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Paradigms and Paradox in the Ethylene Signaling Pathway and Interaction Network

Qiong Zhao and **Hong-Wei Guo**[1]

State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, China

ABSTRACT Phytohormone ethylene plays pivotal roles in plant response to developmental and environmental signals. During the past few years, the emerging evidence has led us to a new understanding of the signaling mechanisms and regulatory networks of the ethylene action. In this review, we focus on the major advances made in the past three years, particularly the findings leading to new paradigms and the observations under debate. With the recent demonstration of the regulation of the protein stability of numerous key signaling components including EIN3, EIL1, EIN2, ETR2, EBF1/EBF2, and ETP1/ETP2, we highlight proteasome-dependent protein degradation as an essential regulatory mechanism that is widely adopted in the ethylene signaling pathway. We also discuss the implication of the negative feedback mechanism in the ethylene signaling pathway in light of ethylene-induced *ETR2* and *EBF2* gene expression. Meanwhile, we summarize the controversy on the involvement of MKK9–MPK3/6 cascade in the ethylene signaling versus biosynthesis pathway, and discuss the possible role of this MAPK module in the ethylene action. Finally, we describe the complex interactions between ethylene and other signaling pathways including auxin, light, and plant innate immunity, and propose that EIN3/EIL1 act as a convergence point in the ethylene-initiated signaling network.

Key words: Ethylene signaling; MAPK; protein turnover; negative feedback; ETP1/2; interplay.

INTRODUCTION

Ethylene, as a simple gaseous phytohormone, plays numerous roles in plant developmental processes and plant reaction to environmental cues. Extensive studies have demonstrated the function of ethylene in the regulation of seed germination, seedling morphology, fruit ripening, fiber elongation, leaf senescence, biotic defense, and abiotic tolerance (Abeles et al., 1992). Based on considerable genetic and biochemical studies in *Arabidopsis thaliana*, a largely linear ethylene signaling transduction pathway from ethylene perception at the membrane to transcriptional regulation in the nucleus has been established (Guo and Ecker, 2004). In plants, ethylene is perceived by a group of membrane-located receptor proteins including ETR1 (ETHYLENE RESPONSE 1), ERS1 (ETHYLENE RESPONSESENSOR 1), ETR2 (ETHYLENE RESPONSE 2), ERS2 (ETHYLENE RESPONSE SENSOR 2), and EIN4 (ETHYLENE INSENSITIVE 4) (Bleecker et al., 1988; Hua and Meyerowitz, 1998). In normal conditions, where the level of ethylene is usually low, the receptors act to suppress ethylene response by activating a downstream negative regulator CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) through direct physical interaction (Clark et al., 1998), and this suppression is relieved upon ethylene binding to the *trans*-membrane domain of the receptors (Wang et al., 2006). CTR1 is a Raf-like MAPKKK (mitogen-activated protein kinase kinase kinase) family protein, acting downstream of receptors and upstream of EIN2 (ETHYLENE INSENSITIVE 2) (Kieber et al., 1993). The membrane-integrated protein EIN2 is an essential transducer of ethylene signal, as its loss-of-function mutant displays little response to exogenous ethylene (Alonso et al., 1999). Recent study found that EIN2 is stabilized at the protein level by ethylene from degradation by two F-box proteins ETP1/2 (EIN2 targeting protein ½) (Qiao et al., 2009). Fluorescence studies showed that EIN2 is localized at the ER (Endoplasmic Reticulum) membrane (Bisson et al., 2009). It was recently reported that EIN2 interacts with the kinase domain of all members of the ethylene receptor family in *Arabidopsis*, and ethylene modulates the EIN2-receptor interaction (Bisson and Groth, 2010). Downstream of EIN2, two plant-specific transcription factors, EIN3 (ETHYLENE INSENSITIVE 3) and EIL1 (EIN3-like 1), are both necessary and sufficient for the activation of ethylene-regulated gene expression and morphological responses (Chao et al., 1997;

[1] To whom correspondence should be addressed. E-mail hongweig@pku.edu.cn, tel. +86 10 6276 7823, fax +86 10 6275 6174.

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Solano et al., 1998). EIN3 is tightly regulated at protein level by SCF complexes containing F-box protein EBF1/2 (EIN3-BINDING F-BOX PROTEIN ½) through a 26S proteasome-mediated protein degradation pathway (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). An RNA decay mechanism seems to modulate ethylene signaling, as the mRNA levels of *EBF1*/*EBF2* were recently found to be negatively regulated by EIN5 (ETHYLENE INSENSITIVE 5), a 5′→3′ exoribonuclease (Olmedo et al., 2006; Potuschak et al., 2006). Recent advances in *Arabidopsis* ethylene research have furthered our understanding on the mode of ethylene action from a largely linear signaling pathway to a more complex regulatory network including feedback regulations, multiple levels of protein stability control, and broad existence of signaling interplay and integration (Figure 1). As several of them have been reviewed and discussed in detail (Kendrick and Chang, 2008; Stepanova and Alonso, 2009; Yoo et al., 2009), and are thus not covered in this review, we will mainly focus on the following four aspects with regard to the emerging mechanisms as well as existing controversy of ethylene signaling: (1) control of protein stability of key signaling components; (2) negative feedback regulation at transcription level of the receptor and EBFs; (3) the controversy of MKK9–MPK3/6 in ethylene signaling versus biosynthesis pathway; (4) interplay between ethylene and light, auxin, and plant innate immunity.

REGULATION OF PROTEIN DEGRADATION IN ETHYLENE SIGNALING

The mechanism of ubiquitin-mediated protein degradation enables plants to respond to developmental and environmental signals quickly and precisely. Studies have shown that the protein turnover plays an important role in ethylene biosynthesis, perception, and response (Guo and Ecker, 2003; Potuschak et al., 2003; Wang et al., 2004; Chen et al., 2007). It has been reported that the production of ethylene is controlled through the negative regulation of ACS5 (ACC synthase 5) protein level by a BTB-domain containing E3 ligase ETO1 (ETHYLENE OVERPRODUCER 1) (Wang et al., 2004). Ethylene was found to induce degradation of ETR2 through the 26S proteasome pathway (Chen et al., 2007), suggesting a desensitization mechanism of ethylene receptor ETR2 via protein turnover. EIN3 transcription factor is also subjected to tight regulation at protein level through EBF1/2-mediated 26S proteasome pathway (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Besides the control of the above proteins, recent studies have demonstrated that EIN3-like 1 (EIL1) is also targeted by F-box proteins EBF1/2 for degradation (An et al., 2010), while EIN2 is destructed by two other F-box proteins ETP1/2 (Qiao et al., 2009). Interestingly, these studies also revealed that both EBF1 and EBF2 proteins are subjected to proteasomal degradation, although the detailed mechanism of the F-box protein turnover is unknown (Qiao et al., 2009; An et al., 2010).

EIN2, an indispensable integral-membrane transducer of ethylene signaling, has recently been found to be a short half-life protein (Qiao et al., 2009). EIN2 protein accumulates upon the treatment of ethylene, and this effect is dependent on the 26S proteasome pathway. Consistent with the genetic position and the positive function of EIN2 in the ethylene response pathway, a higher level of EIN2 protein is detected in the *ctr1* mutants whereas a lower level of EIN2 is observed in the *etr1* plants, but remains normal in *ein3eil1*. These results suggest that the regulation of EIN2 protein turnover might be an activation mechanism of ethylene signal from the receptors/CTR1 to EIN2. Two F-box proteins ETP1 and ETP2 (EIN2-targeting protein 1 and 2) are subsequently identified to form the SCF-type E3 ligases targeting EIN2 for degradation. In yeast cells, either ETP1 or ETP2 physically interacts with the C-terminus of EIN2, and various fragmentation assays show that the highly conserved domain of EIN2, namely the last ~250 amino acids, are both necessary and sufficient for this interaction, suggesting regulation of protein degradation might be evolutionarily conserved. In *Arabidopsis*, knockdown of ETP1/2 by artificial miRNA strategy results in hypersensitivity to ethylene with elevated EIN2 accumulation, while overexpression of either ETP1 or ETP2 leads to insensitivity to ethylene with reduced protein level of EIN2. Although transcription level of neither ETP1 nor ETP2 is altered in response to ethylene, the protein level of both ETP1 and ETP2 are downregulated by ethylene. Therefore, ethylene-induced ETP1/2 turnover seems to be a primary regulatory event that contributes to the stabilization of EIN2, although additional evidence is needed to reinforce this scenario.

EIL1 is a homolog of EIN3 and has been demonstrated to function redundantly with EIN3 in regulating ethylene responses (Chao et al., 1997; Alonso et al., 2003; Binder et al., 2007). Recent study revealed that, like EIN3, EIL1 is also a short half-life protein and accumulates in the nucleus upon ethylene treatment (An et al., 2010). An intact ethylene signaling pathway is required for its stabilization, as EIL1 is barely detected in the *ein2* mutant background (An et al., 2010). Previous biochemical study indicated that F-box proteins EBF1 and EBF2 are able to interact with EIL1, suggesting that F-box proteins EBF1/2 also target EIL1 for degradation (Potuschak et al., 2003; Binder et al., 2007). More definite proof of this regulation came from the observations including the over-accumulation of EIL1 protein in the *ebf1ebf2ein3* triple mutants, and the complete rescue of various defects in *ebf1ebf2ein3* by *eil1* mutation (An et al., 2010).

The next pressing question is how EIN3/EIL1 proteins are stabilized by ethylene. With the identification of $SCF^{EBF1/2}$ as the major E3 ligases to control EIN3/EIL1 stability, this question can be asked as to how ethylene acts to repress EBF1/2-mediated protein degradation. Recently, An et al. (2010) found that EBF1/2 are essential components required for the ethylene signal to stabilize EIN3/EIL1, as a functional EIL1 or EIN3 protein in the *ebf1ebf2* mutant background is unable to further respond to exogenous ethylene in terms of protein accumulation and the induction of downstream gene expression. These results conceivably suggest that EBF1/2 are

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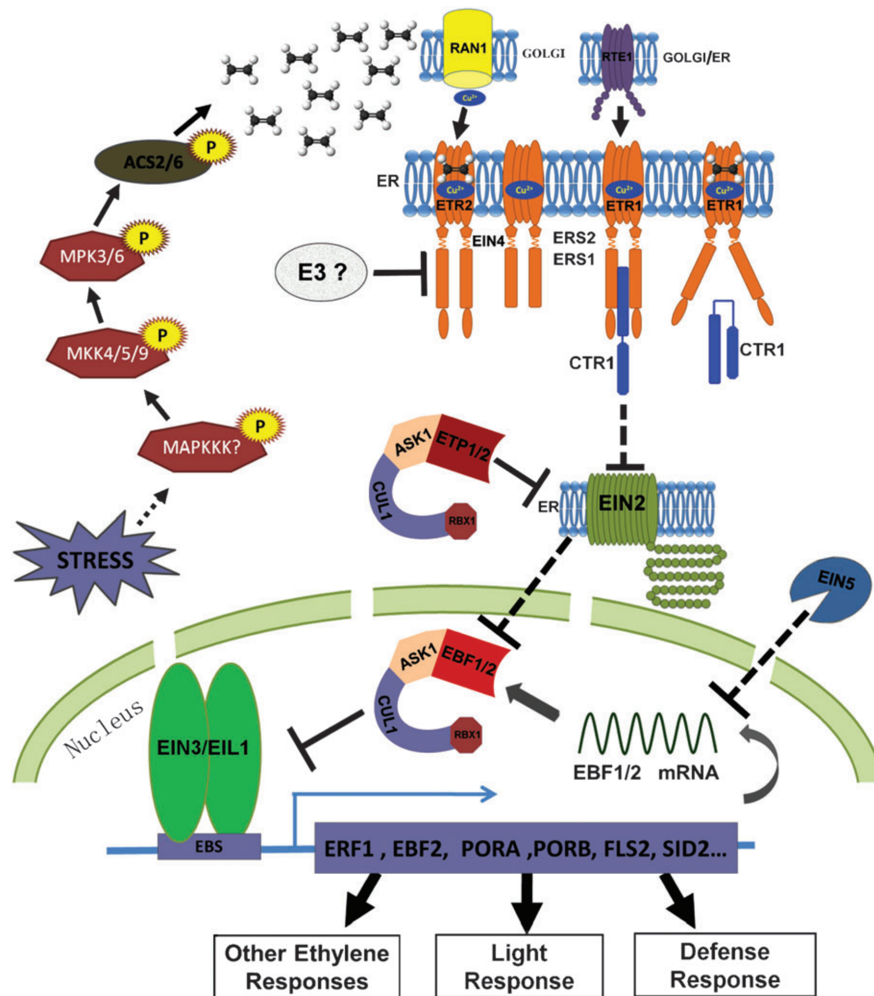

Figure 1. A Schematic Model for Ethylene Signal Transduction and the MAPK Pathway in Ethylene Biosynthesis.

Ethylene gas is perceived by the ER-integrated receptor proteins including ETR1, ETR2, ERS1, ERS2, and EIN4 (Bleecker et al., 1988; Hua and Meyerowitz, 1998; Sakai et al., 1998; Voet-van-Vormizeele and Groth, 2008). A Golgi-localized protein RAN1 (RESPONSIVE-TO-ANTAGONIST 1) is a P-type ATPase copper transporter that delivers the copper ion to the receptors to facilitate ethylene binding (Woeste and Kieber, 2000). RTE1 (REVERSION-TO-ETHYLENE SENSITIVITY 1), another membrane-located protein, promotes the transition of ETR1 from active to inactive state likely through modulating the action of ETR1 N-terminus (Dong et al., 2008; Resnick et al., 2008). In normal growth conditions in which the ethylene level is low, the unoccupied receptors remain in the active state and associate with CTR1, which, in turn, represses the downstream signaling pathway. When plants encounter stress conditions, the MAPK cascade composed of MKK4/5/9 and MPK3/6 can be activated, which then phosphorylates ACS2/6. The phosphorylated ACS2/6 become stabilized and consequently enhance the production of ethylene (Liu and Zhang, 2004). Upon binding by ethylene, the receptor complexes disassociate, and CTR1 released from ER membrane is somehow inactivated (Kieber et al., 1993; Clark et al., 1998; Huang et al., 2003). Therefore, the downstream ethylene signaling pathway including EIN2 is de-repressed (Alonso et al., 1999; Bisson et al., 2009). EIN2 is a short half-life protein targeted by $SCF^{ETP1/2}$ for degradation. Ethylene promotes the accumulation of EIN2 probably by down-regulating the level of ETP1/2 protein through an unknown mechanism (Qiao et al., 2009). In the nucleus, two transcription factors (EIN3 and EIL1) are both necessary and sufficient for the activation of ethylene-regulated gene expression and diverse responses (Chao et al., 1997; Solano et al., 1998; Alonso et al., 2003). EIN3 and EIL1 are also short-lived proteins that are targeted by $SCF^{EBF1/2}$ for degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). The ethylene signal is transmitted via the action of EIN2 to stabilize EIN3/EIL1, probably by promoting the proteasomal degradation of EBF1/2 proteins (An et al., 2010). EBF2 is a direct target gene of EIN3, which activates *EBF2* transcription to form a negative feedback loop (Konishi and Yanagisawa, 2008). *EBF1/2* mRNAs are subjected to negative regulation mediated by a $5' \rightarrow 3'$ exoribonuclease EIN5 (XRN4) (Olmedo et al., 2006; Potuschak et al., 2006). EIN3/EIL1 also directly regulate the expression of a diverse array of genes including *ERF1* (*ETHYLENE RESPONSE FACTOR 1*), *PORA*, *PORB*, *FLS2*, and *SID2*, which initiate various interplays between ethylene and other signals, such as light and innate immunity (Chen et al., 2009; Zhong et al., 2009; Boutrot et al., 2010).

The symbol '?' represents an unknown factor or element. Arrows and T-bars represent positive and negative effects, respectively. Solid lines indicate effects that occur through direct interaction, whereas dotted lines indicate effects that have yet to be shown via direct interaction.

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indispensable signaling transducers for ethylene responsiveness, and their function might be subject to direct control by ethylene (An et al., 2010). In support of this notion, An et al. (2010) found that ethylene was able to down-regulate the protein levels of both EBF1 and EBF2, and blocking ethylene perception by silver ion or *ein2* mutation led to the stabilization of these two F-box proteins.

EIN2 is required for ethylene-induced EIN3/EIL1 stabilization, as no EIN3 or EIL1 accumulation can be detected in the *ein2* mutant (Guo and Ecker, 2003; An et al., 2010). When protein translation is blocked, the turnover rate of EIN3 protein is higher in *ein2* than that in wild-type, suggesting that EIN2 acts to repress the proteasomal degradation of EIN3/EIL1 (An et al., 2010). Together with the finding that EIN2 promotes the proteasomal degradation of EBF1/2 (An et al., 2010), it is likely that one of the EIN2 actions is to modulate the SCF activity. In support of this possibility, the C-terminus of EIN2 has been reported to interact with a putative COP9 signalosome (CSN) component EER5 (Ethylene Enhanced Response 5) (Christians et al., 2008), implying a role of EIN2 in the regulation of SCF activity through the modulation of the CSN function. Further experiments are needed to test this scenario.

THE NEGATIVE FEEDBACK REGULATION AS BRAKES OF ETHYLENE SIGNALING

To fine-tune ethylene response, in spite of multi-step regulations of protein stability mentioned above, feedback regulation is another cellular mechanism adopted by plants to regulate ethylene signaling properly. Binding of ethylene to the receptors inactivates the receptor, and ethylene-induced degradation of ETR2 provides a possible desensitizing mechanism (Chen et al., 2007; Kendrick and Chang, 2008). Meanwhile, the levels of *ERS1* and *ETR2* are transcriptionally induced by ethylene (Hua et al., 1995; Sakai et al., 1998). It is possible that the newly synthesized receptors unoccupied by ethylene are active to inhibit downstream ethylene signaling pathway, and shut off ethylene response quickly. This negative feedback by inducing the synthesis of new receptors helps plants to attenuate ethylene signaling output and restore the ability to respond to subsequent ethylene signal.

Another level of negative feedback regulation occurs at the transcriptional event. Ethylene promotes the accumulation of EIN3/EIL1, which are both required and sufficient for the activation of downstream gene expression and ethylene responses. However, constantly high levels of EIN3 and EIL1 are also deleterious to plant normal growth and development, as evidenced by the severe dwarfism and reduced fertility in the *ctr1* mutant, and, in an extreme case, the growth arrest and seedling lethality in *ebf1ebf2* mutants, both of which constitutively over-accumulate EIN3/EIL1 proteins (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; An et al., 2010). Previous report showed that, upon ethylene treatment, the level of EIN3 protein increases within the first 4 h and decreases thereafter (Guo and Ecker, 2003), implying a signal-damping mechanism in plants to trail off ethylene effects. Such desensitization is likely achieved by the ethylene induction of *EBF2*, which requires EIN3/EIL1 (Guo and Ecker, 2003; Potuschak et al., 2003). Compared with *ebf1*, *ebf2* displays a more hypersensitive phenotype in response to ethylene. One reason for such a difference is that *EBF2* gene expression is dramatically induced by ethylene while *EBF1* is modestly induced (Guo and Ecker, 2003; Potuschak et al., 2003). As such, EBF2 is thought to play a more predominant role in a condition in which the ethylene level is progressively high, which provides an explanation for why *ebf2* shows stronger ethylene hypersensitive phenotype.

Recent work demonstrates a direct negative feedback regulation between EBF2 and EIN3 (Konishi and Yanagisawa, 2008). The *EBF2* mRNA level is elevated in plants overexpressing EIN3, while it is reduced in the *ein3eil1* mutant, indicating that *EBF2* is a target gene of EIN3. Analysis with transgenic plants harboring GUS reporter gene under the control of *EBF2* promoter confirmed that *EBF2* is transcriptionally induced by ethylene. EMSA (Electrophoretic Mobility Shift Assay) analysis with different fragments of *EBF2* promoter as probes identified that EIN3 directly binds to a specific motif 5'-TACAT-3' within the *EBF2* promoter. Furthermore, transformation with *EBF2* coding sequence under the control of mutated *EBF2* promoter in the EIN3 binding motif failed to rescue the ethylene hypersensitive phenotype of *ebf2* mutant, suggesting that EIN3-mediated transcription of *EBF2* is crucial for the control of plant sensitivity to ethylene (Konishi and Yanagisawa, 2008). Therefore, it is likely that ethylene signal leads to quick removal of EBF1/EBF2, permitting the accumulation of EIN3/EIL1 to activate downstream gene expression and response pathways. Meanwhile, EIN3-induced *EBF2* transcription gradually restores the levels of *EBF2*, which, in turn, promotes the turnover of EIN3/EIL1, and dampens ethylene signaling strength. Such a negative feedback loop between EIN3 and EBF2 enables plants to establish a homeostasis in ethylene signaling output, and consequently allows EIN3/EIL1 accumulation to be just adequate for proper growth and defense response but not go uncontrolled to a destructive level.

THE CONTROVERSY OF THE MAPK PATHWAY IN ETHYLENE ACTION

Ever since the isolation and cloning of CTR1, a Raf-like MAPKKK, as an important negative regulator in the ethylene signaling pathway (Kieber et al., 1993), a MAPK (mitogen-activated protein kinase) cascade in ethylene signaling had been proposed and sought after for a long time. However, as forward genetic approaches by extensive screens for ethylene response mutants failed to isolate any kinase-related mutants, alternative efforts to tease out the hidden MAPK module had been made, including biochemical methods and reverse-genetic approaches.

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The first study to indicate the existence of a MAPK module in ethylene signaling came from the observation that ethylene had a positive effect on MAPK-like activity (Novikova et al., 2000). Using MBP (myelin basic protein) as an artificial MAPK substrate, protein extract from ethylene-treated wild-type *Arabidopsis* leaves showed an enhanced phosphorylation activity. Consistently, the putative MAPK activity was higher in *ctr1* and lower in *etr1* (*etr1-1* gain-of-function mutant) when compared with wild-type. Further protein immune-precipitation assay with anti-ERK1 antibody identified a putative MAPK with a molecular weight of 47 kDa.

Three years later, Ouaked et al. (2003) reported that a MAPK module composed of MPK6 (*Arabidopsis* MAPK6) and the related MPK13 (*Arabidopsis* MAPK13) is involved in ethylene signaling. Using different Medicago MAPKs antibodies, immune-precipitated proteins from ACC (1-aminocyclopropane-1-carboxylic acid, the ethylene biosynthetic precursor)-treated cell extract were tested for kinase activity. They identified two ethylene-activated MAPKs that were supposed to be the Medicago SIMK (SALT-STRESS-INDUCIBLE MAPK) and MMK3 (Medicago MAPK3). Subsequently, *Arabidopsis* MPK6, the closest homolog of Medicago SIMK, was found to be activated by ethylene *in vitro*. Consistently, they detected constitutive activation of MPK6 in *ctr1*, and the ethylene-induced activation of MPK6 was independent of EIN2 or EIN3. The transgenic *Arabidopsis* plants overexpressing an active form of SIMKK (SIMK KINASE), which was able to activate Medicago SIMK, showed a *ctr1*-like phenotype in etiolated seedlings. Therefore, unlike other well-studied Raf-like MAPKKKs in mammalian cells, CTR1 had been proposed to repress the activity of MAPKK/MPK6 somehow.

Recently, Yoo et al. (2008) reported that the MKK9–MPK3/MPK6 module functioned downstream of CTR1 and directly phosphorylated EIN3 in ethylene signaling. They found that the kinase activity of MPK3 and MPK6 was enhanced in *ctr1* protoplasts, but suppressed by expressing an active form of CTR1 (CTR1a). Through a cell-based screen, MKK9 and MKK7 were identified to be able to activate MPK3/6 in protoplasts, and the MPK3/6 activation by ethylene was abolished in *mkk9* mutant. In support of this, they showed that *mkk9* displayed slight insensitivity to low concentration of ACC, and overexpression of an active form of MKK9 (MKK9a) resulted in constitutive ethylene response, which could not be suppressed by ethylene receptor mutants *etr1* (*etr1-1* gain-of-function mutant) or application of Ag^+, an inhibitor of ethylene perception. Therefore, the proposed MKK9–MPK3/6 module was positioned downstream of CTR1 and bypassed EIN2. Also, evidence that the MKK9–GFP was translocated into the nucleus in response to ethylene treatment and the ethylene-induced MKK9 nuclear accumulation was abolished in *etr1* (*etr1-1* gain-of-function mutant) protoplast indicated a nuclear phosphorylation event in response to ethylene. Subsequent studies revealed that EIN3 was a substrate of MPK3/6. Two putative MAPK phosphorylation sites were mapped out within EIN3. Phosphorylation at T174 induced by MPK6 led to the stabilization of EIN3, whereas phosphorylation at T592 by an as-yet unknown kinase promoted the degradation of EIN3. In accordance with the report by Ouaked et al. (2003), Yoo et al. (2008) proposed a model in which CTR1 repressed the MKK9–MPK3/6 module that phosphorylated and stabilized EIN3 in an EIN2-independent manner in ethylene signaling.

On the other hand, several groups presented data to evidently suggested that the MKK9–MPK3/6 cascade was actually involved in ethylene biosynthesis (Liu and Zhang, 2004; Joo et al., 2008; Xu et al., 2008; Bethke et al., 2009). In agreement with the previous finding that NtSIPK (the ortholog of AtMPK6) triggered induction of ethylene biosynthesis (Kim et al., 2003), Liu and Zhang (2004) reported that NtMEK2 (the ortholog of AtMKK4/5)-induced ethylene production required MPK6 in *Arabidopsis* plants. Biochemical and functional studies showed that MPK6 phosphorylated ACS2/6 (ACC synthase 2/6), two isoforms of ACC synthase, and stabilized them, which consequently enhanced ethylene production. In contrast to the report by Ouaked et al. (2003), they found that ACC treatment did not promote MPK6 kinase activity in *Arabidopsis* seedlings. Another study by Bethke et al. (2009) also found that MPK6 kinase activity was not enhanced by ACC or in the *ctr1* mutant, and flg22-induced activation of MKK4/5–MPK6 led to ethylene overproduction, which was independent of a functional ethylene signaling pathway. Thus, these results favored that the MKK4/5–MPK6 module played a regulatory role in ethylene biosynthesis. In agreement with this, the ethylene sensitivity was comparable between WT (wild-type) and the *mpk6* mutants, and *mpk6ctr1* double mutants displayed a *ctr1*-like phenotype, which therefore strongly argued against the involvement of MPK6 in ethylene signaling (Joseph, 2004; Menke et al., 2004).

Recently, Xu et al. (2008) found that a dexamethasone-inducible and constitutively active form of MKK9 ($MKK9^{DD}$) was able to active MPK3/MPK6 *in vitro* and *in vivo*. The transgenic plants harboring $MKK9^{DD}$ displayed constitutive ethylene response phenotype in etiolated seedlings, and such phenotype can be reversed by AVG (Aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis) or Ag^+ (an inhibitor of ethylene perception), quite opposite to the report by Yoo et al. (2008). Further study by An et al. (2010) revealed that two alleles of *mkk9* displayed comparable sensitivity to ethylene as WT in all ethylene responses examined, including EIN3 accumulation, downstream gene expression, and triple response phenotype. Double mutants of *mkk9ctr1* displayed a *ctr1*-like phenotype from seedling to adult stages, and the pattern of ethylene-induced EIN3 accumulation and gene expression was similar in *mkk9ctr1* and *ctr1*. The constitutive ethylene response phenotype of $MKK9^{DD}$ can be suppressed by *etr1* (*etr1-1* gain-of-function mutant), *ein2*, or *ein3eil1* mutant (An et al., 2010). Collectively, these studies clearly disfavor a role of MKK9 in ethylene signaling, and again support the engagement of the MKK9–MPK3/6 module in ethylene biosynthesis.

In summary, although it is quite clear that a MAPK pathway including MKK4/5, MKK9, and MPK3/6 is involved in the ethylene biosynthesis pathway, the role of these modules in

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ethylene signaling is still under debate. Data from different groups are not always consistent, sometimes even contradictory, making it difficult to reconcile those observations into a simple model. Given that the MAPK pathway is easily activated by environmental stresses (such as wounding, touch, etc.) (Colcombet and Hirt, 2008), a possible reason for the discrepancies from different biochemical studies is that some observations of MPK6 activation might result from 'treatment' (a touch stress or other types of stress) instead of 'ethylene or ACC treatment'. It is thus necessary to carefully scrutinize the experimental conditions of those studies and further research is needed to clarify this controversial issue.

INTERPLAYS BETWEEN ETHYLENE AND OTHER SIGNALS

Previous physiological and molecular genetic analyses have revealed extensive interactions between ethylene and other signals (Li and Guo, 2007; Zhu and Guo, 2008). In this review, we will focus on the recent advances on the interactions between ethylene and other signaling pathways such as auxin, light, and plant innate immunity signals.

It has been long known that auxin and ethylene, two important plant growth regulators, have wide-ranging and complicated interactions (Stepanova and Alonso, 2009). Auxin has been found to promote the biosynthesis of ethylene by transcriptional induction of *ACS4* (*ACC synthase4*) (Abel et al., 1995). On the other hand, several auxin-resistant mutants showed an ethylene-insensitive root phenotype, suggesting that ethylene inhibits root elongation via the action of auxin (Roman et al., 1995). This notion was confirmed by the identification of two root-specific ethylene-insensitive mutants: *wei2* (*weak ethylene insensitive 2*) and *wei7* (*weak ethylene insensitive 7*) (Stepanova et al., 2005). It was found that ethylene promoted auxin biosynthesis in root meristems by inducing the expression of *WEI2* and *WEI7*, which encode the α and β subunits of anthranilate synthase, a rate-limiting enzyme for biosynthesis of auxin precursor tryptophan (Trp) (Stepanova et al., 2005). Besides the promotion of auxin biosynthesis in root meristems, ethylene also facilitated the transport of auxin (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). Ethylene-promoted transport of auxin from meristem to elongation zone was dependent on activities of auxin influx transporter AUX1 (AUXIN RESISTANT 1) and efflux carrier PIN1 (*ARABIDOPSIS* THALIANA PIN-FORMED 1) (Ruzicka et al., 2007). The characterization of another root-specific ethylene-insensitive mutant, *wei8*, provided further evidence and a direct link between ethylene and auxin biosynthesis in roots (Stepanova et al., 2008). *WEI8* encodes TAA1 (TRYPTOPHAN AMINOTRANSFERASE OF *ARABIDOPSIS*1), a long anticipated tryptophan aminotransferase in the IPA (Indole-3-pyruvic acid) route of auxin biosynthesis. Ethylene treatment promoted the expression of *TAA1* and its homolog *TAR2* (*TAA1-RELATED2*) specifically in the root meristematic region, which enhanced the local production of IAA (Indole-3-acetic acid) responsible for inhibition of root elongation. The ethylene-induced expression of *TAR2* on the inner side of apical hooks, along with the reduced hook curvature observed in *wei8tar2*, indicated that ethylene-regulated auxin gradient was required for the exaggerated hook bending (Stepanova et al., 2008). Meanwhile, ethylene also induced the gene expression of *HLS1* (*HOOKLESS1*), a putative acetyltransferase that acts to modulate the function of auxin response factors (ARFs) in differential hook formation (Li et al., 2004). The coordinated regulation of auxin biosynthesis and signaling pathways by ethylene leads to the establishment of asymmetric distribution of auxin activity and eventually differential growth in the hook region.

Light is one of the most informative environmental signals for plant growth, development, and survival. Previous studies in tobacco revealed that the low red to far-red light (R/FR) ratio promoted the production of ethylene, which partly contributed to shade avoidance response (Pierik et al., 2004). Consistently, ethylene-insensitive tobacco plants showed reduced shade avoidance response in low R/FR ratio, indicating the involvement of ethylene in light response. Conversely, light has also been found to promote ethylene biosynthesis, probably through the action of a basic helix-loop-helix transcription factor PIF5 (PHYTOCHROME INTERACTING FACTOR 5) (Khanna et al., 2007).

Recently, ethylene has been demonstrated to play a key role in the transition from skotomorphogenesis to photomorphogenesis (Zhong et al., 2009). The ethylene -insensitive mutants *ein2* and *ein3eil1* displayed a photo-bleaching phenotype in a prolonged-dark treatment, which resulted from a high level of ROS (reactive oxygen species) accumulation in the cotyledon, as previously observed in *pif1* (*phytochrome interacting factor 1*) and *cop1* (*constitutive photomorphogenesis 1*). Further study found that *ein3eil1* mutant had excessive accumulation of phototoxic intermediate (Pchlide) and reduced expression of *PORA/B* (*PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A/B*), which encode key enzymes for light-initiated chlorophyll synthesis. EIN3 can directly bind to the promoter region of *PORA* and *PORB*, and activated these two genes in response to ethylene. Genetic analysis indicated that EIN3/EIL1 cooperate with PIF1 to prevent photo-bleaching and promote cotyledon greening. Furthermore, activation of EIN3 largely rescued the severe photo-bleaching phenotype of *cop1*, placing EIN3 downstream of COP1. COP1 seemed to up-regulate the stability of EIN3 whereas light destabilizes EIN3 by reversing the effect of COP1 (Zhong et al., 2009). Additionally, genetic studies revealed that EIN3/EIL1 regulated Pchlide accumulation and cotyledon greening partly dependent on PIF3 (PHYTOCHROME INTERACTING FACTOR 3) (Zhong et al., 2010). Therefore, ethylene signaling via EIN3/EIL1 presents a new pathway to repress phototoxic Pchlide accumulation in darkness, and simultaneously facilitate chlorophyll synthesis by inducing *PORA/B* expression. These studies uncovered an essential role of ethylene in regulating the de-etiolation process and

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a complex mode of interactions between ethylene and light-signaling pathways.

Both ethylene and salicylic acid (SA) are major plant defense hormones (Durrant and Dong, 2004). Little is known about how ethylene and SA pathways interact and coordinate during plant immunity response. A recent study provided insight into the molecular mechanism of how ethylene modulates SA biosynthesis (Chen et al., 2009). The biosynthesis of SA was strongly induced upon pathogen infection, and the pathogen-induced SA production was controlled by isochorismate synthesis enzyme SID2 (SALICYLIC ACID INDUCTION DEFICIENT 2) (Wildermuth et al., 2001). The isolation of *ein3* as a resistant mutant to bacterial pathogen infection connected ethylene signaling to the SA pathway. The constitutively high level of SA accumulation in *ein3eil1* suggested a negative role of ethylene signaling in SA biosynthesis. The direct binding of EIN3 to the promoter of *SID2*, with the consequent suppression of *SID2* expression, explained the elevated level of SA and enhanced defense response in ethylene-insensitive mutants including *ein3* and *ein2* (Chen et al., 2009).

In plant innate immunity, the receptor FLS2 (FLAGELLIN-SENSITIVE 2) is responsible for the recognition of bacterial pathogen-associated molecular pattern (PAMP) flagellin or its active epitope flg22 peptide (Zipfel, 2009). Besides the knowledge that the PAMP treatment increases the biosynthesis of ethylene (Felix et al., 1999), little was known on how ethylene modulates the PAMP-induced immune response until the recent isolation of *ein2* as a flagellin-insensitive mutant. The decreased expression level of *FLS2* in *ein2*, *etr1* (*etr1-1* gain-of-function mutant), and *ein3* indicated a transcriptional control of *FLS2* by ethylene signaling. High-affinity binding of EIN3 to the promoter of *FLS2* provided the direct molecular link between ethylene and plant innate immunity (Boutrot et al., 2010). Therefore, EIN3/EIL1 act as a signaling hub that integrates the action of ethylene and plant defense pathways, in a way that EIN3/EIL1 directly repress the *SID2* expression to decrease SA level while directly inducing *FLS2* expression to maintain an optimal level of innate immune receptors.

CONCLUSIONS

Based on dedicated studies on ethylene signaling and interplay between ethylene and other signals, several conclusions can be made: (1) multi-step control of protein stability of EIN3, EIL1, EIN2, ETR2, ETP1/2, and EBF1/2 contributes to a complex and flexible regulation of the ethylene response pathway; (2) the MKK9–MPK3/6 module participates in ethylene biosynthesis, and is still open to debate for its involvement in ethylene signaling; (3) negative feedback regulations enable plants to respond to subsequent ethylene signal as well as establish a homeostasis in the ethylene signaling output; (4) different means of interplay between ethylene and other signals make ethylene signaling pathway an open system that is amenable to regulation executed by various developmental and environmental cues. To summarize these new advances, a model has been proposed in Figure 1, in which ethylene signal is perceived by a linear pathway composed of receptors, CTR1, EIN2, and EBF1/EBF2, to activate EIN3/EIL1 transcription factors, which serve as an integration point for numerous interactions. The MKK9–MPK3/6 module is believed to regulate ethylene production by stabilizing ACS2/6 in response to signals like environmental stress.

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