Mechanistic Insights in Ethylene Perception and Signal Transduction

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The gaseous hormone ethylene profoundly affects plant growth, development, and stress responses. Ethylene perception occurs at the endoplasmic reticulum membrane, and signal transduction leads to a transcriptional cascade that initiates diverse responses, often in conjunction with other signals. Recent findings provide a more complete picture of the components and mechanisms in ethylene signaling, now rendering a more dynamic view of this conserved pathway. This includes newly identified protein-protein interactions at the endoplasmic reticulum membrane, as well as the major discoveries that the central regulator ETHYLENE INSENSITIVE2 (EIN2) is the long-sought phosphorylation substrate for the CONSTITUTIVE RESPONSE1 protein kinase, and that cleavage of EIN2 transmits the signal to the nucleus. In the nucleus, hundreds of potential gene targets of the EIN3 master transcription factor have been identified and found to be induced in transcriptional waves, and transcriptional coregulation has been shown to be a mechanism of ethylene cross talk.

Ethylene, the first gaseous plant hormone that was identified, regulates numerous developmental processes and stress responses in plants. Ethylene is best known for its effects on agronomically important processes such as ripening in climacteric fruits and organ senescence and abscission, but ethylene also mediates numerous other aspects of plant growth and development, including seed germination, root initiation, leaf expansion, flower development, and sex determination (Abeles et al., 1992; Lin et al., 2009). Ethylene also functions as a stress hormone, as its production is elicited in response to biotic and abiotic challenges, such as wounding, flooding, cold, nutrient stress, and pathogen attack (Lin et al., 2009; Iqbal et al., 2013).

Ethylene signal transduction has received intense research attention, in large part due to its wide-ranging effects and critical roles in agronomically important plants. Over the past several decades, the mechanisms of ethylene perception and response have been extensively investigated in the model plant Arabidopsis (Arabidopsis thaliana), as well as in fruit species such as tomato (Solanum lycopersicum; Klee, 2004) and more recently in the rice (Oryza sativa) crop (Yang et al., 2015). The molecular genetic dissection of the ethylene signaling pathway in Arabidopsis has provided major breakthroughs in our understanding of the pathway. The isolation of ethylene response mutants in Arabidopsis was facilitated by a simple genetic screen based on the etiolated seedling triple response phenotype, consisting of a shortened and thickened hypocotyl and root and an exaggerated apical hook (Bleecker et al., 1988; Guzmán and Ecker, 1990; Fig. 1). Mutants that lack the triple response when treated with exogenous ethylene are ethylene insensitive, and mutants that exhibit the triple response even in the absence of ethylene are constitutive response mutants (Fig. 1). The order of action for many of these components in the pathway was determined using double mutant (epistasis) analysis (e.g. Roman et al., 1995).

The key components of the pathway, their mutant phenotypes, and their genetic actions are shown in Figure 1. Notably, Arabidopsis has five ethylene receptor genes (ETR1, ERS1, ETR2, EIN4, and ERS2). As established by genetic studies, the ethylene receptors are negative regulators of ethylene responses (Fig. 1). Dominant gain-of-function mutations in each receptor gene confer ethylene insensitivity (Chang et al., 1993; Hua et al., 1995; Wilkinson et al., 1995; Sakai et al., 1998; Wang et al., 2006; Fig. 1). In contrast, loss-of-function mutations in individual receptor genes confer little or no phenotypes, indicating a significant degree of functional redundancy among the receptors (Hua and Meyerowitz, 1998; Qu et al., 2007). The combination of at least two or three loss-of-function ethylene receptor mutations, however, confers constitutive ethylene responses (Hua and Meyerowitz, 1998). In the ethylene-signaling pathway, the ethylene molecule represses ethylene receptor signaling (Hua and Meyerowitz, 1998), thereby preventing the next downstream component, negative regulator CTR1 (Kieber et al., 1993), from repressing the pathway (Fig. 1); EIN2, a central positive regulator of ethylene responses (Alonso et al., 1999), is then free to signal to the nucleus where the transcription factors EIN3 and its homolog EIL1 (Chao et al., 1997; An et al., 2010) initiate a transcriptional

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Despite the conservation of the ethylene-signaling pathway in the aquatic ancestor of land plants prior to the colonization of land, evolutionarily, this is the first known appearance of the ethylene-signaling pathway. Although the seedling triple response is highly specific to ethylene, the response involves coordinated regulation with other hormones, including auxin, GA, brassinosteroid, and jasmonate (JA) in apical hook development (Li et al., 2004; Vriezen et al., 2004; Vandenbussche et al., 2010; Zádníková et al., 2010; An et al., 2012; M ud ay et al., 2012; Song et al., 2014; Zhang et al., 2014) and auxin in hypocotyl and root elongation (Swarup et al., 2007; Strader et al., 2010; M ud ay et al., 2012; Xu et al., 2012). An understanding of ethylene signal transduction therefore provides a foundation for identifying mechanisms of hormone cross talk.

This Update summarizes recent advances in the ethylene-signaling pathway with an emphasis on mechanisms in ethylene signaling and downstream cross talk.

**Figure 1.** Genetic diagram of the core ethylene signaling pathway. The ethylene signal represses the function of the five ethylene receptor genes (ETHYLENE RESPONSE1 [ETR1], ETHYLENE RESPONSE SENSOR1 [ERS1], ETR2, ETHYLENE INSENSITIVE4 [EIN4], and ERS2), which otherwise repress ethylene responses through the negative regulator CONSTITUTIVE RESPONSE1 (CTR1). Ethylene responses are positively regulated by EIN2, EIN3, and downstream primary and secondary ethylene-responsive genes, such as ETHYLENE RESPONSE FACTOR1 (ERF1). Representative seedling phenotypes in the triple response assay (ethylene insensitivity or constitutive ethylene response) are shown for the dominant gain-of-function mutations in the ethylene receptor genes and the loss-of-function mutations in CTR1, EIN2, and EIN3/ETHYLENE INSENSITIVE3-LIKE1 (EIL1). Arrows indicate activation, and T-bars indicate repression of the pathway.

**ETHYLENE RECEPTOR SIGNALING AT THE ER MEMBRANE**

**Ethylene Receptor Domain Structure**

Plants possess a small family of ethylene receptors (Gallie, 2015) related to prokaryotic two-component His protein kinase (HK) receptors (Schaller et al., 2011). As in typical HK receptors, the ethylene receptors have an N-terminal ligand-binding domain that is connected to a HK-like domain via a GAF domain (named for the proteins in which it was first characterized: cyclic guanosine monophosphate phosphodiesterase, adenylyl cyclase, and formate hydrogen lyase transcriptional activator). At the C terminus of some ethylene receptors, there is also a receiver domain (the second component of the two-component system; Gallie, 2015). The ethylene receptors fall into two subfamilies based on their phylogeny and shared sequence features. In contrast to subfamily I members, subfamily II receptors generally have degenerate HK domains and typically have an additional transmembrane domain at the N terminus that possibly serves as a signal sequence (for review, see Binder et al., 2012; Shakeel et al., 2013). In Arabidopsis, ETR1 and ERS1 are in subfamily I, and ETR2, EIN4, and ERS2 are in subfamily II (Chang et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998). One receptor in each Arabidopsis subfamily (ERS1 and ERS2) lacks the receiver domain, but this arrangement varies among plant species (Gallie, 2015).

The ethylene receptors are disulfide-linked homodimers (Schaller et al., 1995; Müller-Dieckmann et al., 1999; Hall et al., 2000; Mayerhofer et al., 2015) with the ethylene-binding domain lying within the ER membrane and the signaling domains residing in the cytoplasm (Chen et al., 2002, 2007; Ma et al., 2006; Fig. 2). Three conserved N-terminal transmembrane domains

The conservation of the ethylene-signaling pathway, the responses mediated by the pathway are often quite different among various species. Recent examples include differences in seedling growth kinetics and other ethylene responses between eudicots and monocots (Kim et al., 2012; Yang et al., 2015).
in each monomer form the ethylene-binding domain (Schaller and Bleecker, 1995; Hall et al., 2000; Ma et al., 2006). Ethylene binding requires a copper ion cofactor (Rodriguez et al., 1999), which is supplied by the P-type adenosine triphosphatase copper transporter RAN1 (Hirayama et al., 1999; Fig. 2). The copper cofactor is also required for ethylene receptor biogenesis (Hirayama et al., 1999; Woeste and Kieber, 2000; Binder et al., 2010).

The receptor dimers form clusters that are mediated in part by GAF-GAF domain interactions (Gao et al., 2008; Grefen et al., 2008). Besides interacting among themselves, the receptors interact with other components in the ethylene-signaling pathway, such as CTR1

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**Figure 2.** Schematic model of the ethylene signaling pathway. In the absence of ethylene perception (left), the formation of functional ethylene receptors depends on a copper cofactor provided by the copper transporter RESPONSIVE TO ANTAGONIST1 (RAN1), as well as activation by REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1), which depends on cytochrome b5 (Cb5). The ethylene receptors (represented here by ETR1 and ERS1 homodimers) at the endoplasmic reticulum (ER) membrane are in a protein complex with downstream components EIN2 and CTR1. The receptors associate with and activate (by an undefined signaling mechanism) the CTR1 protein kinase domain (KD), which phosphorylates the EIN2 C-terminal domain. Phosphorylation prevents EIN2 from signaling, and EIN2 is targeted for 26S proteasomal degradation by F-box proteins ETHYLENE INSENSITIVE2 TARGETING PROTEIN1 (ETP1) and ETP2. Meanwhile, in the nucleus, the F-box proteins ETHYLENE INSENSITIVE3 BINDING F-BOX1 (EBF1) and EBF2 target the EIN3/EIL1 transcription factors for 26S proteasomal degradation (only EIN3 is shown), preventing induction of gene expression such that there is no ethylene response. Additionally, there is a postulated secondary pathway from the receptors involving autophosphorylation of the His by the receptor His kinase (HK) domain, and transfer of the phosphate to the receiver (R) domain, followed by transfer of the phosphate to ARABIDOPSIS HIS PHOSPHOTRANSFER (AHP), which is released by a conformational change in the receptors (indicated by the altered shapes of the HK and R domains between the left and right sides) when they bind ethylene (right). The binding of ethylene (right) inactivates ethylene receptor signaling (indicated by the altered shapes of the HK and R domains between the left and right sides). In addition, the levels of ERS1 and other ethylene receptor isoforms (not shown) increase (represented by the darker color on the right side relative to the left side) due to transcriptional induction, but reach an equilibrium state due to being degraded by the 26S proteasome. CTR1 levels increase in the complex as well (represented by the darker color on the right side relative to the left side) due to the increased level of ethylene receptors and protect the ETR1 receptor from proteolysis. However, the ethylene receptors no longer activate CTR1, and therefore, EIN2 is no longer phosphorylated. Instead, a cytoplasmic portion of EIN2 is proteolytically cleaved by an unidentified protease, and the liberated C-terminal portion of EIN2 (C-END) moves into the nucleus where signal transmission results in EIN2-dependent 26S proteasomal degradation of the F-box proteins EBF1/2 and, consequently, the stabilization and accumulation of master transcription factors EIN3/EIL1. EIN3/EIL1 activate a transcriptional cascade that includes the downstream ERF1 transcription factor gene. An exoribonuclease (EXORIBONUCLEASE4 [XRN4]) also plays an indirect role in the repression of EBF1/2 mRNA.
and EIN2 (Gao et al., 2003; Bisson et al., 2009; Bisson and Groth, 2010; Dong et al., 2010; Ju et al., 2012; Shakeel et al., 2015; Fig. 2). The receptors have been observed in high-molecular-mass protein complexes (Chen et al., 2010), indicating the likely presence of additional unidentified proteins.

Although the ethylene receptor isoforms have been shown to have overlapping functions (e.g. Hua and Meyerowitz, 1998), it has become increasingly clear that the isoforms also have distinct roles (Shakeel et al., 2013). For example, Arabidopsis subfamily II receptors cannot functionally substitute for subfamily I receptors (Wang et al., 2003). Arabidopsis subfamily I has a larger role than subfamily II (Qu et al., 2007), whereas in tobacco (*Nicotiana tabacum*), subfamily II appears to play a greater role than subfamily I (Chen et al., 2009). Within Arabidopsis subfamily I, ETR1 and ERS1 differentially repress ethylene responses (Liu and Wen, 2012), and ERS1 even appears to positively regulate ethylene responses in an ETR1-dependent manner (Liu et al., 2010). Among the Arabidopsis ethylene receptors, only ETR1 is required for ethylene-induced nutational bending in the hypocotyl (Kim et al., 2011), and ETR1 plays a major role in mediating the effects of silver, which blocks ethylene responses (McDaniel and Binder, 2012). In addition, Arabidopsis ETR1 and ETR2 act oppositely in abscisic acid-mediated seed germination under salt stress (Wilson et al., 2014).

### How Do the Ethylene Receptors Signal?

An important question that remains largely unanswered concerns the biochemical mechanism(s) of ethylene receptor signaling. In the canonical two-component signaling system, His autophosphorylation by the receptor is followed by phosphotransfer to an Asp residue in the associated receiver domain, which then mediates downstream responses (Schaller et al., 2011). Although Arabidopsis ETR1 displays HK activity in vitro (Gamble et al., 1998; Moussatche and Klee, 2004), there is no evidence that this is the primary receptor signaling mechanism (Binder et al., 2012; Shakeel et al., 2013). In fact, the HK activity of ETR1 is dispensable in ethylene signaling (Wang et al., 2003), playing only a minor role in modulating the level of ethylene receptor signaling (Hall et al., 2012). There is also no evidence that phosphotransfer to the receiver domain of the receptors is required for ethylene responses. The presence of the receiver domain is, however, important in the growth recovery response upon the removal/dispersal of ethylene (Binder et al., 2004; Kim et al., 2011). Adding to the confusion, ETR1 exhibits in vitro association with ARABIDOPSIS HIS PHOSPHOTRANSFER (AHP) proteins (Scharein et al., 2008), which are downstream elements in the two-component multistep (His-Asp-His-Asp) phosphorelay (Schaller et al., 2011), raising the possibility of a two-component multistep phosphorelay downstream of the ethylene receptors (Fig. 2). Interestingly, the ETR1-AHP1 interaction appears to be controlled by the phosphorylation state of ETR1 (Scharein and Groth, 2011). Given that the cytokinin pathway utilizes His-Asp-His-Asp pathway components (Schaller et al., 2011), an intriguing possibility is that the AHP pathway is involved in ethylene cross talk with cytokinin signaling.

Instead of HK activity, subfamily II receptors in Arabidopsis (Moussatche and Klee, 2004), tobacco (Chen et al., 2009), and rice (Wuriyanghan et al., 2009) all exhibit Ser/Thr kinase activity in vitro, whereas Arabidopsis ERS1 (in subfamily I) is bifunctional, displaying both His and Ser/Thr kinase activity in vitro (Moussatche and Klee, 2004). Overexpression of the tobacco subfamily II receptor NTHK1 in Arabidopsis conferred ethylene insensitivity that was abolished by a mutation blocking kinase activity (Chen et al., 2009), suggesting that Ser/Thr kinase activity plays a role in tobacco ethylene signaling. Moreover, upon ethylene treatment or salt stress, NTHK1 phosphorylates an ankyrin repeat protein named NTHK1 ETHYLENE RECEPTOR-INTERACTING PROTEIN2, which inhibits ethylene responses and improves plant growth through its interaction with NTHK1 (Cao et al., 2015).

Another question is the relation between receptor kinase activity and ethylene binding. In vitro biochemical data suggest that ethylene binding inhibits HK activity in Arabidopsis ETR1 (Voet-van-Vormizeelee and Groth, 2008), whereas genetic data indicate that ethylene perception activates ETR1 kinase activity (Hall et al., 2012). In tomato, the ethylene receptors SIETR4 (in subfamily II) and Never Ripe (in subfamily I) were found to be phosphorylated on multiple sites in vivo, and the level of phosphorylation was reduced during ethylene treatment and fruit ripening (Kamiyoshihara et al., 2012), supporting an inverse relationship between ethylene binding and kinase activity. What remains unclear is whether the observed phosphorylation is due to autokinase activity of SIETR4 and Never Ripe. Overall, further experimentation is needed to reconcile the biochemical versus genetic relationship between ethylene binding and receptor signaling activity, and to resolve the significance of receptor kinase activity in ethylene signaling. Toward this goal, a crystal structure of the ethylene-binding domain would be highly valuable.

### REVERSION-TO-ETHYLENE SENSITIVITY/ GREEN-RIPE and Cytochrome b5 Promote ETR1 Receptor Signaling

ETR1 signaling activity in Arabidopsis is dependent on an upstream unique protein called RTE1. RTE1 localizes to the Golgi/ER membrane (Dong et al., 2008) and represses ethylene responses by promoting ETR1 signaling (Resnick et al., 2006, 2008). Although the biochemical function of RTE1 is unknown, its action in ethylene signaling seems to involve a physical interaction with ETR1 (Dong et al., 2010) and requires the
N-terminal domain of ETR1 (Zhou et al., 2007; Qiu et al., 2012). Interestingly, RTE1 appears to have little or no effect on the other Arabidopsis ethylene receptors (Resnick et al., 2006; Rivarola et al., 2009), but the basis for this specificity is unknown. Expression of the RTE1 gene is somehow regulated by HYPER RECOMBINATION1 (HPR1), a component of the THO/Transcription Export complex that is related to mRNA processing (Xu et al., 2015). HPR1 has been previously implicated in ethylene responses (Pan et al., 2012).

Recently, Arabidopsis RTE1 was found to interact with ER-localized isoforms of Cb5. Genetic analyses suggest that Cb5 proteins act via RTE1 to promote ETR1 signaling (Chang et al., 2014; Fig. 2.). Since Cb5 proteins carry out oxidation/reduction reactions in other organisms (Schenkman and Jansson, 2003), RTE1 might be activated by Cb5-mediated redox modification. Another possibility is that Cb5s and RTE1 are involved in redox modification of ETR1, providing a mechanistic link between oxidative stress and ethylene signaling.

Plants carry two or three members of an RTE1 gene family. Arabidopsis REVERSION-TO-ETHYLENE SENSITIVITY1-homolog (AtRTH) has no apparent role in ethylene signaling and, instead, might share an unidentified function with that of metazoan RTE1 homologs. The tomato RTE1 homologs GREEN-RIPE (GR) and GREEN-RIPE LIKE1 (SIGR1; Barry and Giovannoni, 2006) repress distinct but overlapping sets of ethylene responses, whereas SIGR2, which is more similar in sequence to AtRTH, does not appear to have a role in ethylene responses (Ma et al., 2012). Among the three rice RTE1 homologs (OsRTHs), only OsRTH1, which is most similar to AtRTE1, conferred ethylene-insensitive phenotypes when overexpressed in rice (Zhang et al., 2012). In petunia (Petunia hybrida), a knock-down mutant of PhGRL2, which lies in the AtRTH/SIGR2 group, conferred enhanced flower senescence; although this phenotype would be consistent with RTE1 function, the authors have raised the possibility that PhGRL2 prevents ethylene biosynthesis (Tan et al., 2014).

Dynamic Associations of the Ethylene Receptors with the Downstream Proteins CTR1 and EIN2

The ethylene receptors signal to CTR1, a Ser/Thr protein kinase that negatively regulates ethylene responses (Kieber et al., 1993; Wang et al., 2013; Fig. 2). Genetic evidence indicates that the CTR1 kinase is activated by the receptors in the absence of ethylene and is inactive in the presence of ethylene (Kieber et al., 1993; Huang et al., 2003). CTR1 has an N-terminal regulatory domain and a C-terminal protein kinase domain (Kieber et al., 1993). A physical association between the receptor signaling domains (the HK-like and receiver domains) and the CTR1 regulatory domain is essential for activating the CTR1 kinase domain (Gao et al., 2003; Huang et al., 2003), but the mechanism of activation is unknown. Since ETR1 HK activity is nonessential for ethylene signaling and there is no evidence that the receptors phosphorylate CTR1, the activation of CTR1 potentially involves a noncatalytic steric interaction between the receptors and CTR1. Crystal structure analysis suggests that the Arabidopsis CTR1 kinase domain is active when dimerized, and oligomerization of the CTR1 dimer might help to bring the ethylene receptors together, reinforcing the receptor complex (Mayerhofer et al., 2012). Increased levels of ER-associated CTR1 are correlated with ethylene-induced expression of ethylene receptors due to their physical interaction (Shakeel et al., 2015). The increase in ethylene receptors is countered by ethylene-induced receptor turnover, except for the ETR1 receptor, which appears to be protected from turnover by its tight association with CTR1 (Shakeel et al., 2015). Several Arabidopsis and tomato ethylene receptors have been shown to undergo ligand-induced degradation by the 26S proteasome-dependent pathway (Chen et al., 2007; Kevany et al., 2007; Shakeel et al., 2015; Fig. 2).

The ethylene receptors also interact with EIN2 (Bisson et al., 2009; Bisson and Groth, 2010, 2015), a central positive regulator in the ethylene-signaling pathway (Alonso et al., 1999) that acts downstream of CTR1 (Roman et al., 1995; Ju et al., 2012). Arabidopsis ein2 mutants are completely insensitive to ethylene (Alonso et al., 1999), and mutants of EIN2 orthologs in rice (Ma et al., 2013), Medicago truncatula (Pennetsa et al., 2008), and Lotus japonicus (Miyata et al., 2013) also display ethylene-insensitive phenotypes. EIN2 has an ER membrane-localized N-terminal domain that has sequence similarity to the widely conserved Nramp (Natural resistance-associated macrophage protein) metal ion transporters, although metal transport has not been demonstrated for EIN2. The C-terminal portion of EIN2 consists of a plant-specific hydrophilic domain of unknown biochemical function that is required for the activation of ethylene responses (Alonso et al., 1999). In Arabidopsis, it was shown that the HK-like domain of the ethylene receptors interacts with the C-terminal domain of EIN2 (Bisson et al., 2009; Bisson and Groth, 2010). Blocking or mimicking His phosphorylation on ETR1 resulted in reduced or increased affinity with EIN2, respectively (Bisson and Groth, 2010), and a nuclear localization signal in the EIN2 C-terminal domain is important for this interaction (Bisson and Groth, 2015). The direct interaction of EIN2 with the receptors could be consistent with an alternative pathway of ethylene receptor signaling that bypasses CTR1; such a pathway has been implicated by the finding that overexpression of the ETR1 N-terminal domain partially suppresses the constitutive ethylene response phenotype of the Arabidopsis ctr1-1 mutant (Qiu et al., 2012).

The above findings indicate that there is dynamic regulation of proteins within the ethylene receptor complex in response to ethylene. Recently obtained crystal structures for the Arabidopsis ETR1 cytosolic domain and catalytic ATP-binding domain, as well as for the dimerization domain of the Arabidopsis ERS1 HK domain (Mayerhofer et al., 2015), should facilitate...
the modeling of conformational states of ethylene receptor signaling and receptor interactions with CTR1, EIN2, AHPs, and other proteins.

**THE CENTRAL REGULATOR, EIN2, IS CONTROLLED BY THE CTR1 PROTEIN KINASE AND DELIVERS THE SIGNAL FROM THE ER TO THE NUCLEUS**

Until recently, there have been major gaps in our understanding of ethylene signaling between CTR1 and events in the nucleus. Because CTR1 is most similar in sequence to the Rapidly accelerated fibrosarcoma family of mitogen-activated protein kinase (MAPK) kinase kinases (Kieber et al., 1993), the expectation for many years had been that EIN2, which is the next known downstream element in the pathway, would be regulated by a MAPK pathway. To date, however, there are no known MAPK kinases or MAPKs acting together with CTR1. Meanwhile, it had long been proposed that EIN2 resides at the nuclear membrane to signal to the nucleus where changes in gene expression were known to take place. When EIN2 was instead found to localize to the ER membrane (Bisson et al., 2009), this raised the key question of how the ethylene signal could be transmitted from EIN2 into the nucleus.

Recently, there has been substantial progress in understanding how EIN2 both receives and then relays the ethylene signal, even as the biochemical functions of EIN2 remain elusive. In terms of how EIN2 is regulated, proteomic studies of ethylene responses in Arabidopsis revealed that the EIN2 C-terminal portion is phosphorylated on multiple Ser and Thr residues in the absence, but not presence, of ethylene (Chen et al., 2011; Qiao et al., 2012). Considering that CTR1 is known to be active in the absence of ethylene, CTR1 was a likely candidate for being the kinase responsible for phosphorylating EIN2. Indeed, CTR1 was found to phosphorylate the C-terminal portion of EIN2 on multiple residues in vitro (Ju et al., 2012), and these residues matched those that had been identified in vivo by Chen et al. (2011). Ala substitutions blocking phosphorylation at two highly conserved serines at positions 645 and 924 resulted in constitutive ethylene responses similar to those exhibited by ctr1 mutants (Ju et al., 2012; Qiao et al., 2012), leading to the conclusion that EIN2 signaling is repressed by phosphorylation at these residues in the absence of ethylene (Fig. 2). These findings also suggest that signaling from CTR1 to EIN2 does not require a MAPK pathway. It will be interesting to see whether this phosphorylation is connected to the proteasomal degradation of EIN2 via two F-box proteins, ETP1/2, observed in the absence of ethylene (Qiao et al., 2009).

Closing the physical gap in ethylene signaling from the ER membrane to the nucleus, it was discovered that a portion of the EIN2 C terminus (C-END) is proteolytically cleaved from the ER-anchored N-terminal domain of EIN2 and then translocated into the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012; Fig. 2). Nuclear localization of the C-END is required for the activation of ethylene responses (Qiao et al., 2012; Wen et al., 2012), and the phosphorylation of EIN2 by CTR1 is a key regulatory mechanism of this translocation, as the translocation occurs constitutively in ctr1 mutants or when Ser phosphorylation of EIN2 is blocked by Ala substitutions (Ju et al., 2012; Qiao et al., 2012). Cleavage of EIN2 reportedly occurs at Ser-645 (Qiao et al., 2012), although there is some conflicting evidence on the importance of this specific site (Cooper, 2013; Qiao et al., 2013). Final confirmation of the cleavage site(s) will likely require the identification of the protease responsible for the cleavage. It will also be interesting to see how both the EIN2 N-terminal domain and dynamic interactions between the ethylene receptors and EIN2 (described in the previous section) are involved in regulating EIN2 signaling.

Once in the nucleus, the biochemical mechanisms of EIN2 C-END signaling are still unknown. EIN2 might be involved in either directly activating the transcription factors EIN3/EIL1 or stabilizing EIN3/EIL1 via repression of EBF1 and EBF2, which target EIN3/EIL1 for degradation (described in the next section). Interestingly, the EIN2 C-terminal domain can interact with subunits of the CONSTITUTIVE PHOTOMORPHOGENIC9 signalosome (Christians et al., 2008), although the biological significance of these interactions is unknown.

In the current model of EIN2 regulation, CTR1 phosphorylates the EIN2 C-terminal domain at the ER to prevent ethylene signaling in the absence of ethylene. Protein turnover of EIN2 also appears to play a role in preventing EIN2 from signaling (Qiao et al., 2009). In the presence of ethylene, the absence of EIN2 phosphorylation results in the activation of downstream signaling via the cleavage of EIN2 and translocation of the C-END into the nucleus (Fig. 2).

**THE EIN3/EIL1 TRANSCRIPTION FACTORS INITIATE A TRANSCRIPTIONAL CASCADE**

The EIN3 transcription factor and its homolog EIL1 are master positive regulators of ethylene responses in Arabidopsis (Chao et al., 1997; Solano et al., 1998; An et al., 2010; Chang et al., 2013). Together, EIN3 and EIL1 cooperatively and differentially regulate the full array of ethylene responses, with EIN3 mainly controlling seedling responses and EIL1 having a greater role in adult leaves and stems (An et al., 2010). EIN3/EIL1 activate a transcriptional cascade by binding as homodimers (Solano et al., 1998; Yamasaki et al., 2005) to the promoters of ERF genes, such as ERF1 (Solano et al., 1998), ETHYLENE AND SALT INDUCIBLE1 (Zhang et al., 2011), and other ERF genes (Chang et al., 2013; Figs. 2 and 3A) in the APETALA2-ETHYLENE RESPONSE ELEMENT BINDING PROTEIN transcription factor family. The ERFs in turn bind to the GCC box element in the promoters of additional ethylene-responsive genes (Fujimoto et al., 2000; Fig. 2), such as stress-response
genes (e.g. Wu et al., 2008; Zhang et al., 2011). Additionally, EIN3 has been found to bind to the promoter of various target genes (described in the text) that control a diverse array of responses. B. The EIN3 protein can physically associate with other transcriptional activators or repressors, including DELLA, JASMONATE ZIM-DOMAINs (JAZs), MYC2, and FER-LIKE FE DEFIciENCY-INDUCED TRANSCRIPTION FACTOR (FIT), which are regulated by GA, JA, and iron, respectively, to coactivate (arrows) or corepress (T-bars) transcription in various processes.

Although EIN3/EIL1 are constitutively expressed, their protein products are degraded in the absence of ethylene via the 26S proteasomal pathway (Guo and Ecker, 2003; An et al., 2010; Fig. 2). Two F-box proteins, EBF1/2 (mentioned earlier), are responsible for targeting EIN3/EIL1 for degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007; An et al., 2010). When ethylene is perceived, the EBF1/2 F-box proteins themselves are turned over, resulting in the stabilization and accumulation of EIN3/EIL1 (An et al., 2010). The ethylene-induced degradation of EBF1/2 and concomitant EIN3/EIL1 stabilization are EIN2 dependent (An et al., 2010; Fig. 2). At the mRNA level, EIN3 activates the expression of EBF2, providing a negative feedback (Konishi and Yanagisawa, 2008; Chang et al., 2013). XRN4 represses the level of EBF1/2 transcripts (Fig. 2), although the mechanism appears to be indirect (Olmedo et al., 2006; Potuschak et al., 2006). EIN3 turnover and stability might involve phosphorylation (Yoo et al., 2008); a conserved phosphorylation site in tomato SlEIL1 has been implicated in having a role in SlEIL1 dimerization and signaling (Li et al., 2012).

EIN3/EIL1 ARE AN INTEGRATION NODE FOR SIGNAL CROSS TALK

The EIN3/EIL1 transcription factors serve as a major integration point for ethylene cross talk with other signals, and this could largely explain the involvement of ethylene in diverse responses (Fig. 3). The Chang et al. (2013) ChIP-seq study of EIN3 transcriptional targets revealed numerous genes either within or downstream of essentially all other plant hormone pathways (e.g. Fig. 3A). For example, EIN3/EIL1 bind to the promoters of HOOKLESS1 (HLS1) and HLS1-LIKE HOMOLOG2 (HLH2; An et al., 2012; Chang et al., 2013), which are positive regulators of apical hook formation and thought to be part of the mechanism underlying the coregulation of ethylene and auxin in plant growth and development (Chang et al., 2013; Fig. 3A). Additional studies have identified EIN3/EIL1 target genes involved in cross talk with abiotic signals. For example, ethylene plays an essential role in seedling deetiolation in coordination with light; EIN3/EIL1 activates expression of the PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A and B (PORA/B) genes, which encode rate-limiting enzymes in the chlorophyll biosynthesis pathway (Zhong et al., 2009; Fig. 3A). In freezing tolerance, which is negatively regulated by ethylene (e.g. Zhao et al., 2014), EIN3 binds to the promoters of cold-induced C-repeat Binding Factor/Dehydration-Responsive Element (CRE/DERE) genes to prevent their transcription (Shi et al., 2012; Fig. 3A). High salinity enhances proteasomal degradation of F-box proteins EBF1/2, resulting in the accumulation of EIN3/EIL1 proteins, which then activate expression of salt tolerance genes.
(Zhang et al., 2011; Peng et al., 2014), as well as peroxidase genes whose products scavenge reactive oxygen species to reduce the damage imposed by high salt (Peng et al., 2014).

Recent studies have also uncovered a mechanism of transcriptional coregulation that involves physical interactions between EIN3/EIL1 and other transcription factors (Fig. 3B). For example, in the coordinated regulation of apical hook formation in Arabidopsis seedlings by ethylene and GA3, the GA3-repressible DELLA transcriptional regulators interact with the DNA-binding domains of EIN3/EIL1 to attenuate transcription of HLS1 in the absence of GA3, and EIN3/EIL1 are derepressed in the presence of GA3 (An et al., 2012). Similarly, there is evidence that JA signaling leads to the removal of JAZ transcriptional repressors that physically interact with and repress EIN3/EIL1 activity in root development and necrotrophic pathogen defense (Zhu et al., 2011). On the other hand, a reciprocal inhibitory interaction between EIN3 and the JA-activated transcription factor MYC2 was found to underlie the antagonistic roles of ethylene and JA in the regulation of apical hook curvature and herbivory defense (Song et al., 2014). In contrast, EIN3/EIL1 play a stabilizing role, rather than an inhibitory role, in the ethylene-dependent response to iron deficiency in roots by interacting with FIT, a positive regulator of iron uptake (Lingam et al., 2011).

**CONCLUSION**

Critical advances in recent years have elucidated several key aspects of ethylene signal transduction, providing a more comprehensive view of the pathway, particularly with respect to mechanistic and dynamic properties. We now have greater insight into (1) the ethylene receptors, their roles, and their dynamic interactions with other signaling proteins; (2) the direct role of CTR1 in regulating EIN2; (3) the dynamics of EIN2 regulation and shuttling to the nucleus; (4) how diverse ethylene responses are achieved via an extensive EIN3-regulated transcriptional network; and (5) how cross talk signaling is achieved via EIN3/EIL1.

There are still a number of underlying mechanisms in the pathway that are poorly understood. Major questions surround EIN2, its biochemical activities, the relationship between the EIN2 N-terminal Nramp-like domain and ethylene signaling by the C-END, how EIN2 is cleaved, and the functions of EIN2 C-END in the nucleus. In addition, the signaling mechanisms leading to the turnover of F-box proteins EBF1/2, which are critical to controlling EIN3/EIL1 levels, are unknown. Mechanistic details need to be resolved in the early part of the pathway as well, such as the biochemical mechanisms of ethylene receptor signaling and the regulation of CTR1. Structural protein analyses will be essential in providing insight into the signaling dynamics of the ethylene receptor complex, which could also advance our understanding of how plants respond to ethylene with various sensitivities depending on the tissue and/or developmental stage. Together with mechanistic insights, additional signaling elements are likely to be discovered, such as the protease responsible for cleaving EIN2, the signaling pathway(s) that bypass CTR1, and as yet uncloned genes (e.g. ENHANCING CTR1-10 ETHYLENE RESPONSE2, reported by Xu et al., 2014).

A broader challenge that is gaining more attention is to understand how ethylene signaling results in so many diverse responses in different species in a variety of tissues and stages. This can be addressed by further studies to elucidate the transcriptional networks of ethylene signaling while also carrying out in vivo analyses of the numerous targets already uncovered (e.g. by EIN3 ChIP-seq). Continued technological improvements for transcriptomic and proteomic analyses will also help to elucidate ethylene signaling networks, and greater insights into these networks should facilitate the eventual modeling of specific ethylene responses, integrated with a diversity of signals, including environmental stresses and other plant hormones. The application of such knowledge has the potential to tremendously impact agriculture and to provide ways of addressing pressing global concerns, such as the changing climate and the increasing demand for food.

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