ethylene signaling. *EER3* encodes a prohibitin, AtPHB3, and its mode of action remains to be determined [43]. EER4 is a TATA-box-binding TFIID-associated transcription factor (*Arabidopsis* TAF12b) that is homologous to human TAF12 and yeast TAF61. Interestingly, it interacts with EIN3, ERF1 and two PP2A catalytic subunits, and is required for ethylene and jasmonate induction of *ERF1* expression [44]. It seems that EER4 might act not only as a positive regulator to bridge EIN3 and ERF1 to the TFIID complex to activate ethylene-responsive transcription but also to control genes that antagonize ethylene growth responses. It is essential to identify more EER4-interacting nuclear proteins and specific target genes using a combination of whole-genome microarray and chromatin immunoprecipitation to reveal the complex physiological functions and molecular actions of EER4.

Despite enhanced ethylene inhibition in seedling growth, the *eer5* mutant also fails to induce a subset of ethylene-responsive genes, which could be crucial to reset the ethylene signal transduction pathway. EER5 encodes a protein with a proteasome CONSTITUTIVE PHOTOMORPHOGENESIS9 (COP9) initiation factor-associated module (PAM) domain often found in components of the COP9 signalosome (CSN). Intriguingly, in yeast two-hybrid analyses, EER5 interacts directly with the C terminus of EIN2 and CSN8–COP9, and EIN2 interacts with CSN3, CSN6A and CSN6B [45]. Future research will determine whether EER1, EER3, EER4, EER5, EIN2 and CSN are all part of large signaling complexes that act between the ER and the nucleus to control transcription factors and to modulate ethylene signaling and its connections to other regulatory pathways.

Functional mode of ethylene receptor complexes

The first plant hormone receptor was identified from a screen for ethylene response mutants and provided the evidence that plant small molecules act through specific ligand–receptor functional modes [3,9,12–14,20,21,46]. Although all receptors have similar ethylene-binding abilities, different receptors might direct different signaling outputs in *Arabidopsis* [20,35,46]. Among the five *Arabidopsis* ethylene receptors, ETR1 and ERS1 in subfamily I contain highly conserved signature motifs for HK and have a predominant role in ethylene perception in a redundant manner. For example, the loss-of-function double *etr1 ers1* mutant exhibits severe growth inhibition and strong constitutive ethylene signaling and cannot be fully complemented by subfamily II receptors [19,22,23,46]. All of the defects exhibited by *etr1 ers1* depend on *EIN2*, because *ein2* is fully epistatic to the *etr1 ers1* double mutant [47]. It was previously suggested that the canonical HK activity of the receptors is not crucial in ethylene signaling because the HK-dead etr1 mutant protein can restore the ethylene response in the loss-of-function *etr1* or *etr1 ers1* mutants [19,22,23,46–48]. However, the mutants tested have

residual ERS1 activity. In addition, when the truncated etr1(1-349) without the HK and receiver domains is expressed in the null *etr1-9 ers1-3*, the transgene function cannot fully restore ethylene responses [22,23]. Thus, it remains to be determined whether the HK-dead ETR1 or ERS1 can complement the *etr1 ers1* null mutant and the degree to which HK contributes to ethylene signaling.

The receptor subfamily II in *Arabidopsis* comprises ETR2, ERS2 and EIN4. Because the *etr2 ers2 ein4* triple mutant also displays a constitutive ethylene responsive phenotype in the presence of full subfamily I receptor activities, their functions in ethylene signaling are indispensable [20,22,49,50]. Moreover, the null *etr1-9 ers1-3* mutant can still respond to ethylene through subfamily II receptors without HK activity [23]. Interestingly, by using tandem affinity purification (TAP)-tagged versions of ERS1, ETR2, ERS2 and EIN4 expressed in loss-of-function mutants, it was shown that ETR1 preferentially associates with the subfamily II receptors and could form multimeric receptor clusters through the GAF domain, a domain found in cGMP-specific and stimulated phosphodiesterase, adenylate cyclases and the *Escherichia coli* protein FhlA [35]. Extensive protein–protein interaction analysis also supports all possible ethylene receptor combinations [51]. The available information indicates that the two subfamilies are not functionally equivalent but act synergistically for the full activity of the ethylene receptor complexes in *Arabidopsis* [22,23,35,46]. Further investigation of the physiological significance of protein–protein interactions and multimeric receptor complexes will be important to elucidate the molecular mechanisms underlying ethylene signaling.

Based on a suppressor screen for a specific dominant ethylene-insensitive receptor mutation, *etr1-2*, the *rte1* mutant has been isolated and characterized [12,38]. *RTE1* encodes a novel protein with a tomato ortholog, *GREEN-RIPE* (*GR*), and is evolutionarily conserved in plants and animals. Overexpression of *RTE1* reduces ethylene signaling, and ectopic *GR* expression inhibits fruit ripening in tomato [12,38,52,53]. RTE1 is localized in the Golgi and ER and acts specifically with ETR1 in suppressing ethylene signaling (Figure 2) [12,38,53,54]. Genetic analysis of the ability of *rte1* to suppress a variety of *etr1* ethylene-binding domain mutations indicates that RTE1 is involved in promoting or stabilizing the 'on' conformational state for ETR1 signaling [55].

Although both subfamilies of ethylene receptors exist in other plants, their functions and regulations might not be identical to those found in *Arabidopsis*, given that ethylene signaling mechanisms are diverged in different plants and can use more or fewer conserved components. For instance, the tomato subfamily II receptor LeETR4 displays functional specificity but can also compensate for reduction of a subfamily I receptor [8]. Interestingly, four *Arabidopsis* ethylene receptors (excluding ETR1) and a tobacco homolog, NtHK1, show strong preference for Ser–Thr autophosphorylation, even in the HK-dead mutant forms [8,56–58]. It will be important to explore the roles of the Ser–Thr PK activities of these receptors in ethylene signaling in addition to the two-component phospho-relay initiated by HK activity [35,46,56]. Furthermore, the HK activity of ETR1 might have ethylene-independent functions in plant growth regulation [48], and the C65 in ETR1 seems to be crucial for H_2O_2 responses in *Arabidopsis* guard cells [59].

A single but extended study coupling extensive mutagenesis and ethylene-binding analysis in the predicted ethylene-binding domain of ETR1 has identified residues in the transmembrane Helices I (D25, Y32, I35 and P36) and II (I62, C65 and H69) as the core ethylene-copper binding pocket [21]. Consistent with the inverse-agonist model, mutations eliminating ethylene binding confer constitutive receptor signaling and dominant ethylene insensitivity in plants [21]. Surprisingly, a large number of mutations clustered near the cytoplasmic side of membrane-spanning Helices I and III show wild-type levels of ethylene-binding activities but still confer constitutive repression of ethylene signaling [21]. These residues must therefore be crucial for switching off the transmitter domain of the receptor in response to ethylene binding [21]. The study suggests the existence of an intermediate state between the 'on' and 'off' states of the receptor and provides a possible explanation of how the dominant truncated etr1-1 receptor can cause ethylene insensitivity in multiple receptor complexes [21,22,35,46,60].

Ample genetic evidence, direct physical association and ER co-localization have suggested that CTR1 is part of the ethylene receptor complexes and mediates convergent signaling output from multiple ethylene receptors that activate its PK activity in the absence of a ligand [24–26,61,62]. In response to ethylene, CTR1 is likely to be inactivated through a receptor-mediated conformational change. It has become clear that regulation of CTR1 by the receptors is probably nonstoichiometric, and its membrane association is not always correlated with the level of signaling. Although all receptors might interact and modulate CTR1, ETR1 or ERS1-associated CTR1 might have higher activities in suppressing ethylene signaling by as yet unknown biochemical mechanisms [25,26,46,61]. A recent study suggests that an important lipid secondary messenger phosphatidic acid (PA) can bind directly to CTR1 through the PK domain and interfere with its activities *in vitro* [63]. However, the specificity and physiological significance of PA binding and signaling in ethylene responses or for the regulation of CTR1 activity require further investigation.

Nuclear events in ethylene signaling

Ethylene signaling induces an immediate early transcription response in plants. Such fast nuclear responses to cellular signals often utilize protein modification strategies without *de novo* protein synthesis. Independent studies have converged on the central finding that the protein stability control of the key transcription factor EIN3 is the primary nuclear event in ethylene signaling [28–34,64]. Although *EIN3* transcription is not affected, EIN3 accumulates in the nucleus in response to ethylene or the immediate ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) within 1 h [29–32]. Without exogenous ethylene induction or application, EIN3 is degraded constantly through the 26S proteasome. Two partially redundant EIN3-binding F-box proteins (EBF1

and EBF2) serve as the substrate recognition subunits of the E3 ubiquitin ligases to target EIN3 for degradation. Consistently, EIN3 protein accumulates in *ebf1 ebf2* double mutants, which exhibit strong constitutive ethylene signaling phenotypes [29,31,32,64]. The constitutive ethylene response of null *ebf1 ebf2* mutants causes seedling lethality. However, the severe phenotype is rescued by the loss-of-function *ein3 eil1* double mutant. EBF1 and EBF2, therefore, act mainly with EIN3 and EIL1 in ethylene signaling [64]. The distinct phenotypes of *ctr1 ebf1* and *ctr1 ebf2* suggest that EBF1 and EBF2 also have non-overlapping functions. Careful analysis of growth dynamics of *ebf1* and *ebf2* single mutants has indicated that EBF1 acts in the air during the initial phase of ethylene signaling, whereas EBF2 probably has a prominent role during the later stages of ethylene responses and growth reset after ethylene removal [64]. This model is consistent with the ethylene induction of *EBF2* through direct EIN3 regulation of the *EBF2* promoter as a negative-feedback loop [29,31,34,64].

Intracellular signaling via dual MAPK cascades

Despite the identification of many key components in ethylene signaling, it remains unclear how the receptor–CTR1 complex is connected to the nuclear EIN3 and EIL1 transcription factors. Although the negative regulator CTR1 is a putative MKKK inactivated by ethylene, independent biochemical studies have shown that ethylene or the *ctr1* mutant induces but does not inhibit MAPK-like activities in *Arabidopsis* and alfalfa (*Medicago sativa*) [65–67]. The recent identification of the MKK9 (MAPK kinase 9)–MPK3 (MAPK3) and -6 cascade activated by ethylene or in the *ctr1* mutant provides molecular and genetic evidence for the involvement of an unexpected MAPK cascade acting positively in ethylene signaling downstream of CTR1 [67]. Combining cell-based and genetic screens using immunocomplex MAPK activity, ethylene-specific reporter activation and triple-response seedling assays, MKK9 and MPK3 and -6 have emerged as positive regulators in ethylene signaling [67]. The role of MKK9 in linking the ER-located receptor–CTR1 complex to the nuclear events is further supported by the observation that MKK9–GFP (green fluorescent protein) is enriched in the nucleus by ACC treatment and in the constitutively signaling *ctr1* cells but dispersed in the ethylene-insensitive *etr1-1* cells [67]. Consistently, loss-of-function *mkk9* exhibits a broad spectrum of phenotypes associated with ethylene insensitivity, including reduced seedling triple response, abolished primary gene and MPK3 and -6 activation and decreased suppression of leaf expansion and chlorophyll accumulation in response to ethylene or ACC, as well as hypersensitivity to high glucose and salt stress [67]. The relatively moderate phenotypes in *mkk9* are probably due to the remaining CTR1 pathway that can still respond to ethylene independently of the MKK9–MPK3 and -6 cascade (Figure 2).

The current model for ethylene signaling suggests simultaneous actions of the dual MAPK cascades, the activation of the positive-acting MKK9–MPK3,6 cascade and the inhibition of the negative-acting CTR1 pathway by ethylene (Figure 2). Strong evidence supporting the functions of the dual MAPK cascades has also come from studies on EIN3 regulation in ethylene signaling. Several research groups have provided compelling data that the control of EIN3 stability is a key mechanism in ethylene signaling [29–32]. Prompted by the observation that the nuclear translocation of MKK9 depends on ethylene signaling and that the MKK9 substrates MPK3 and -6 are present in the nucleus, the hypothesis of direct MAPK phosphorylation and regulation of EIN3 was tested [67]. Computational search and mutagenesis analysis confirmed the importance of two predicted MAPK phosphorylation sites in modulating EIN3 stability, Thr^{174} for promoting EIN3 stabilization and Thr^{592} for enhancing EIN3 degradation [67]. Both *in vitro* and *in vivo* assays provided further evidence that the MKK9–MPK3 and -6 cascade directly phosphorylates and stabilizes EIN3. Analysis of the EIN3 phosphorylation mutants also supports the essential but opposite roles of the two MAPK phosphorylation sites in ethylene signaling in transgenic *Arabidopsis* [67].

Many stress signals not perceived via ethylene receptors can also activate MKK7 and -9–MPK3 and -6 or MKK4 and -5–MPK3 and -6 cascades [68–71] and can potentially phosphorylate Thr^{174} in EIN3. However, without ethylene binding, the receptors cannot shut down CTR1, which constantly promotes EIN3 degradation through the phosphorylation of Thr^{592}. Therefore, only when the activation of the MKK9–MPK3 and -6 cascade and the suppression of CTR1 pathway occur simultaneously can specific activation of ethylene signaling be properly executed. This model explains the specificity of ethylene signaling using MAPK cascades shared by other pathways and why *mkk9* lacking only one part of MAPK cascades involved in ethylene signaling exhibits broad but relatively moderate ethylene insensitivity [67]. A recent study shows that inducible overexpression of MKK9–MPK3 and -6 can activate ethylene and the biosynthesis of camalexin, a phytoalexin [72]. However, the activation of MPK3 and -6 by ethylene is much weaker than that caused by wounding or by a microbe-associated molecular pattern, flg22 [67,70,71], and is not closely correlated with the production of ethylene or camalexin. Also, such a strong activation of the MKK9 cascade seems to trigger a plethora of signaling cascades that complicate the analysis [67–72]. It will be informative to examine how MAPK phosphorylation influences EIN3 affinity toward EBF1 and -2 [29,31,32] to further clarify PK functions in ethylene signaling.

Integration of ethylene and GA in growth, stress and flowering

Recent research has suggested that ethylene modulates plant growth and development via signaling networks involving other plant hormones [73–76]. Under favorable growth conditions, ethylene inhibits root, stem and leaf growth and delays flowering, which are all promoted by the GA growth hormones (Figure 3a). However, under stress, ethylene signaling is essential to maintain plant growth and survival [75]. Two important molecular mechanisms of ethylene interaction with GA are mediated by modulating bioactive GA levels and the stability of DELLA proteins, including GA INSENSITIVE (GAI) and REPRESSOR OF

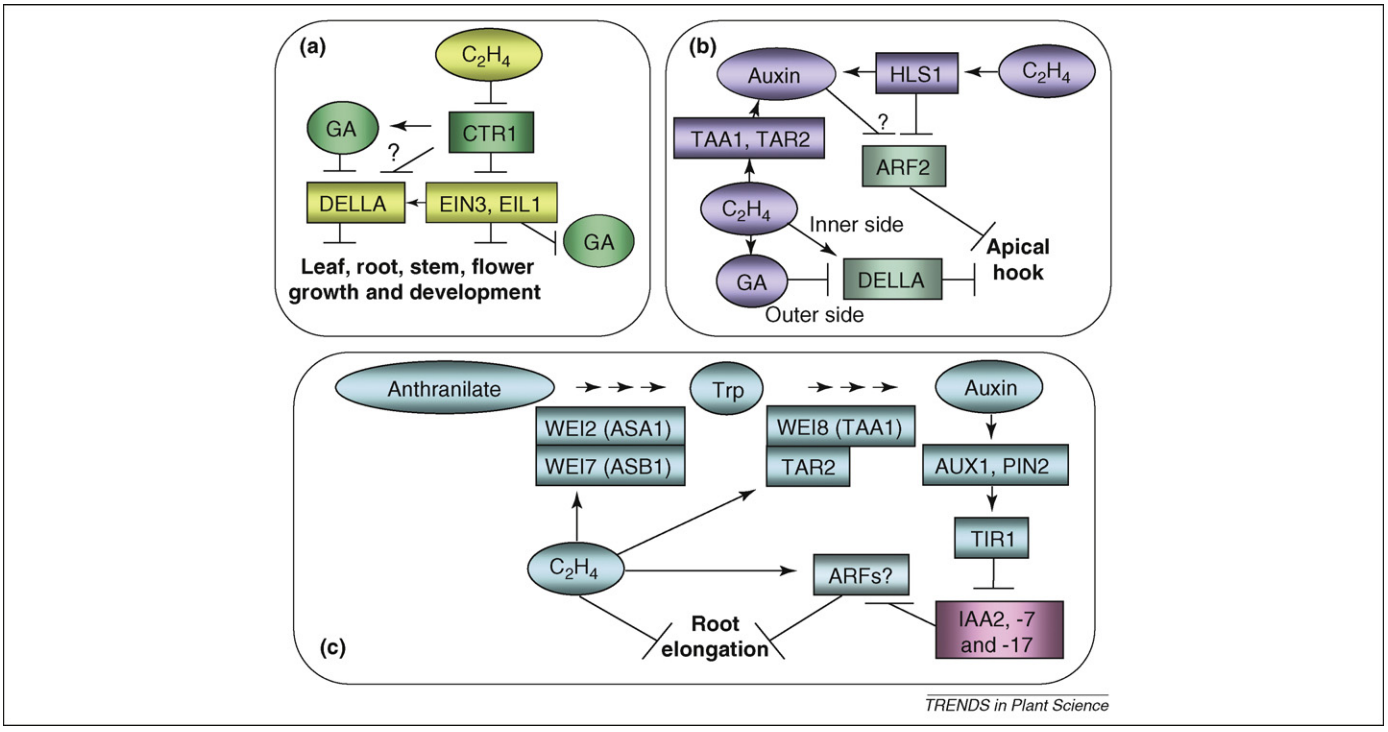

Figure 3. Molecular interactions among ethylene, GA and auxin in growth control. Ethylene interactions with GA and auxin are contextual and differ across tissues and organs. **(a)** Ethylene and GA have antagonistic roles in leaf, root and stem growth and the modulation of flowering time [79–81]. Ethylene can inhibit bioactive GA accumulation through the actions of EIN3, EIL1 and CTR1. It also delays the degradation of DELLA proteins as general growth repressors. GA signaling degrades DELLA proteins as negative regulators. **(b)** Ethylene promotion of the apical hook formation requires GA and auxin in etiolated seedlings [79,82–84]. Ethylene activates *HLS1* expression, which decreases ARF2 protein levels but enhances auxin transport and response. ARF2 is a repressor in auxin signaling [82]. Ethylene activates *TAA1* and *TAR2* expression, which is important for auxin biosynthesis and hook formation [83]. Ethylene probably not only delays DELLA protein degradation to inhibit cell elongation in the inner side of the hook but also alters GA metabolism. GA represses DELLA proteins and promotes cell division and elongation in the outer side of the hook [79,82,84]. **(c)** Ethylene inhibition of root elongation is mediated by both auxin-independent and -dependent pathways [83,85–88]. Ethylene activates the expression of WEI2 (ASA1), WEI7 (ASB1), WEI8 (TAA1) and TAR2 in the root apex to enhance local auxin biosynthesis [83,85,86]. Newly synthesized auxin flows in a basipetal (i.e. tip to distal) direction through AUX1 and PIN2 to activate auxin signaling in the transition and elongation root zones to inhibit root growth. The ethylene-activated local auxin signaling requires the auxin receptor TIR1, which inhibits the repressors IAA2, -7 and -17 and might activate ARFs [86–88]. Three different colors in (a), (b) and (c) for C2H4 represent three different C2H4 signaling conditions in different organs. The other colors, green (a), blue (b) and pink (c), mark the components playing negative roles in C2H4 (ethylene) signaling. Abbreviation: Trp, tryptophan.

ga1-3 (RGA) in *Arabidopsis* (Figure 3a) [76–81]. For example, bioactive GA levels are low in the *ctr1* mutant and after ACC treatment but increase in the ethylene-insensitive *etr1-2* mutant [77,78,81]. DELLA proteins contain specific DELLA domains at their C-terminal ends among the molecularly related GRAS (GAI, RGA and SCARECROW) members. The functionally redundant DELLA proteins are nuclear regulators acting as conserved growth repressors and negatively regulate all aspects of GA responses [74,76,79–81]. GA promotes plant growth mainly through proteasome-mediated DELLA protein degradation, but ethylene significantly delays GA-induced DELLA degradation and sustains its growth repression [74,76,79]. Interestingly, the stabilization of DELLA in *ctr1* increases the expression of the DELLA target genes *GA3ox1* and *GA20ox1*, which are involved in bioactive GA synthesis. However, the bioactive GA levels are still low in *ctr1* mutants, in which GA is probably repressed by a DELLA-independent pathway through the regulation by CTR1 or EIN3 (Figure 3a) [81].

As an environmental signal integrator, ethylene also transmits salt stress signaling and enhances plant survival in a DELLA-dependent manner [80]. However, because the *ctr1 gai rga* triple mutant still maintains a significant survival rate on a high salt medium, DELLA proteins can only partially account for the enhanced salt tolerance in *ctr1* (Figure 3a) [80]. A recent study suggests that ethylene signaling significantly delays *Arabidopsis* flowering time through repression of the floral meristem identity genes *LEAFY* (*LFY*) and *SUPRESSOR OF OVEREXPRESSION CONSTANS1* (*SOC1*) [81]. Consistent with the inhibition of bioactive GA accumulation in *ctr1*, the flowering time can be shortened by GA treatment in *ctr1* or by genetically removing GAI and RGA in *ctr1 gai rga* [81]. Because GA treatment promotes early flowering without altering EIN3 stability in the constitutive ethylene signaling mutant *ebf1 ebf2*, it is proposed that ethylene inhibits GA levels downstream of EIN3. The experiment does not exclude the possibility that CTR1 directly modulates GA biosynthesis enzymes (Figure 3a). However, the mechanism delaying flowering time of dominant ethylene insensitive *etr1* mutants remains to be resolved [81].

Ethylene modulation of GA and auxin in tissue-specific growth regulation

In etiolated *Arabidopsis* seedlings, the ethylene triple response, which includes apical hook formation and inhibition of hypocotyl and root elongation, is also a coordinated event among ethylene, GA and auxin in differential cell growth. Although ethylene normally antagonizes the effects of GA and auxin (Figure 3a) [5,6,73–76], in etiolated seedlings, the promotion of apical hook formation

(Figure 3b) and the inhibition of root elongation (Figure 3c) by ethylene require the synthesis, transport and actions of the plant growth hormones [73–76,79,82–88].

Molecular genetic analysis has identified HOOKLESS1 (HLS1, which encodes a putative *N*-acetyltransferase) as a positive regulator and AUXIN RESPONSE FACTOR2 (ARF2, which encodes a nuclear transcription factor) as a negative regulator in ethylene promotion of apical hook formation in dark-grown etiolated seedlings [82]. Ethylene rapidly enhances HLS1 levels, whereas both ethylene treatment and HLS1 overexpression decrease ARF2 protein levels [82]. The *arf2* mutant seems to promote more local and differential expression of an auxin reporter, *DR5::GUS*. The hookless phenotype is phenocopied by inhibitors of auxin transport [79], and *hls1* also alters spatial expression patterns of the auxin response reporter. It is proposed that HLS1 promotes differential growth in the apical hook by modulating localized auxin transport and responses (Figure 3b) [82]. It will be important to elucidate how a putative *N*-acetyltransferase, HLS1, controls differential auxin distribution and ARF2 stability in response to ethylene.

Recent characterization of a *weak ethylene insensitive8* (*wei8*) mutant has revealed the unexpected role of ethylene in controlling the indole-3-pyruvate acid (IPA) branch of auxin biosynthesis through the localized activation of tryptophan aminotransferase by *WEI8* (*TAA1* [*TRYPTOPHAN AMINOTRANSFERASE of ARABIDOPSIS1*]) and *TAA-RELATED2* (*TAR2*) expression (Figure 3b) [83]. Furthermore, the examination of GA synthesis and signaling mutants supports a crucial role of GA in ethylene-mediated hook formation [79,84]. Ethylene is likely to enhance bioactive GA levels and DELLA degradation in the outer side of the hook to promote cell division and elongation but to stabilize DELLA in the inner side of the hook to inhibit cell elongation (Figure 3b) [84]. More detailed spatial and temporal characterization of reporter genes for ethylene, auxin and GA will facilitate the molecular and cellular understanding of differential growth in the apical hook. It will provide the best case study of localized hormone sensitivity and differential hormone responses though multiple hormone interactions that are prevalent in whole plant growth and development.

Ethylene also has a primary role in modulating root growth and morphology in *Arabidopsis* [83,85–89]. It inhibits root growth by hampering cell elongation rather than by interfering with cell-cycle regulation [85–88]. Using a set of reporter genes, a combination of ethylene and auxin mutants, and microarray-based gene expression profile analysis, three groups have reported comprehensive studies on the interconnected roles of auxin synthesis, transport and signaling in contributing to ethylene-mediated root growth inhibition [85–88]. An integrated model can now be proposed to provide a spatial and temporal framework of ethylene response in etiolated seedling roots [83,85–88] (Figure 3c). The molecular genetic analysis of three *wei* mutants displaying root-specific ethylene insensitivity enabled the discovery that the expression of genes involved in tryptophan (Trp)-mediated auxin biosynthesis, such as *WEI2* (*ASA1*), *WEI7* (*ASB1*), *WEI8* (*TAA1*) and *TAR2*, are activated by ethylene or ACC in the root apex [83,85]. *ASA1* and *ASB1* encode anthranilate synthase α1 and β1, which are subunits of a rate-liming enzyme for Trp biosynthesis [83]. TAA1 and its redundant member TAR2 are Trp aminotransferases that are essential in the IPA branch of auxin biosynthesis [85] (Figure 3c). Ethylene also upregulates the auxin efflux component PIN-FORMED2 (PIN2) and the influx carrier AUXIN RESISTANT1 (AUX1) to promote basipetal flow of auxin to the transition and elongation zones via the lateral root cap [86–88]. Ethylene-stimulated auxin signaling is observed at the root apex and the transition and elongation zones through the receptor TIR1 and the combined actions of IAA2, -7, -17 and ARF2 [86–88]. Auxin, PIN2 and AUX1 mediated ethylene signaling sensitizes and activates the ethylene reporter *EBS::GUS* in the root transition zone [86]. However, all studies suggest that the pathways of auxin- or ethylene-mediated root inhibition are not identical, and some ethylene-mediated inhibition of root growth is auxin independent (Figure 3c) [86–88]. Auxin-mediated root growth inhibition can also occur in ethylene-insensitive *etr1-3* and *ein2* mutants [88]. This function model is supported by extensive microarray analyses of the roots of wild-type plants and *ein2* and *aux1* mutants that have identified groups of genes that can be classified as auxin-independent, auxin-mediated or auxin-dependent ethylene responses or ethylene-independent, ethylene-mediated or ethylene-dependent auxin responses [86]. Several auxin biosynthesis genes in the quiescent center at the root tip have been observed to be activated by ethylene [83,85], suggesting that ethylene might be able to modulate stem cell division in roots in response to diverse stimuli, although this hypothesis needs further confirmation [89]. It is likely that the ethylene and auxin interactions uncovered in recent studies are pieces of a larger signaling network integrating complex developmental and environmental signaling processes to determine plant growth and survival.

Perspectives

Integrative approaches combining biochemical and cellular assays and genetic and genomic tools have provided the means to establish a new conceptual framework to enhance our understanding of the mode and actions of receptor complexes, intracellular MAPK cascades and the control of nuclear transcription factors in ethylene signaling [8,12,21,34–38,46,55,64,67]. Molecular details of signaling convergence and synergism between ethylene and other plant hormones, such as GA and auxin, have emerged as the regulatory basis of organ-specific growth and development in response to environmental stimuli [73,74,76,80–89]. Future work is likely to yield new information about the regulation of the receptor–CTR1 complexes [12,46,61,63] and resolve the roles of HK and Ser–Thr PK activities in the signal transduction process of ethylene receptors in *Arabidopsis* and other plants [57,58,60]. It will be exciting to gain a more mechanistic understanding of the actions of diverse negative modulators, including RTE1/GR for the ETR1 receptor [12,38,52–55] and EERs in the ethylene responses [42–45]. The genetic analysis of *ein5* mutants has led to unexpected findings for the new regulatory mechanism of RNA metabolism and silencing

pathways [39–41]. Elucidating the biochemical function of EIN2 as a central regulator in ethylene and stress signaling could reveal innovative mechanisms for transcription factor regulation between the ER and the nucleus [27,29,45]. More detailed molecular analysis of early ethylene response genes, such as *EBF2*, also offers an opportunity to unravel new regulatory mechanisms of transcriptional and post-transcriptional regulation in ethylene signaling [34]. More molecular understanding of ethylene interactions with other hormones and nutritional, stress and microbial signals is necessary to obtain a holistic view of the signal transduction networks that govern every aspect of plant life.

The involvement of MAPK cascade signaling in ethylene responses has been proposed as a result of the identification of CTR1 as a putative MKKK [24,26]. New evidence has emerged to explain the ethylene signaling specificity through the actions of dual MAPK cascades that control the stability of EIN3 and EIL1 [65–67]. It will be important to determine whether CTR1 binds and inhibits MKK9 directly in the absence of ethylene and whether ethylene inactivation of CTR1 releases MKK9 to the nucleus to activate MPK3 and -6. It is also possible that the inactivation of CTR1 activates another MKKK to then activate MKK9 (Figure 2). Identification of the MKKs and MPKs in the CTR1 cascade will provide additional insight into functional modes and regulatory roles of the MAPK pathway.

MAPK cascade signaling is an evolutionarily conserved information-processing machinery that transmits various extracellular signals, including abiotic and biotic stresses, hormones and peptides in plants [65–72]. Careful dissection of MPK3 and -6 activation at different amplitudes, durations and subcellular locales in diverse cell types will be essential to characterize thoroughly their various physiological triggers and diverse functions [67–72].

Given that a broad range of important agronomic traits, such as plant yield, organ freshness–senescence and fruit ripening–abscission, are regulated by ethylene, the physiology of this hormone has been an attractive target for biotechnological applications [3,8,10]. Understanding ethylene signaling in molecular and cellular details in *Arabidopsis* and crop plants will provide innovative tools for improving plant adaptability against unfavorable and unexpected environmental fluctuations to enhance plant fitness and yield for food, fiber and fuel.

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