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REVIEW

Emerging Complexity of Ethylene Signal Transduction

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Abstract The plant hormone ethylene modulates growth and development and mediates diverse stresses and pathogens. Genetic studies with a laboratory reference plant, *Arabidopsis*, enabled researchers first to identify and place several key signaling components in a linear pathway for hormone signaling. Biochemical and cellular investigations have now led us to integrate functionally these genetically identified factors within a signaling context. Multi-step regulation of protein stability that accompanies phosphorylation/de-phosphorylation appears to be a central and underlying molecular mechanism. Here, we briefly summarize recent findings in such post-translational regulation of ethylene signaling factors. Based on this, we can postulate a new framework and formulate specific questions to unravel the emerging dynamics and complexity of ethylene signaling.

Keywords Ethylene · Intracellular signaling · Protein stability · EIN2 · ETP1 · ETP2 · EIN3 · EBF1 · EBF2 · Protein phosphorylation/de-phosphorylation · MKK9 · MPK3 · MPK6

Ethylene in Plant Biology

Ethylene (C_2H_4, Eth) is produced in most, if not all, living plant cells (Abeles et al. 1992). This hormone has important roles in various physiological processes, such as germination, growth, development (e.g., fruit ripening), senescence, and abscission, as well as in defense and resistance (Abeles et al. 1992; Wang et al. 2002). Because of its wide range of functions in the most important traits for agriculture, Eth physiology has been intensively investigated in many crop species and model plants (Schaller and Kieber 2002; Klee 2004).

The Eth signaling pathway has been studied mainly through a genetics approach. For the reference plant *Arabidopsis*, etiolated mutant seedlings have been screened for a hormone-insensitive or constitutive triple response that is manifested by an exaggerated apical hook, inhibited root growth, and enhanced hypocotyl radial growth in the presence of Eth (Alonso and Stepanova 2004). Corresponding genes responsible for these mutant phenotypes have been discovered, and the molecular identities of positive and negative regulators have been revealed for hormone signaling. Current biochemical studies are connecting these genetic factors into a hormone-signaling context through their cellular functions (Kendrick and Chang 2008; Yoo et al. 2009). Here, we will focus on summarizing recent biochemical findings and unraveling the dynamic and complex multi-step regulation of intracellular Eth signaling.

Genetic Pathway of Ethylene Signaling

When endogenous developmental signals, diverse environmental stresses, or pathogen infections promote Eth synthesis, plant cells perceive the hormone through multiple membrane receptors, including ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4) (Fig. 1; Bleecker et al. 1988; Chang et al. 1993; Hua et al. 1995, 1998; Hua and Meyerowitz 1998; Sakai et al. 1998). These receptors are localized to the ER and can bind Eth

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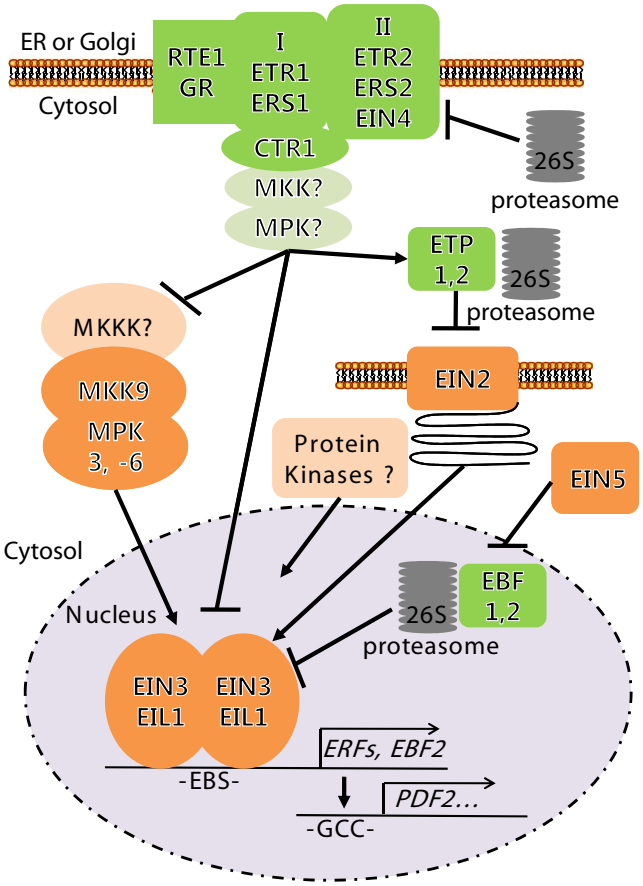

Fig. 1 Updated model for ethylene intracellular signaling. Ethylene receptor complexes comprising five partially redundant members act together but differentially to activate CTR1 PK activity at the ER in the absence of ethylene. *Arabidopsis* RTE1 is ETR1 receptor modulator localized to ER and Golgi. CTR1 is putative MAPKKK that might activate multiple MKKs and MPKs as cascades and phosphorylate EIN3 and EIL1 in the nucleus. This might enhance their affinity toward F-box proteins EBF1 and -2 to promote protein degradation through 26S proteasome and to suppress ethylene signaling. Upon ethylene binding to receptors, negatively acting receptor–CTR1 complexes are inactivated, resulting in activation of MKK9–MPK3/6 cascade, which phosphorylates EIN3 and EIL1 in the nucleus. MKK9–MPK3/6-dependent phosphorylation of EIN3 and EIL1 probably increases their stability by reducing their affinity toward EBF1 and -2. It is unclear whether MKK9–MPK3/6 is activated by a MAPKKK that differs from CTR1. EIN2 is membrane protein that accumulates upon ethylene signaling. Its stability is under control of two F-box proteins: ETP1 and -2. Intact EIN2 functioning is crucial for accumulation of EIN3. EIN5 indirectly affects *EBF1,2* transcript levels

(O'Malley et al. 2005; Wang et al. 2006). In the absence of Eth, ETR1 and other receptors play negative roles in Eth signaling, probably together with another genetically identified negative regulatory component, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), that encodes a putative Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK) (Kieber et al. 1993; Clark et al. 1998; Huang et al. 2003). An ER- and Golgi-localized membrane protein, REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1), modulates ETR1 receptor complexes (Resnick et al. 2006; Zhou et al. 2007; Dong et al. 2008). In the presence of Eth, the negative function of receptor–CTR1 complexes is released (Hua and Meyerowitz 1998; Wang et al. 2006). For instance, Eth can cause protein degradation of ETR2 and promote hormone responses in *Arabidopsis* (Chen et al. 2007). Downstream of those complexes, intact functioning of the membrane-integrated transporter-like EIN2 appears to be necessary for Eth signaling (Alonso et al. 1999; Guo and Ecker 2003). EIN2 accumulates in response to Eth through the regulation of two E3 ligases, EIN2 TARGETING F-box PROTEIN1 (ETP1) and ETP2 (Qiao et al. 2009).

EIN3, a key transcription factor in Eth signaling, also accumulates in the presence of Eth via the control of two E3 ligases, EIN3 BINDING F-BOX PROTEIN1 (EBF1) and EBF2 (Chao et al. 1997; Guo and Ecker 2003; Potuschak et al. 2003; Yanagisawa et al. 2003; Gagne et al. 2004). EIN3 and EIN3-like 1 (EIL1) are two main transcription activators for the expression of Eth-responsive genes, such as ETHYLENE RESPONSE FACTOR1 (ERF1), that contain EIN3-binding sites (Solano et al. 1998; Alonso et al. 2003). ERF1 is also a transcription activator and is involved in secondary responsive gene expression through a GCC element. Cascades of Eth-responsive transcription produce cellular effectors that modulate plant growth and development. To attenuate the perceived signaling as feedback regulation, expression of *ERS1* and *ETR2* is induced as a primary response to Eth. These receptors that are not bound by Eth suppress hormone signaling and diminish that signaling effect.

EBF2 is also highly induced transcriptionally upon such signaling, which may contribute to a negative feedback mechanism by enhancing the degradation of EIN3 (Guo and Ecker 2003; Potuschak et al. 2003; Konishi and Yanagisawa 2008). More complicatedly, the accumulation of *EBF1* and *EBF2* transcripts are under the control of a 5′ to 3′ exoribonuclease, EIN5 (Olmedo et al. 2006; Gregory et al. 2008). These newly identified signaling factors and their functions demonstrate that the Eth signaling pathway is constantly checked and finely tuned in plants. Conceptually, multi-step regulation of protein stability by hormone signaling factors, via proteasome activities, appears to have a central mechanistic role that underlies signal transduction.

Regulation of Protein Stability in Ethylene Signaling

The nuclear response in Eth signaling is mainly controlled by two transcription activators, EIN3 and EIL1 (Fig. 1; Chao et al. 1997; Alonso et al. 2003). Protein levels of those two factors, but not their transcript levels, are tightly regulated by hormone signaling (Yanagisawa et al. 2003).

EIN3 and EIL1 proteins accumulate to a certain amount as early as 1 h after treatment with Eth or ACC, and are closely related to Eth-responsive transcriptome changes (Guo and Ecker 2003; Potuschak et al. 2003; Yanagisawa et al. 2003; Gagne et al. 2004; Binder et al. 2007). EIN3 and EIL1 are constantly degraded through 26S proteasome activities in the absence of Eth, but stabilized in its presence, at least under darkness. Light seems to be another factor that can stabilize EIN3/EIL1 (Lee et al. 2006). Thus, proteasome inhibitors mimic the hormone effect and stabilize these proteins in the absence of Eth. Three groups of Eth researchers have independently identified two F-box ubiquitin E3 ligases, EBF1 and -2, that bind EIN3 in yeasts (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004). In the *ebf1 ebf2* double knockout mutant, EIN3 accumulates and causes a constitutive Eth phenotype even in the absence of Eth. Because EBF1 and -2 interact with EIN3 in yeasts in the absence of Eth, the E3 ligases might recognize the unmodified form of EIN3. Those results might imply that protein modification of EIN3 is a biochemical mechanism for the protein to be stabilized in response to Eth.

The stability of EIN3 largely depends on EIN2 (Guo and Ecker 2003). In *ein2*, EIN3 protein is hardly detected; this lack is highly correlated with the strongest Eth-insensitive phenotype of the mutant. EIN2 is another fast turnover protein, for which stability is under the control of proteasome activities, and is accumulated upon Eth signaling (Fig. 1; Qiao et al. 2009). Two ubiquitin E3 ligases, ETP1 and 2, regulate the degradation process of EIN2. In *etp1 etp2* double knockout mutants, the accumulation of EIN2 protein can cause a constitutive Eth response in the absence of Eth. Consistently, transgenic plants that overexpress *ETP1* or *ETP2* show Eth insensitivity. Nevertheless, it is also notable that *etp1 etp2* is not saturated for the Eth response and still reacts to ACC. Likewise, *ETP1* or *ETP2* transgenic lines show a relatively weak Eth-insensitive phenotype. These results demonstrate that Eth signaling confers both EIN2-dependent and -independent pathways.

In addition to the regulation of positively acting EIN3, EIL1, and EIN2 in hormone signaling, the level of a negatively acting ETR2 protein is also controlled via proteasome activities upon Eth signaling (Chen et al. 2007). Similarly, the tomato ortholog NEVER RIPE (NR) is degraded by the developmental signal in ripening fruits in response to Eth (Zhong et al. 2008). The decline in negatively acting receptors enhances tissue sensitivity toward Eth signaling. So far, ETR2 is the only *Arabidopsis* Eth receptor that has ligand-induced protein degradation, but none of the E3 ligases responsible for this process has been identified.

Taken together, it is obvious that multi-step regulation of protein stability for Eth signaling factors has an important role in such signaling. This type of cellular mechanism is well established in many eukaryotic signaling pathways. For NF-kB signaling, multi-layer regulation of protein stability is a central mechanism that underlies innate immunity from fly to human (Wullaerdt et al. 2006; Bhoj and Chen 2009). It will be interesting to examine whether other protein stability regulators might be functionally involved in Eth signaling. We must also elucidate how those regulator activities are modulated within the context of hormone signaling.

Protein Phosphorylation and De-phosphorylation in Ethylene Signaling

Unlike the abundance of biochemical and genetic analyses for membrane receptors and nuclear transcription factors in Eth signaling (reviewed by Yoo et al. 2009), studies of the intracellular pathways that connect the functions of membrane receptors to those of nuclear factors are rare in the field of hormone signal transduction. Biochemical characterization of Eth intracellular signaling has shown that the hormone activates rapid and transient protein phosphorylation, which is required for the induction of Eth-responsive gene expression in tobacco, mung bean, and pea (Raz and Fluhr 1993; Kim et al. 1997; Kwak and Lee 1997). Eth-responsive transcription in tobacco becomes robust when tissues are pre-treated with protein phosphatase1 (PP1) and PP2A inhibitor okadaic acid (Raz and Fluhr 1993). Within the same line of evidence, *enhanced ethylene response1* (*eer1*), which encodes one of three PP2A-A subunits, *ROOTS CURL IN NPA1* (*RCN1*), has been isolated as a second enhancer of *ctr1* (Larsen and Chang 2001; Larsen and Cancel 2003). Because RCN1 is a positive regulator of PP2A activity (Deruère et al. 1999), that fact might implicate the functional involvement of protein phosphorylation/de-phosphorylation in the hormone signaling process. More recently, a systematic survey of Eth-induced phosphorylation/de-phosphorylation of *Arabidopsis* proteins has been conducted using a phosphor-proteomics method (Li et al. 2009). In *ein2*, 224 phosphopeptides have been recovered as candidates for Eth-responsive protein phosphorylation, and PRD/Gx and PDYxx have been deduced as two highly conserved phosphorylation target motifs. These results indicate that Eth might induce protein phosphorylation independent of or in parallel with EIN2 during Eth signaling.

As protein kinases responsible for phosphorylation upon Eth signaling, MAPK function has been unequivocally demonstrated in *Arabidopsis* (Novikova et al. 2000; Ouaked et al. 2003; Liu and Zhang 2004; Yoo et al. 2008; Xu et al. 2008). Initially, the activation of protein kinase phosphorylating myelin basic protein (MBP) was reported for the detached leaves from 6-week-old *Arabidopsis* that

responded to 1 ppm Eth within 1 h. The protein kinase was then probed with mammalian MAPK antibodies, indicating that Eth-induced protein kinase activities were most likely those of MAPK in *Arabidopsis* (Novikova et al. 2000). Their activities were elevated in the constitutive Eth-responsive mutant *ctr1-1*, but reduced in the insensitive mutant *etr1-1*. Because CTR1, an Eth-signaling negative regulator, encodes a MAPKKK, the elevated level of MPAK activities in *ctr1-1* might imply the existence of a distinct MPAK cascade that serves as a positive regulator of Eth signaling. Using the immediate Eth precursor ACC as signal, Ouaked et al. (2003) also have identified Eth-inducible MPK6 and other unknown MPKs through their evaluation of *Arabidopsis* culture cells and a sensitive immunocomplex MAPK assay with an MPK6-specific antibody. In *ctr1-1*, MPK6 activity is elevated, implying that the constitutive Eth responses in mutants might be mediated by such activity. ACC-dependent MPK6 activation is blocked by the receptor mutant *etr1-1*, but not by *ein2-1* and *ein3-1* mutants, indicating that MPAK acts between the receptor and EIN2 in the Eth signaling pathway.

To determine the specific MAPK cascades involved in Eth, our research group has developed a more sensitive and specific assay that uses leaf mesophyll protoplasts to accommodate diverse biochemical, molecular, cellular, and genetic analyses that explore the genomics and proteomics information available for *Arabidopsis* (Yoo et al. 2008). A cell-based MAPK activity screen and an Eth-specific reporter assay that is facilitated by constitutively Eth-responsive *ctr1-1* cells have identified two antagonistic MAPK cascades in Eth signaling, MKK9–MPK3/6, which comprises positively acting MAPK cascades, and CTR1, which initiates negatively acting cascades (Fig. 1; Yoo et al. 2008). Likewise, loss-of-function *mkk9* shows a broad spectrum of Eth insensitivity, including a typical triple response: inhibition of Eth-dependent growth, promotion of senescence, and hypersensitivity to glucose and salt stresses. Epistatic analysis using a transgenics approach has indicated that MKK9 modules act downstream of the receptor complexes, but upstream or independently of EIN2. MKK9 localization in the nucleus upon signaling, as well as MAPK cascade-dependent EIN3 regulation via biochemical and site-directed mutagenesis analyses, has demonstrated that the activities of two antagonistic MAPK cascades are integrated into EIN3 through alternative phosphorylation and that they modulate protein stability and downstream transcription cascades.

Significantly, our study has yielded a new paradigm for linking complex MAPK cascades to control quantitative hormonal responses. Because several hormone, stress, and defense signals can activate MKK9, MPK3, and MPK6 in plants, it has long been questioned how converged MAPK signaling can secure their specificity. We have shown that Eth signaling particularly activates those MKK9–MPK3/6 modules and phosphorylates T174 of the EIN3 protein to stabilize it. However, that signaling suppresses the CTR1-dependent cascades that phosphorylate T592 of EIN3 to enhance its degradation. Our results indicate that only when both MAPK modules are cooperatively and simultaneously regulated can Eth signaling be appropriately executed in plants. This explains the broad but relatively weak Eth insensitivity of *mkk9*, which lacks only one part of the two MAPK cascades. Likewise, *mkk9 ctr1* double mutants display partial but clear Eth insensitivity in the presence of light but in the absence of nutrients. Moreover, *ctr1* shows a rather stronger constitutive Eth signaling phenotype, which most likely is contributed by the activation or de-repression of the MKK9–MPK3/6 cascade in addition to the loss of CTR1-dependent MAPK cascade activity.

Endogenous signals such as Eth probably do not provoke drastic MAPK activation, as unlike exogenously derived pathogenic elicitors or chemicals and thus it might be challenging to monitor such a low level of MAPK activation. This subtle and transient activation by Eth could be due to dynamic control through both positive and negative regulators as well as by multiple feedback modulators (Fig. 1). Because the specific amplitude and duration of MAPK activities are involved in determining the signaling specificity of MAPK cascade signaling

Table 1 Ethylene-specific signaling components in *Arabidopsis* and maize

Arabidopsis	Maize
Class I ethylene receptors	
ETR1 At1g66340	ZmERS1-14 (AY359577)
ERS1 At2g40940	ZmERS1-25 (AY359578)
Class II ethylene receptors	
EIN4 At3g04580	ZmETR2-9 (AY359580)
ERS2 At1g04310	ZmETR2-40 (AY359581)
ETR2 At3G23150	
Intracellular components	
CTR1 At5g03730	ZmCTR1 (T233377,TC203507)
EIN2 At5g03280	ZmEIN2 (AY359584)
ETP1 At3g18980	
ETP2 At3g18910	
Nuclear effectors	
EIN3 At3g20770	ZmEIL1-1 (AX077258)
EIL1 At2g27050	ZmEIL1-3 (AX077260)
EBF1 At2g25490	ZmEBF1 (AAN23093)
EBF2 At5g25350	
Primary response factors	
ERF1 At3g23240	ZmERF1 (AAT75013.1)

(Schwartz and Madhani 2004), it will be critical to evaluate or modulate MAPK activities quantitatively to clarify MAPK function in specific signaling.

Prospects

Although many key signaling components in the Eth signal transduction pathways have been described over the past two decades, our understanding of the signaling mechanisms is still limited by poor biochemical connections among signaling factors. MKK9–MPK3/6 have now been identified as positive MAPK cascades, but the entire group of cascades is not yet known because its MAPKKK member(s) are still missing (Fig. 1). As for negative MAPK cascades, a genetically identified negative regulator, CTR1, has a MAPKKK role in which it most likely suppresses the positively acting MKK9 modules (Fig. 1). However, the molecular identities of MKKs and MPKs in CTR1 cascades are yet to be determined. In our preliminary studies, two MKKs have demonstrated suppression of EIN3 accumulation and Eth-specific reporter activity (Yoo, unpublished data). Cell-based research has implicated these MKKs might act downstream of CTR1.

The protein function of EIN2, which is genetically positioned between CTR1 and EIN3, remains to be addressed (Fig. 1). We only know that the level of EIN2 protein is under the control of an Eth-dependent protein degradation process (Qiao et al. 2009). This protein shows high similarity to members of the NRAMP family of metal transporters, but transport activity has never been demonstrated for EIN2 function in Eth signaling.

Eth-dependent EIN3 stabilization in the nucleus has been established (Fig. 1; Guo and Ecker 2003; Potuschak et al. 2003; Yanagisawa et al. 2003; Gagne et al. 2004), but it is still unknown how hormone signaling can modulate that stability. It is appealing that MAPK-dependent phosphorylation of EIN3 may change its affinity toward cellular proteasome processes.

The identification and functional analyses of Eth signaling components in other crop plants have revealed a high degree of conservation among many species. For example, genes encoding almost every player in the Eth signal transduction pathway in the dicot *Arabidopsis* have now been found in the expressed gene databases of the monocot maize, although *ZmCTR1* and *ZmEBF1* show high divergence in their sequences (Table 1). In those plants, Eth signaling components also have similar biochemical functions (Gallie and Young 2004). Therefore, with this high level of conservation in components and regulatory mechanisms, our understanding of the signaling pathway for Eth via the reference plant *Arabidopsis* might provide an ideal blueprint for engineering crop genomes with a custom-designed Eth signaling regime. This will then maximize plant adaptability and productivity under unfavorable or unexpected environmental fluctuations.

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