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The ethylene signaling pathway: new insights

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During the past decade, molecular genetic studies on the reference plant *Arabidopsis* have established a largely linear signal transduction pathway for the response to ethylene gas. The biochemical modes of action of many of the signaling components are still unresolved. During the past year, however, progress in several areas has been made on several fronts. The different approaches used have included a functional study of the activity of the receptor His kinase, the determination of the ethylene receptor signaling complex at the endoplasmic reticulum and of the regulation of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) activity by these receptors, the identification of a unique MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascade, the cloning and characterization of numerous ETHYLENE INSENSITIVE3 (EIN3)/EIN3-like (EIL) transcription factors from many plant species, and the integration of the ethylene and jasmonate response pathways via the ETHYLENE RESPONSE FACTOR (ERF) family of transcription factors. The elucidation of the biochemical mechanisms of ethylene signal transduction and the identification of new components in the ethylene response pathway in *Arabidopsis* are providing a framework for understanding how all plants sense and respond to ethylene.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ctr1	constitutive triple response1
EIL	EIN3-like
ein2	ethylene insensitive2
eir1	ethylene insensitive root1
ER	endoplasmic reticulum
EREBP	ETHYLENE RESPONSE ELEMENT BINDING PROTEIN
ERF1	ETHYLENE RESPONSE FACTOR1
ERS1	ETHYLENE RESPONSE SENSOR1
eto1	ethylene overproduction1
etr1	ethylene receptor1
JA	jasmonate
LOF	loss-of-function
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase

MPK6	<i>Arabidopsis</i> MAPK6
NtMEK2	<i>Nicotiana tabacum</i> MAPK/ERK KINASE2
PDF1.2	PLANT DEFENSIN1.2
ran1	responsive to antagonist1
SIMK	SALT-STRESS-INDUCIBLE MAPK
SIMKK	SIMK KINASE
SIPK	SALICYLIC-ACID-INDUCED PROTEIN KINASE

Introduction

Ethylene is a gaseous plant hormone that affects myriad developmental processes and fitness responses, including germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and responsiveness to stress and pathogen attack [1,2]. A well-known effect of ethylene on plant growth is the so-called ‘triple response’ of etiolated dicotyledonous seedlings. This response is characterized by the inhibition of hypocotyl and root cell elongation, radial swelling of the hypocotyl, and exaggerated curvature of the apical hook. This highly specific ethylene response occurs at an early developmental stage (3 days post-germination), permitting large mutant populations of seedlings to be screened rapidly for ethylene response defects. Over the past decade, genetic screens that are based on the triple-response phenotype have been extensively conducted on *Arabidopsis* by many laboratories. More than a dozen unique mutants have been identified and these can be divided into three distinct categories: constitutive triple-response mutants (i.e. *ethylene overproduction1* [*eto1*], *eto2*, *eto3*, *constitutive triple response1* [*ctr1*] and *responsive to antagonist1* [*ran1*]/*ctr2*); ethylene-insensitive mutants (i.e. *ethylene receptor1* [*etr1*], *etr2*, *ethylene insensitive2* [*ein2*], *ein3*, *ein4*, *ein5*, and *ein6*); and tissue-specific ethylene-insensitive mutants (i.e. *hookless1* [*hls1*], *ethylene insensitive root1* [*eir1*], and several auxin-resistant mutants) [1–3].

A combination of genetic and molecular analyses of these mutants has defined a largely linear ethylene response pathway leading from hormone perception at the membrane to transcriptional regulation in the nucleus. Briefly, ethylene is perceived by a family of membrane-associated receptors, including ETR1/ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1)/ERS2 and EIN4 in *Arabidopsis* [4–7]. Ethylene binds to its receptors via a copper co-factor, which is probably delivered by the copper transporter RAN1. Genetic studies predict that hormone binding results in the inactivation of receptor function [8]. In the absence of ethylene, therefore, the receptors are hypothesized to be in a functionally active form that constitutively activates a Raf-like serine/threonine (Ser/Thr) kinase, CTR1, which is also a negative

regulator of the pathway [9]. EIN2, EIN3, EIN5, and EIN6 are positive regulators of ethylene responses, acting downstream of CTR1. EIN2 is an integral membrane protein whose function is not understood [10]. EIN5 and EIN6 have not yet been characterized at the molecular level. The nuclear protein EIN3 is a transcription factor that regulates the expression of its immediate target genes such as *ETHYLENE RESPONSE FACTOR1* (*ERF1*) [11,12]. ERF1 belongs to a large family of APE-TALA2-domain-containing transcription factors that bind to a GCC-box present in the promoters of many ethylene-inducible, defense-related genes [13]. Thus, a transcriptional cascade that is mediated by EIN3/EIN3-like (EIL) and ERF proteins leads to the regulation of ethylene-controlled gene expression. Several comprehensive reviews on the ethylene signaling pathway have been published recently [14–16]. Our intent in this review is not to redundantly describe aspects of ethylene biology that have been covered previously, but rather to discuss the new discoveries on ethylene signaling mechanisms that have emerged during the past two years.

Ethylene perception: the role of receptor kinase activity?

In *Arabidopsis*, ethylene is perceived by a family of five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) that share similarity with bacterial two-component regulators [17]. On the basis of structural similarities, the receptor family can be divided into two subfamilies. Members of the type-I subfamily, which include ETR1 and ERS1, contain an amino-terminal ethylene-binding domain (also called the sensor domain) and a well-conserved carboxy-terminal histidine (His) kinase domain. The type-II subfamily receptors, which include ETR2, ERS2 and EIN4, contain an amino-terminal ethylene-binding domain and a degenerate His kinase domain that lacks one or more elements that are necessary for catalytic activity. ETR1 (type I), ETR2 and EIN4 (type II) also have an additional receiver domain at their carboxyl termini whose function is unknown.

Genetic and biochemical studies have revealed that the ethylene receptors function as negative regulators of ethylene responses and that ethylene binding inactivates them [1,17]. Dominant mutations in each of the five receptors confer ethylene-insensitivity, suggesting that all five receptors participate in ethylene perception. Dominant ethylene-insensitivity mutations in *ETR1* have been found to abolish ethylene binding, allowing the mutant ETR1 receptor to escape from inactivation by the hormone and leading to constitutive receptor signaling [18]. Loss-of-function (LOF) mutations in *ETR1*, *ETR2*, *EIN4*, and *ERS2* have been previously isolated [8]. Single and double LOF mutants have no appreciable ethylene response phenotypes, suggesting a high degree of functional overlap among the receptors. In contrast to the single and double mutants, however, triple and quad-

ruple receptor mutants show constitutive-ethylene-response phenotypes. If signaling from one receptor is sufficient to repress the pathway, then the constitutive-ethylene response phenotypes of the triple and quadruple receptor mutants are rather puzzling: the remaining receptors should theoretically be active (i.e. in the default state) and fully capable of repressing the downstream ethylene pathway. One resolution of this paradox may lie in the observation that dominant mutations in *ETR1* result in the elevated expression of receptor protein, leading to an increase in signal output that is sufficient to repress all ethylene responses [19*]. An alternative (or additional) explanation is that each receptor provides a similar contribution to the 'repression' signal; thus, the lower levels of total signal (receptor) result in increased sensitivity to ethylene whereas the activity of more receptors would reduce sensitivity. In this respect, the triple and quadruple mutants exhibit a constitutive ethylene response because the basal levels of ethylene that are constitutively produced in these plants are sufficient to inactivate the remaining receptor 'repressive' activity. In support of this notion, a careful re-examination of *etr1* LOF mutants has shown that these plants exhibit a weakly enhanced response to ethylene [20*].

Although comprehensive genetic studies have been performed with *etr1*, *etr2*, *ers2* and *ein4* mutants, LOF mutations in *ERS1* gene (type II) were isolated only recently [19*,21**]. Like all other single-receptor LOF mutants, *ers1* plants showed no obvious constitutive-ethylene-response phenotypes. However, double LOF *etr1 ers1* mutants exhibit strong constitutive-ethylene-response phenotypes, comparable to those of quadruple LOF receptor mutants [21**]. These phenotypes are also seen in plants that carry a strong allele of *ran1*, which is predicted to cause a LOF of all ethylene receptors [22]. *etr1 ers1* double mutants display myriad phenotypes, including defects in floral organ development, infertility, an extremely compact rosette and late flowering, all of which are more severe than the same phenotypes in the constitutive mutant *ctr1* [23*]. Importantly, these defects were completely suppressed by the *ein2* mutation, suggesting that these newly described developmental defects are EIN2 dependent and therefore define previously unknown roles of ethylene in these developmental processes.

Interestingly, the *etr1 ers1* double mutant phenotypes can be rescued by the transgenic expression of *ETR1* or *ERS1* (type I), but not of *ETR2*, *ERS2*, or *EIN4* (type II) [21**]. Considering the fact that only type-I receptors contain a functional His kinase domain, it is reasonable to suspect that the unique role of type-I receptors in ethylene signaling could lie in the His kinase activity that is missing from type-II receptors. To test this possibility, a mutant ETR1 was created in which the His kinase activity was eliminated, and this construct was introduced

into *etr1 ers1* plants [21**]. Like wildtype ETR1, the His-kinase-dead ETR1 was capable of rescuing the double mutant phenotypes. Thus, the His kinase activity is not required for the function of ETR1 in repressing ethylene responses.

An independent line of evidence that supports this claim comes from mutational studies of the dominant ethylene-insensitivity mutant *etr1-1* [24*]. A mutant form of *etr1-1* that eliminates *in-vitro* His kinase activity was able to evoke ethylene insensitivity to the same degree as that observed in *etr1-1*. This result indicates that His kinase activity is not required for the ethylene-insensitivity phenotypes conferred by *etr1-1*. Furthermore, a truncated form of *etr1-1* that lacks the entire carboxy-terminal region, including both the His kinase domain and the receiver domain, was still able to confer ethylene insensitivity. This finding is surprising because the carboxy-terminal half of the receptor was thought to play a role in signal output, potentially through interaction with downstream signaling components such as CTR1 (see below). One possibility is that the *etr1-1* amino-terminal domain remains functional because it is associated with other intact receptors, although only homodimerization of ethylene receptors has been observed to date [25].

It has been shown that ETR1 has *in-vitro* His kinase activity [26]. As His kinase activity is not important for receptor function, why do the ethylene receptors (type I) maintain this activity? One explanation may be that the His kinase activity is required for other aspects of receptor functionality, such as localization, protein stability, or interaction with other factors, to fine-tune signal output. The more degenerate His kinase domains in type-II receptors might, in fact, have evolved into a Ser/Thr kinase, as shown in the case of His kinase domains in phytochrome, a family of red/far-red photoreceptors. These proteins also contain degenerate His kinase domains but behave functionally as a Ser/Thr kinase [27]. Recently, a type-II ethylene receptor from tobacco *Nicotiana tabacum* HISTIDINE KINASE1 (NTHK1) was shown to possess Ser/Thr kinase activity but not His kinase activity [28]. It will be intriguing to see whether *Arabidopsis* type-II receptors also function as Ser/Thr kinases. Nonetheless, on the basis of multiple-receptor mutant studies, neither His kinase nor Ser/Thr kinase activity is essential for ethylene receptor signaling, raising the possibility that these receptors function through a non-kinase mechanism to sense and transmit the hormone signal (see below).

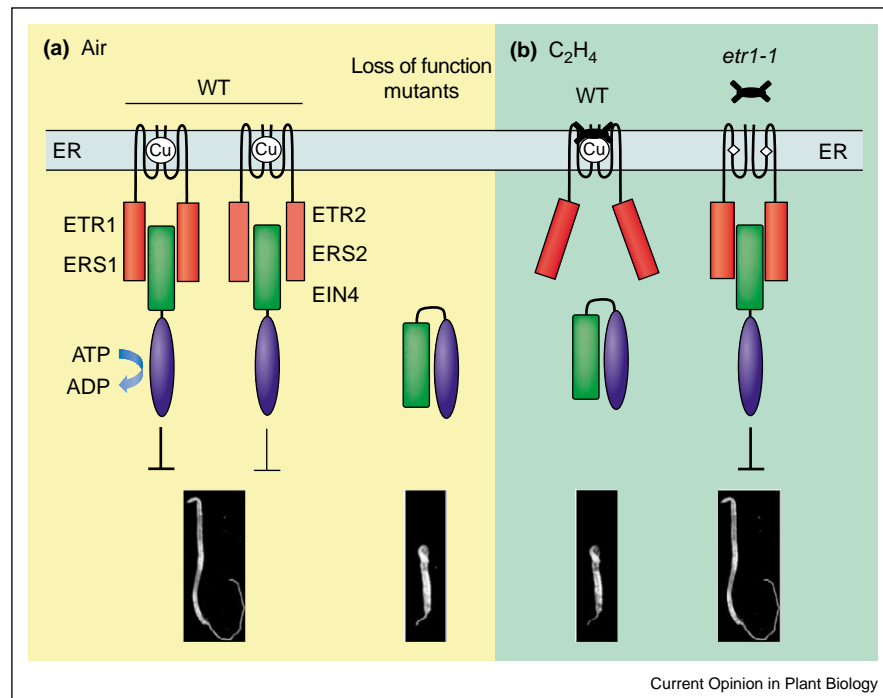
Early signaling events: a receptor–CTR1 complex at the ER

The recent finding that ethylene perception and signaling occur at the endoplasmic reticulum (ER) is a major breakthrough in understanding the mechanism of ethylene signaling [29**,30**]. Previous studies suggested that

the ethylene receptors may be localized at the plasma membrane. Recently, however, *Arabidopsis* ETR1 has been shown convincingly, on the bases of both sucrose density-gradient fractionation and immunogold electron microscopy, to associate with the ER [29**]. The amino-terminal membrane-spanning sensor domain of ETR1 is sufficient for the ER association. Ethylene treatment or introduction of the *etr1-1* mutation did not affect the ER localization of ETR1. Importantly, CTR1 has also been found to be localized at the ER [30**]. CTR1 is composed of an amino-terminal domain of unknown function, and a carboxy-terminal kinase domain that is most related to Raf-like mitogen-activated protein kinase (MAPK) kinase kinases (MAPKKKs). Previous yeast two-hybrid and pull-down experiments showed that the amino-terminal domain of CTR1 can interact with the His kinase domains of ETR1 and ERS1 [31]. Because CTR1 has no obvious trans-membrane domain or membrane attachment motifs, it is likely that its ER localization might result from direct interaction with the ER-associated receptors. This hypothesis predicts that the removal of ethylene receptors or disruption of the interaction between CTR1 and these receptors would decrease the level of ER-associated CTR1. To test this possibility, a series of single, double, and triple receptor mutants have been examined to assess the effect of functional receptors on the ER localization of CTR1 [30**]. Whereas most single receptor mutations had little effect on the level of ER-bound CTR1, double and triple combinations of receptor mutations resulted in reduced levels of ER-bound CTR1. Moreover, the levels of ER-bound CTR1 correlated with the strength of the constitutive-ethylene-response phenotypes in the multiple-receptor mutants. In addition, a missense mutation in the amino-terminal domain of CTR1 (CTR1-8) was found to disrupt the interaction of CTR1 with ETR1 [32**]. This mutation did not affect the kinase activity or the level of the CTR1 protein, but conferred phenotypes that were as severe as those of CTR1-kinase-dead mutants. Accordingly, the CTR1-8 protein was primarily soluble, suggesting that an association with ER-bound receptors is required for CTR1 function.

Direct proof of CTR1–ETR1 interaction *in vivo* comes from two lines of evidence. Affinity purification of CTR1 from the *Arabidopsis* ER-membrane fraction results in the co-purification of ETR1, demonstrating the presence of ETR1 and CTR1 in the same protein complex [30**]. Genetic evidence is also available to support the proposition that CTR1 function may be regulated by its association/dissociation with the ethylene receptors ([32**]; Figure 1). If this is the case, the overexpression of the amino-terminal domain of CTR1 (which is responsible for receptor binding) might confer a dominant negative effect by preventing endogenous CTR1 from associating with the receptors. Consequently, this overexpression may cause a LOF *ctr1* mutant phenotype. In contrast, we would expect that overexpression of a CTR1-8 mutant

Figure 1



Proposed signaling mechanisms of the ethylene receptors and CTR1 at the ER. There are two types of ethylene receptors in *Arabidopsis*. Type-I receptors (ETR1 and ERS1) contain a conserved histidine kinase domain (red), whereas type-II receptors (ETR2, ERS2 and EIN4) contain a degenerate histidine kinase domain (pink) that is predicted to lack catalytic activity. The histidine kinase activity observed in type-I receptors is not required for receptor signaling. The amino-terminal sensor domains of the receptors contain a copper cofactor (Cu) that is needed for ethylene binding and are associated with the ER membrane. The amino-terminal domain of CTR1 (green) interacts with the histidine kinase domain of the receptors, at a higher affinity with type-I members than with type-II members. This interaction allows CTR1 to be localized to the ER. **(a)** In the absence of ethylene, the receptors are predicted to remain in a functionally active state, which is able to interact with CTR1. CTR1 is activated by association with the ER-bound receptors and represses the downstream ethylene responses by a mechanism that requires its carboxyl terminal Ser/Thr kinase domain (blue). Loss of the function of multiple receptors (in LOF mutants) leads to the dissociation of CTR1 from the ER. The soluble CTR1 is inactivated, possibly through self-association with a negative regulatory domain as has been shown for some Raf kinases. As a result, the repression of downstream ethylene responses is relieved. The loss of two type-I receptors results in ethylene response phenotypes that are more severe than those that result from the mutation of any other combination of two LOF receptors. The explanation for this greater response may be unrelated to the fact that type-I receptors possess histidine kinase activity. Instead, it could be due to the fact that the affinity of type-I receptors for the amino-terminal domain of CTR1 is stronger than that of type-II receptors. **(b)** When ethylene is present, it binds to the sensor domain of the receptor and presumably causes a conformational change, resulting in an inactive receptor. CTR1 is then released from the ER and also becomes inactivated. In contrast, dominant mutations in the receptor that disrupt ethylene binding may lead to constitutive receptor-CTR1 interaction and the repression of downstream components in the ethylene signaling pathway. Seedling photographs were taken by Hongwei Guo.

version of the CTR1 amino-terminal domain would not produce the *ctr1* phenotype because this amino-terminal domain is incapable of interacting with the receptors. These elegant experiments have been carried out by Huang and co-workers [32^{••}] and their results confirmed these predictions.

Taken together, both genetic and biochemical studies indicate that the function of CTR1 depends upon both its carboxy-terminal Ser/Thr kinase activity and the association of its amino-terminal domain with ER-bound ethylene receptors. CTR1 is also able to interact weakly with carboxy-terminal domain of ETR2 (type II) [20[•]]. Thus, it is hypothesized that all five ethylene receptors are able to interact with CTR1 via their carboxy-terminal kinase

domains. However, type-I receptors (i.e. ETR1 and ERS1) have a high affinity for CTR1, whereas type-II receptors (at least ETR2) possess a low binding affinity for CTR1. Although the His kinase domain of ETR1 is sufficient for CTR1 binding, this His kinase activity is not required for the interaction of these proteins [30^{••}], further supporting the idea that His kinase activity is not required for receptor function. These findings provide an explanation for the observation that type-I receptors play a special role in ethylene signaling. They suggest that this unique role is not due to the His kinase activity of type-I receptors; rather, the difference between type-I and type-II receptors may lie in the strength of their physical association with a downstream signaling factor, CTR1 (Figure 1).

Signal transduction: a unique MAP kinase cascade uncovered?

Although CTR1 was shown to encode a protein with highest homology to Raf-like MAPKKs a decade ago [9], its kinase activity has not been demonstrated until very recently [32^{••}]. As predicted from sequence, the CTR1 protein expressed by *Escherichia coli* behaves as a Ser/Thr kinase and can phosphorylate myelin basic protein, an artificial substrate for numerous MAPKs. Ever since CTR1 was identified as a MAPKK-like protein, it has been proposed that the ethylene signaling pathway includes a MAPK cascade. However, extensive genetic studies failed to identify any MAPK kinase (MAPKK) or MAPK in the ethylene response pathway. It seems, therefore, either that no authentic MAPKK/MAPK module is involved in ethylene signaling or that obstacles to the genetic approaches taken, such as functional redundancy or mutational lethality, have prevented its identification.

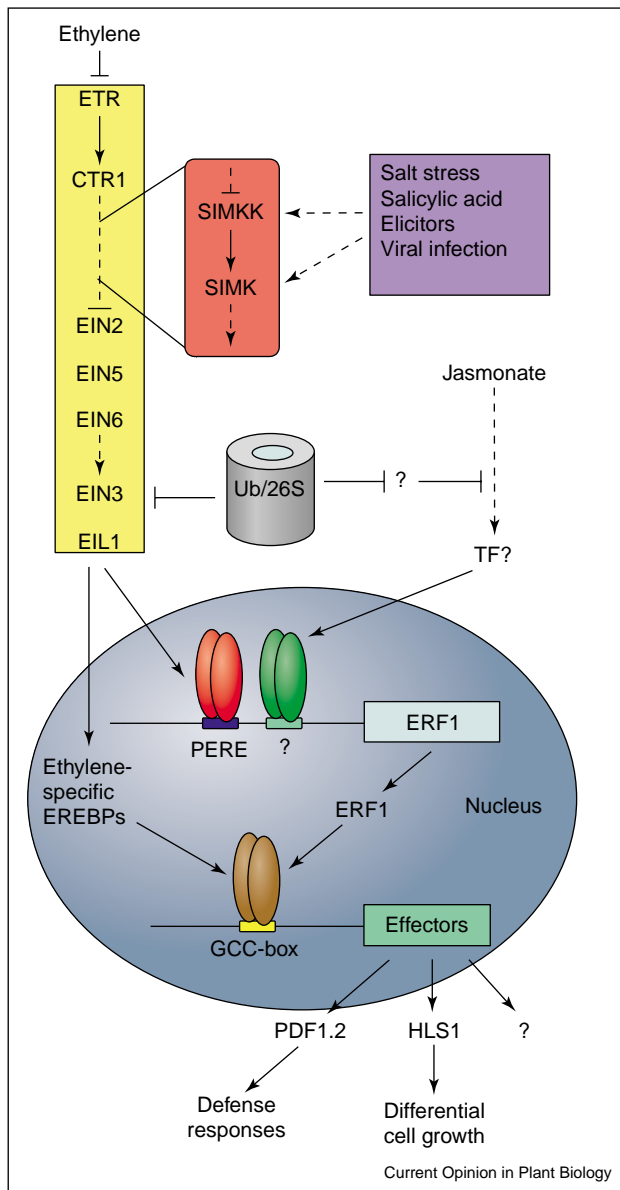
In support of the presence of a MAPK cascade in the ethylene response pathway, it has recently been shown that ethylene can activate an *Arabidopsis* 47-kDa protein that has properties characteristic of the MAPKs [33]. In addition, the expression of CTR1 in maize protoplast activates a 44-kDa MAPKK whose identity is unknown [34]. Further convincing evidence of a MAPK cascade in ethylene response pathway comes from a recent study by Ouaked *et al.* [35^{••}]. This group initially identified several *Medicago* MAPK kinases that respond to various biotic and abiotic stresses. In particular, two MAPKs, SALT-STRESS-INDUCIBLE MAPK (SIMK) and *Medicago* MAPK3 (MMK3), were found to be activated by a MAPKK (SIMK KINASE [SIMKK]) in response to salt stress or treatment with a pathogen elicitor [36]. Similarly, treatment with 1-aminocyclopropane-1-carboxylic acid (ACC) also induced the activation of SIMK and MMK3 within a few minutes, and this activation was mediated by SIMKK [35^{••}]. Importantly, SIMKK activity was stimulated by treatment with ACC. Ethylene-induced activation of *Arabidopsis* MAPK (MPK6), an ortholog of *Medicago* SIMK, has also been observed [37]. The ACC-induced activation of MPK6 required functional ethylene receptors and CTR1, but not EIN2 and EIN3. These biochemical studies hint that a MAPK cascade consisting of SIMKK and SIMK (MPK6) is involved in the ethylene response pathway, and acts downstream of the receptors and CTR1. Because overexpression of the *Medicago* SIMKK can activate SIMK without stimulation by upstream factors, transgenic *Arabidopsis* plants that overexpress SIMKK (instead of SIMK/MMK3) have been constructed [35^{••}]. These transgenic lines exhibited constitutive-ethylene-response phenotypes. Like the *ctr1* phenotype, the phenotypes of these transgenics were not suppressed by the ethylene-biosynthesis inhibitor aminoethoxyvinylglycine (AVG), indicating the occurrence of enhanced ethylene signaling rather than ethyl-

ene overproduction in these plants. In keeping with the constitutive-ethylene-response phenotype, the activation of several pathogen-associated ethylene response genes was observed in the SIMKK-overexpressing plants. Moreover, AtMPK6 activity was constitutively induced in SIMKK-overexpressing plants. These results indicate that the SIMKK-MPK6 pathway is a positive regulator of ethylene responses.

Although a more compelling genetic study of LOF MAPKK/MAPK mutations is required before final conclusions can be drawn, the proposed positive regulatory role of a MAPK module in the ethylene response pathway is intriguing. For instance, if CTR1 is the upstream MAPKK for SIMKK, and given that CTR1 is a negative ethylene-signaling component, then CTR1 must negatively regulate the SIMKK-MPK6 module. Such a scenario has not been reported for any of the known MAPK cascades in animals or plants [38]. The biochemical connection between CTR1 and SIMKK needs to be examined to clarify this puzzle. In addition, because the SIMKK-overexpression phenotype has not been examined in an *ein2* or *ein3* background, it will be important to ascertain whether the SIMKK-MPK6 pathway works upstream of or independently of EIN2 and EIN3, given that EIN2 and EIN3 are essential components of the ethylene-response pathway.

Recently, *Nicotiana tabacum* MAPK/ERK KINASE2 (NtMEK2) and *N. tabacum* SALICYLIC-ACID-INDUCED PROTEIN KINASE (NtSIPK), the tobacco orthologs of SIMKK and SIMK, respectively [37], have been demonstrated to participate in the induction of ethylene biosynthesis in response to wounding and viral infection [39[•]]. Transient overexpression of an active form of NtMEK2 in tobacco (and the consequent activation of NtSIPK) resulted in a dramatic increase in ethylene production, possibly because of a rise in ACC synthase expression/activity. It is not clear how the same MAPK cascade is involved in both the ethylene response and biosynthesis pathways. One explanation for these results is that the SIMKK-MPK6 module may represent a branch-point in the ethylene signaling pathway. Although its activity is regulated by the ethylene receptors/CTR1, the SIMKK-MPK6 module controls other stress responses (which are not dependent upon EIN2 or EIN3), including ethylene production. Consistent with this view, SIMKK, MPK6 and SIMK are induced by many stimuli, including pathogen elicitors, wounding, salt stress, and viral infection ([37]; Figure 2). In light of this model, the *ctr1*-like phenotype observed in SIMKK-overexpressing plants could be the consequence of elevated ethylene biosynthesis, although the available data do not support this possibility [35^{••}]. Further experiments, such as the overexpression of SIMKK or the active form of NtMEK2 in *etr1-1* or *ein2* mutants, are required to solve this enigma.

Figure 2



A model for the ethylene response pathway in the regulation of gene expression. Ethylene gas is perceived by a family of ER-associated receptors (ETR). Ethylene binding is proposed to inhibit receptor function. CTR1 is proposed to be activated by the unoccupied receptors via physical interaction with them, and is inhibited upon binding of ethylene by the receptor. A MAPK module, consisting of SIMKK and SIMK, is proposed to act downstream of CTR1, although the biochemical consequence of this MAPK pathway is not evident. Because many biotic and abiotic stimuli activate the SIMKK/SIMK pathway, it remains to be determined whether their activation is dependent upon the functions of the ethylene receptors and CTR1. Downstream components in the ethylene pathway include several positive regulators (EIN2, EIN5, EIN6 and the transcription factors EIN3 and EIL1). The level of EIN3 protein is controlled by ethylene, possibly via the proteasome (Ub/26S). The primary ethylene signaling pathway components (indicated in yellow) are required for all known ethylene responses and, to date, none have been found to respond to signals other than ethylene. Branch points in the ethylene response pathway may lie downstream of EIN3/EIL1. Several EREBP transcription

Primary transcription factors: the action of EIN3/EIL proteins

Differential gene expression has been reported in many ethylene responses. The molecular characterization of *EIN3* has provided direct evidence of ethylene-controlled transcriptional regulation [11]. In *Arabidopsis*, there are six members of the EIN3 family, in which EIN3 and EIL1 are the most closely related proteins [40**]. LOF mutations in the *EIL1* gene have recently been isolated [40**]. Like *ein3* mutants, *eil1* plants show incomplete ethylene insensitivity, albeit *eil1* plants are more sensitive to ethylene than *ein3* mutants. The weaker ethylene-insensitivity phenotype caused by *eil1* mutations can be explained by the lower expression level of *EIL1* compared with that of *EIN3* [41**]. Interestingly, *ein3 eil1* double mutants show complete ethylene insensitivity in all known ethylene responses, including the triple response and pathogen resistance, as well as the ability to suppress the *ctr1* mutation fully [40**]. This raises the question of the role of EIL2, EIL3, EIL4 and EIL5 in ethylene responses.

The overexpression of EIN3 and EIL1, but not of other EILs, confers a constitutive-triple-response phenotype in wildtype plants or in the *ein2* background ([11]; H Guo, JR Ecker, unpublished). In the *Arabidopsis* EIN3 family, therefore, only EIN3 and its closest relative EIL1 have been shown conclusively to function in the ethylene response pathway. Together, these two transcription factors could mediate most, if not all, aspects of seedling growth responses to ethylene. The more distantly related members of the EIN3 family (EIL2–5) might either play a minor role in the ethylene response in specific tissue types or developmental stages or function in totally different pathways that are unrelated to ethylene responses. Supporting evidence for this hypothesis comes from the characterization of EIN3-like transcription factors in other plant species. At least five tobacco *EIN3*-like (*NtEIL*) genes, three tomato *EIL* (*LeEIL*) genes, and two Mung bean *EIL* (*VR-EIL*) genes have been identified

factors are known to be immediate targets of EIN3/EIL1, which can bind to a primary ethylene response element (PERE) in the promoters of *EREBP* genes. One EREBP, called ERF1, is also involved in JA-mediated gene regulation. It is likely that an as yet unidentified JA-regulated transcription factor (TF) may also bind to the promoter of *ERF1* to activate its expression. Therefore, the promoter of *ERF1* might function to integrate signals from both the ethylene and JA signaling pathways. Other EREBP proteins may act in a similar manner to integrate the actions of ethylene with developmental signals and/or other hormone signals. Many EREBP proteins are known to regulate gene expression through interaction with a *cis*-element called the GCC-box, which is found in several ethylene-responsive genes including *PDF1.2* and *HOOKLESS1* (*HLS1*). These genes encode effector proteins that are needed to execute a wide variety of ethylene responses, from disease resistance to differential cell growth. '?' 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 not yet been shown to occur through direct interaction.

[42–44]. A few of the proteins encoded by these genes are known to be equivalent to EIN3 in terms of their biological function and DNA-binding properties [42,43,45]. Interestingly, all of these EIL proteins are more closely related to *Arabidopsis* EIN3 and EIL1 than to EIL2–5. The *EIN3/EIL1*, *NtEIL*, *LeEIL* and *VR-EIL* genes are ubiquitously expressed throughout the plants, although the levels and patterns of their expression differ slightly [42–44]. Transgenic studies on tomato plants that expressed antisense RNAs for each *LeEIL* gene provided persuasive evidence for functional redundancy of the EILs [43].

None of *EIN3/EIL* genes identified to date is transcriptionally regulated in response to ethylene [11,42–44]. This suggests that changes in *EIN3/EIL* mRNA levels are not required for the activation of ethylene responses, and that the activities of these genes are regulated by ethylene through a posttranscriptional mechanism. On the other hand, the overexpression of several *EIN3/EIL* members, including *EIN3*, *EIL1*, *NtEIL1* and *VR-EIL1/2*, confers constitutive ethylene responses [11,42,45]. Thus, the increased expression of *EIN3/EIL* is sufficient to induce an ethylene response. One explanation to reconcile these findings is that the levels of the EIN3/EIL protein, rather than the levels of *EIN3/EIL* mRNA, are subject to ethylene-induced regulation. In support of this hypothesis, Yanagisawa *et al.* [46[•]] recently reported that the level of EIN3 protein in seedlings is affected by treatment with ethylene for 5 days. However, more extensive studies of the ethylene response carried out in protoplasts revealed only a minor enhancement of EIN3 levels after treatment with ACC [46[•]]. The EIN3 protein may be degraded after *de-novo* protein synthesis is inhibited. This degradation process could be mediated by a ubiquitin/proteasome pathway because several proteasome-specific inhibitors can stabilize EIN3 [46[•]]. ACC treatment was found to inhibit the degradation of EIN3, but the signaling mechanism through which ethylene acts to stabilize the EIN3 protein is unknown [46[•]]. Interestingly, glucose enhances EIN3 degradation, and this effect requires a functional glucose-sensing hexokinase (AtHXK1) [46[•]]. It is not clear whether the acceleration of EIN3 degradation by glucose signaling is the consequence of decreased ethylene production, of a repressed ethylene signaling pathway, or of a more global pleiotropic effect on plant growth exerted by the general requirement to integrate glucose metabolism with hormone-regulated developmental processes.

Secondary transcriptional regulation: integration of ethylene responses with other signals

The current information points towards a model in which the components of the primary ethylene signaling pathway, from the receptors to EIN3/EIL, are common to all ethylene responses (Figure 2). However, exposure to

exogenous ethylene or increases in the synthesis of endogenous ethylene do not always induce the same ethylene responses in different tissue types or at different developmental stages. It could be that ethylene-independent signals are integrated with the primary ethylene signaling pathway to modify the downstream steps of ethylene responses. Biochemical and genetic studies have identified the ethylene-responsive gene *ERF1* as an immediate target for EIN3 [12]. ERF1 was originally identified as a *trans*-factor that binds to the GCC-box, a promoter motif that is present in many ethylene- and pathogen-induced genes [47]. ERF1 belongs to a large family of plant-specific transcription factors that were initially referred to as ETHYLENE RESPONSE ELEMENT BINDING PROTEINS (EREBPs) but later found to function in a diverse range of processes [48]. Overexpression of *ERF1* can rescue the loss of many *EIN3* functions [12]. In addition, *ERF1*-overexpressing plants mimic *EIN3*-overexpressing plants in a subset of ethylene responses, indicating that ERF1 regulates only one branch of the EIN3-mediated ethylene response [12].

Recently, it has been shown that ERF1 also regulates other hormone responses, particularly the jasmonate (JA)-mediated defense response [49,50^{••}]. Ethylene and JA mediate defense responses against pathogen attack partly by inducing the expression of defense genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*). Recently, the GCC-box required for ERF1 binding in the *PDF1.2* promoter has also been identified as a JA-responsive element [51]. These findings suggest that ERF1 might be an essential factor for both hormone signals. Like ethylene, JA is a volatile signal that rapidly induces the expression of *ERF1*, and *ERF1* expression is activated synergistically by treatment with both hormones [50^{••}]. Both signaling pathways are required concurrently for the induction of *ERF1* expression and the activation of its target gene *PDF1.2* [50^{••}]. Blocking either pathway by genetic means prevents *ERF1* induction by the two hormones either alone or in combination [50^{••}]. Constitutive overexpression of *ERF1* is sufficient to restore *PDF1.2* gene expression and to rescue the defense response defects of either ethylene-insensitive or JA-insensitive mutants [12,50^{••}]. Thus, ERF1 functions as a transcription factor that integrates signals from the ethylene and JA pathways. This notion is further supported by a microarray analysis in which a large number of ethylene/JA-responsive genes were co-induced in *ERF1*-overexpressing plants [50^{••}]. The mechanism of the concomitant requirement for both pathways to activate *ERF1* expression is unclear. It is plausible that an unknown JA-induced transcription factor interacts cooperatively with EIN3 in the promoter of *ERF1* (Figure 2). Either transcription factor alone, in a situation in which either the ethylene or the JA signal is blocked, is not sufficient to activate *ERF1* transcription. In wildtype plants, however, the presence of a basal level of either signal (JA or ethylene) is sufficient to allow

ERF1 expression in response to treatment with ethylene or JA alone.

Conclusions

Significant progress toward the delineation of ethylene action in plants has been made during the past decade using a combination of genetic and molecular-biology approaches. This work has made the ethylene pathway one of the most well-defined signaling pathways in plants. However, the detailed biochemical mechanisms of action of each of the known components of the ethylene signaling pathway are just beginning to be understood. Despite our current understanding of the mechanisms of ethylene perception, signal transduction and transcriptional regulation, many questions remain to be answered. It is not clear which 'activity' of ethylene receptors is regulated by ethylene nor how ethylene binding modulates the function of these receptors. Further characterization of the receptor complex would no doubt provide new insights into the mode of ethylene perception.

Despite the increasing evidence that *CTR1* is directly regulated by ethylene receptors, the biochemical events that control such regulation are not well understood. The involvement of a MAPK cascade and its role in the transmission of the ethylene signal are still vague, and more conclusive genetic evidence is needed. Another major unresolved mystery is the function of *EIN2*, a protein that plays an essential role in mediating all known ethylene responses. As for nuclear events, determining how ethylene controls the stability/activity of the primary transcription factors *EIN3/EIL* represents another challenge, as does ascertaining the downstream steps in gene regulation. Global gene expression profiling experiments show that hundreds of genes are induced or repressed by ethylene [41^{••}]. It will be important to discern how many of these genes are immediate targets of *EIN3/EIL1* and, in the long term, to categorize the transcriptional networks in the continuum of ethylene responses.

Ethylene modulates responses to other plant hormones, such as JA, salicylic acid, auxin, abscisic acid (ABA), and cytokinin, but the mechanisms that control each of these critical hormone-hormone interactions are largely unknown. Clearly, an important challenge is to begin to integrate these different hormone signaling pathways, identifying their points of intersection and determining what 'cross-talk' means at the biochemical level. It is certain that the next few years will bring about many more groundbreaking findings in this exciting field of plant hormonology.

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The authors isolated a LOF T-DNA insertion mutation in *ERS1*. The loss of two type-I receptors in *etr1 ers1* double mutants conferred strong constitutive-ethylene response phenotypes, implying that type-I receptors play a special role in ethylene signaling. Because all type-II receptors are predicted to lack a histidine kinase activity, the *etr1 ers1* double mutant provided a platform from which to test whether a histidine kinase activity is required for hormone perception. The expression of each of the five ethylene receptors under an *ETR1* promoter in the *etr1 ers1* background indicated that only type-I receptors (i.e. *ETR1* or *ERS1*) can rescue the double mutant phenotype. However, a mutant form of ETR1 that lacks the histidine kinase activity was able to function like wildtype ETR1, ruling out the requirement of a histidine kinase activity for type-I receptor signaling.

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A convincing study that takes three independent approaches to address the subcellular localization of ETR1. An aqueous two-phase partitioning

experiment suggested that ETR1 is localized in the ER (but not the plasma membrane). To confirm these results, the authors performed sucrose density-gradient centrifugation of *Arabidopsis* microsomes, and the fractions were analyzed by immunoblots. ETR1 co-fractionated with the ER marker BiP (a HSP70 protein) and exhibited the same diagnostic Mg²⁺-dependent density shift as BiP. The third line of evidence came from immunogold electron microscopy. Gold granules representing ETR1 were detected on the ER. The authors further showed that the ER localization of ETR1 was determined by its amino-terminal ethylene-binding domain, but was not affected by ethylene binding or *etr1-1* dominant mutation.

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This work provides possible mechanistic insight into the regulation of CTR1 by the ethylene receptors. Analysis that was based on sucrose-density-gradient fractionation showed that CTR1 was primarily localized to the ER. The ER localization of CTR1 was dependent upon the presence of ethylene receptors. A mutation in CTR1 that eliminates ETR1 binding resulted in the dissociation of CTR1 from the ER. An *in-vivo* association between ETR1 and CTR1 was demonstrated by affinity co-purification. Moreover, an *in-vitro* pull-down assay revealed that CTR1 binds to the carboxy-terminal half of ETR1, but a histidine kinase activity was not required for CTR1-ETR1 interaction. The authors proposed a model for signaling by the ethylene receptor-CTR1 complex in which CTR1 is activated by association with the ER-bound receptors.

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Ethylene and JA have been shown to induce several plant defense genes synergistically. This study defined a previously isolated ethylene response factor, ERF1, as a common downstream component of two hormone response pathways. *ERF1* expression was induced by both hormones, and this induction required the concurrent existence of both signaling pathways. Overexpression of *ERF1* suppressed the defense response defects observed in JA-insensitive mutants. Global gene expression analysis showed that many ethylene/JA-regulated genes are constitutively activated in *ERF*-overexpressing plants. Thus, ERF1 is demonstrated to be a key factor in the integration of two hormone signals.

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