The Role of Ethylene in Host-Pathogen Interactions

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Abstract

The phytohormone ethylene is a principal modulator in many aspects of plant life, including various mechanisms by which plants react to pathogen attack. Induced ethylene biosynthesis and subsequent intracellular signaling through a single conserved pathway have been well characterized. This leads to a cascade of transcription factors consisting of primary EIN3-like regulators and downstream ERF-like transcription factors. The latter control the expression of various effector genes involved in various aspects of systemic induced defense responses. Moreover, at this level significant cross-talk occurs with other defense response pathways controlled by salicylic acid and jasmonate, eventually resulting in a differentiated disease response.

INTRODUCTION

In order to survive the continuous threat of diverse pathogenic microorganisms in their immediate vicinity, plants have developed efficient defense mechanisms. These include physical barriers and the production of antimicrobial components, in both a preformed and an induced manner. Induced mechanisms involve the specific perception of pathogen attack and the subsequent build-up of appropriate defense responses. Among the early steps of induced defense responses are the generation of reactive oxygen species (such as $O_2^{\cdot -}$ and H_2O_2) and nitric oxide. Depending on the extent of the response, these reactive oxygen species can lead to a hypersensitive response (HR) characterized by a rapid programmed death of host cells (reviewed in 100, 166). Although HR is generally effective in halting further ingress by biotrophic pathogens, which need living host cells for nutrient supply, it usually does not affect, or even promote, infection by necrotrophs. More downstream induced responses rely on a network of cross-communicating signaling pathways of which salicylic (SA), jasmonic (JA) acid, and ethylene (ET) are the principal mediators (reviewed by 36, 150). Different studies indicate that JA- and ET-signaling often operate synergistically to induce the effector genes of induced defense responses (40, 117, 119, 134).

In this chapter we focus on the possible role of ET in the plant's interaction with microbial pathogens. Although ET is involved in very different aspects of plant life, a major part of the ET-pathway seems to be conserved in all ET-mediated responses. Many excellent reviews have been published on general ET biosynthesis (12, 132, 162) and downstream signaling events (3, 29, 59, 162), as well as on overall aspects of signaling in plant disease responses including ET-controlled mechanisms (53, 134, 143, 150). Here we aim to combine current understanding of both general ET biosynthesis and downstream ET-signaling and ET involvement in host-pathogen interactions. This broad scope limits us to summarizing generally accepted processes and elaborating only on recent findings. Moreover, because basic aspects of ET-mediated responses appear relatively well conserved among plant species, we focus mainly on the model plant *Arabidopsis thaliana*, and refer readers to an excellent recent review by Anderson et al. (6) for extrapolation to other plants.

Hypersensitive response (HR): a programmed cell death in plants that occurs locally in response to attempted invasion by some pathogens. It is characterized by the rapid production of reactive oxygen intermediates and collapse of the plant cell, thereby preventing further infection specifically by biotrophic pathogens

SA: Salicylic Acid

JA: Jasmonic Acid

ET: Ethylene

AdoMet: *S*-adenosyl-methionine

ADS: *S*-AdoMet synthase

ACS: 1-aminocyclopropane-1-carboxylate synthase (ACC synthase)

ACC: 1-aminocyclopropane-1-carboxylic acid

ACO: 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)

ETHYLENE BIOSYNTHESIS

Pathogen challenge of plant tissues in many cases triggers enhanced ET production (13, 34, 91, 118, 128, 158). The early steps that precede activation of ET biosynthesis genes or enzymes, next to a range of other cellular responses, involve and ensure recognition of pathogen-derived elicitor molecules and/or avirulence molecules by plant receptors. These early responses have been reviewed extensively elsewhere (64, 102, 105) and thus are not, or only briefly, discussed here. Instead we focus on the different levels of transcriptional and posttranscriptional activation of genes and gene products involved in ET biosynthesis (**Figure 1**).

The biosynthetic pathway of ET was unraveled to a large extent by the pioneering work of Yang and co-workers in the 1970–1980s (81). ET is synthesized from the amino acid methionine, which is converted to *S*-adenosyl-methionine (AdoMet) by the enzyme *S*-AdoMet synthase (ADS). AdoMet is the major methyl donor in plants and is involved in the methylation reactions of lipids, proteins, or nucleic acids (45). AdoMet is converted by the enzyme ACS to 5′-methylthioadenosine (MTA), which is converted back to methionine via the Yang-cycle and to 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ET. ACC is finally oxidized by ACC oxidase (ACO) to form ET, cyanide, and carbon dioxide. The conversion of AdoMet to ACC by ACS is the first committed and generally considered as the rate-limiting step in ET biosynthesis, and consequently has been most intensively studied.

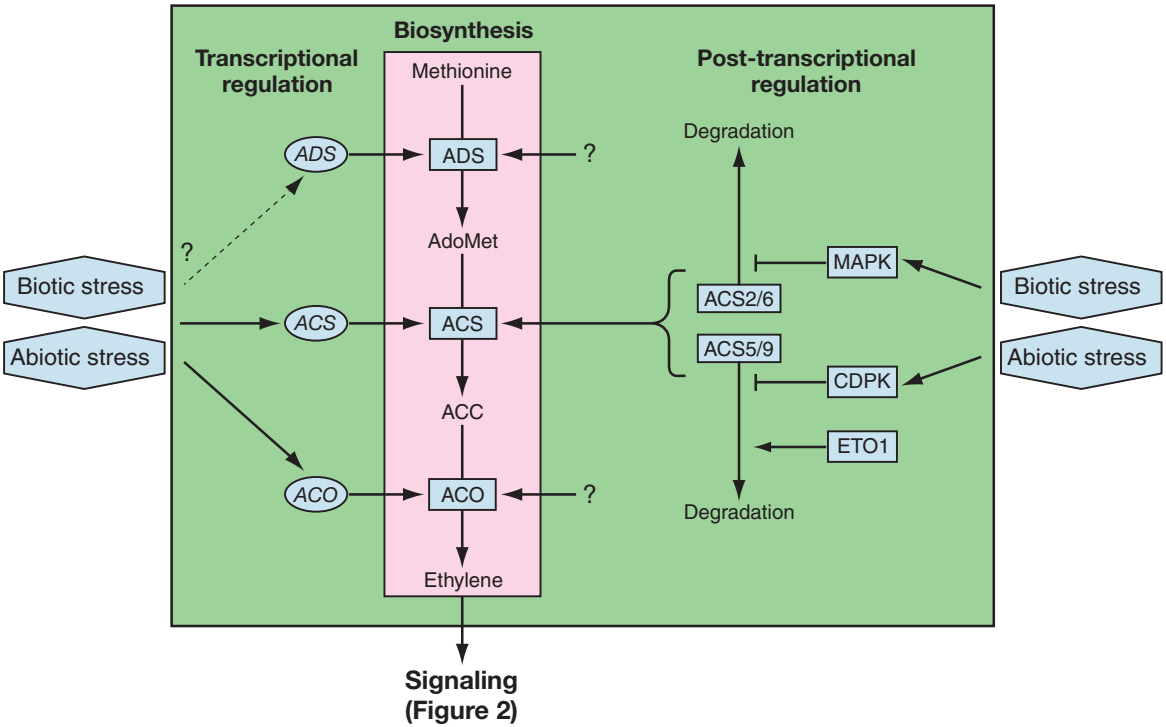

Figure 1

Simplified schematic representation of the ET biosynthesis pathway. Arrows and end-blocked lines indicate positive and negative regulation, respectively. Genes and proteins are represented in light blue ovals and boxes, respectively.

Transcriptional Regulation of Ethylene Biosynthesis

Genes encoding plant ADS have been cloned from various plant species (including Arabidopsis), and their characterization has revealed that they are highly conserved in all organisms and encoded by a gene family (125). ADS is involved in many more metabolic pathways than just ET biosynthesis (125). Apparently, no specific induction of ADS genes upon pathogen attack or abiotic stress stimuli has been reported. Thus, in an analysis of publicly available gene expression data (using "Genevestigator"; 177) we were not able to detect any pronounced pathogen-mediated gene induction or repression of ADS genes.

ACOs are encoded by multigene families in all plant species studied so far (162). The members of ACO gene families are differentially expressed during plant development or in response to pathogen attack and abiotic stress stimuli such as wounding, flooding, or ozone exposure (34, 94, 98, 101, 169). Very recently, Cohn & Martin (34) showed the involvement of the virulence/avirulence factors AvrPto and AvrPtoB from the bacterial pathogen *Pseudomonas syringae* pv. *tomato* in ET biosynthesis in a susceptible tomato cultivar. More particularly, these pathogen-derived proteins up-regulated two specific ACO genes (*LeACO1/2*), whereas other tomato ACO genes remained unaffected. Screening for all Arabidopsis ACO-encoding genes in reported expression data (177) also demonstrated significant abiotic and biotic stress-mediated gene regulation for particular subsets of ACO genes. As an example, the ACO genes at loci At1g62380

and At1g05010 in particular appear to be up-regulated upon treatment of Arabidopsis with ET and *Botrytis cinerea*, whereas the ACO gene at At1g12010 tends to be down-regulated following inoculation with *P. syringae* and *Alternaria brassicicola*. The other ACO-encoding genes are not affected under these experimental conditions. Taken together, the differential expression patterns of *ACO* genes suggest that transcriptional control of these genes contributes to the regulation of ET production. The control of ET production remains, however, largely attributed to the *ACS* genes, as discussed below.

ACSs are encoded by multigene families in plants (12, 78). The majority of *ACS*-expression studies have been conducted in tomato, which contains at least 10 *ACS* genes, and in *Arabidopsis thaliana*, which contains 9 *ACS* genes. Because of their pivotal role in ET biosynthesis, the regulation of *ACS* has been thoroughly studied. A first level of ET biosynthesis regulation occurs at the ACS gene expression level. Indeed, many studies have demonstrated that differential transcription of the various members of the *ACS*-gene families is an important factor regulating ET production in response to different stimuli (7, 113). Recently, Tsuchisaka & Theologis (154) examined the spatio-temporal expression patterns of the *ACS* gene family members in Arabidopsis during plant growth and development and under different abiotic stress stimuli, and they showed specific and partially overlapping patterns of expression among the various *ACS* gene family members in specific tissues. For instance, wounding of hypocotyl tissue inhibited the constitutive expression of *AtACS1* and *AtACS5* in this tissue and induced the expression of *AtACS2*, *4*, *6*, 7, *8*, and *11*. Unfortunately, expression patterns of the different *ACS* genes upon pathogen infection have not yet been examined. It would be of great interest to explore whether the different key genes show a specific expression profile after various pathogen inoculations, including viruses, bacteria and different nectrotrophic and biotrophic fungi. In a preliminary search (177) on the expression patterns obtained for all 9 Arabidopsis ACS-encoding genes, we found cases of significant pathogen-induced gene up-regulation, pathogen-induced repression, or invariant gene expression following various pathogen interactions. As an example, *AtASC2* in particular is strongly up-regulated upon challenge of Arabidopsis with *P. syringae*, *B. cinerea*, and *Alternaria brassicicola*, whereas *AtACS5* and *AtACS11* tend to be down-regulated following inoculation with *P. syringae*. The other ACS-encoding genes appear not to be affected under these experimental conditions.

Posttranscriptional Regulation of Ethylene Biosynthesis

Whereas transcriptional regulation of *ACS* gene family members is central for enhancement of ET production, recent findings indicate the pivotal role of ACS protein turnover as a key regulator of ET production in plants (reviewed in 22). Studies on the Arabidopsis *ethylene-overproducer* (*eto*) mutants (61, 82) have provided compelling evidence that ACS protein turnover is indeed regulated posttranscriptionally and that this regulation mechanism provides a handle on ET biosynthesis (22, 168). The molecular mechanisms regulating AtACS5 activity and turnover were finally elucidated by cloning of the gene responsible for the *eto1* mutation (163). ETO1 is a member of a novel protein family, unique to the plant kingdom, featuring some distinct protein-protein interaction motifs including a BTB (Broad-complex, Tram-track, Bric-à-brac) domain. BTB domain–containing proteins have been shown to link CUL3-based ubiquitin ligase to substrate proteins (122). Wang et al. (163) have demonstrated direct interaction of ETO1 with both AtACS5 and CUL3 using in vitro pull-down assays, suggesting that ETO1 acts as a substrate-specific adaptor protein for AtACS5 and possibly also

for other ACS isozymes, thereby targeting these ACS proteins for degradation by the 26S proteasome. Moreover, ETO1 inhibits the activity of wild-type AtACS5 but not *eto2* mutant AtACS5 in vitro (163). Together, this study suggests that ETO1 directly inhibits AtACS5 enzymatic activity in addition to its effect on the proteolysis-mediated stability of the ACS5 protein.

Although there is increasing insight into the regulation of ET biosynthesis via proteolysis of ACS, an important question regarding the control of this mechanism is not fully answered. A mechanism for ACS breakdown would involve the modification of the ACS proteins themselves in such a way that they become targeted for degradation by the ubiquitin-26S-proteasome machinery. One probable candidate for such a modification is protein phosphorylation. Several studies in tomato and Arabidopsis provide explicit indications that protein phosphorylation regulates the turnover of the ACS proteins. For example, a serine residue in the carboxy terminus of LeACS2 was found to be phosphorylated by a calcium-dependent protein kinase (CDPK) present in extracts of wounded tomato fruits (142). This study, in combination with protein kinase and phosphatase inhibitor studies (138, 155), suggest that ACS protein turnover is regulated through phosphorylation by CDPK. A more intriguing question is by which stimuli, including challenge by pathogens, CDPK phosphorylation and consequently ACS protein turnover and ET biosynthesis are regulated. Another recent study in Arabidopsis revealed that some ACS proteins are substrates for mitogen-activated protein kinase (MAPK) phosphorylation and that this phosphorylation regulates their stability (88). In particular, activation of a MAPK pathway involving MPK6 led to in vivo stabilization of the AtACS6 protein and to in vitro phosphorylation of three conserved serine residues of both AtACS2 and AtACS6. AtACS4, -5, -8, and -9 isozymes do not have these conserved serine residues. Moreover, AtACS5 is not phosphorylated by MPK6 in vitro (88). These findings suggest that MPK6 phosphorylation inhibits the degradation of AtACS2 and AtACS6 proteins and that both enzymes are stabilized in response to pathogens and other stresses through direct phosphorylation by MPK6 (22). Numerous studies have previously demonstrated the role of MAPK cascades in defense signaling and plant immunity (recently reviewed by Pedley & Martin, 114)

As a conclusion, at least two parallel signaling pathways, involving either a MPK6 or a CDPK, appear to play a role in modulation of ACS function and regulation of ET biosynthesis. In both cases, phosphorylation could block the interaction with the ETO1-CUL3 ubiquitin ligase and 26S-proteasome-directed breakdown of the ACS proteins. However, the exact link between pathogen recognition and control of ACS protein stability remains to be elucidated. Ludwig et al. (90) demonstrated in tobacco that CDPK and MAPK signaling trails do not function independently and that a concerted activation of both pathways controls response specificity to abiotic and biotic stresses. Such parallel signaling branches not only offer a back-up system to assure multiple activation events as a reaction to one stimulus, but also allow fine-tuning of responses by regulating different sets of partly overlapping reactions. ET biosynthesis and perception are thought to fulfill a central role in this cross-talk. In the case of ET biosynthesis, different ACS members become activated depending on the signaling branch (**Figure 1**).

A final remark concerns the positioning of the MPK6 pathway in the overall ET-mediated response. This was originally proposed by Ouaked et al. (112) to be downstream of ET perception (discussed below). As discussed above, the ACS proteins are the direct interaction partner of MPK6, and a null MPK6 mutation affects only ET biosynthesis and not ET responsiveness. Therefore, it is more likely that MPK6 is situated upstream of ET perception (37).

Proteasome: A protein complex that is a part of a major catabolic pathway that degrades intracellular proteins after they have outlived their usefulness. The degradation process occurs in both the nucleus and the cytoplasm, and involves marking the target proteins through the addition of ubiquitin molecules (ubiquitination) for complete hydrolysis

MAPK: mitogen-activated protein kinase

CDPK: calcium-dependent protein kinase

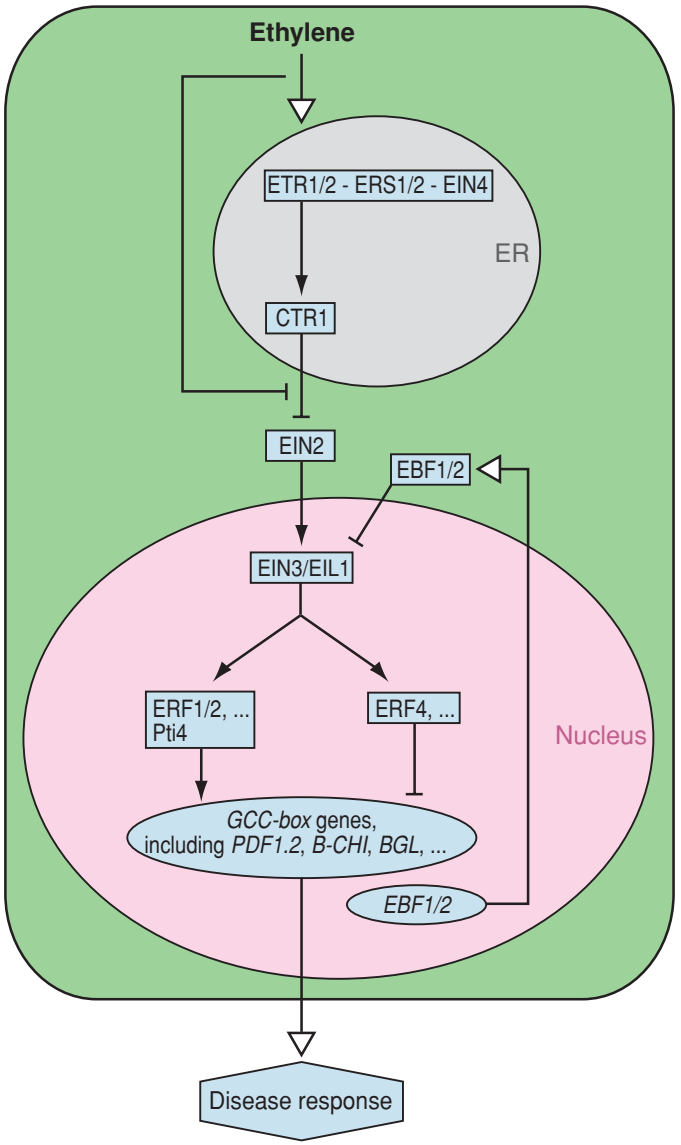

Figure 2

Simplified schematic representation of the ET downstream signaling pathway mentioned in the text. Arrows and end-blocked lines indicate positive and negative regulation, respectively. White arrows indicate signal direction. Genes and proteins are represented in light blue ovals and boxes, respectively.

PERCEPTION AND CYTOPLASMIC SIGNALING

ET produced after either internal or external stimuli is perceived by the cell and this signal is further transmitted through a single well-conserved signaling cascade (for recent reviews see 29, 59, 139). Most of the components of this pathway have been discovered using simple genetic screens focused on Arabidopsis mutants with an impaired ET response, such as the morphological "triple response" of seedlings to ET exposure (61). The triple response is characterized by an inhibition of hypocotyl and root cell elongation, a radial thickening of the hypocotyl and a hypercurving of the apical hook. Mutants resulting from these screenings could be categorized as either ET-insensitive mutants or constitutive ET-response mutants. Both classes of mutants have proven to be invaluable tools for unraveling the ET perception and signaling cascade (**Figure 2**).

Ethylene Perception by Endoplasmatic Reticulum Receptors

Based on the analysis of ET-insensitive mutants impaired in early hormone perception, a family of five receptors (ETR1, ETR2, ERS1, ERS2, EIN4) was identified (11, 24, 70, 72, 129) sharing sequence similarities with bacterial two-component regulators (123). Among all five receptors the most conserved domain is their N-terminal part, consisting of three or four transmembrane regions in subfamily 1 (ETR1, ERS1) and subfamily 2 (ETR2, ERS2, EIN4) receptors, respectively. This N-terminal domain has been shown to be responsible for ET binding for at least ETR1 and ERS1 (62, 127, 131). It has been further demonstrated that ETR1 and ERS1 are present in the endoplasmic reticulum (ER) membrane as disulfide-linked homodimers (62, 133). Each homodimer forms a hydrophobic pocket constituting one ET-binding site and ET binding to this pocket is mediated through a single copper cofactor (74, 167).

In contrast to the well-conserved ET-binding region, conservation of the remaining domains of the receptors appears to decrease from the N to the C terminus. Other domains present in the ET receptors include a

potential cGMP binding site (25) and a histidine kinase region that is believed to be functional only in subfamily 1 ET receptors (50, 72). However, the role of these domains in ET perception or further signal transduction is not clear (51, 164). Based on in vitro assays, serine kinase activity has been suggested for subfamily 2 receptors as well as for ERS1 as an alternative to the histidine kinase mechanism for transducing the phosphorylation signal to downstream components of the ET signaling cascade (95). Whether both types of kinase activities are also significant in vivo needs to be further investigated.

Even less is known about the specific role of the C-terminal part of the receptors, consisting of a specific receiver domain in ETR1, ETR2, and EIN4 receptors. Along with the kinase domain, the receiver moiety of ETR1 appears to participate in the interaction of the receptor with CTR1, a downstream component of the ET signaling cascade (33). It has been postulated that the ERS-type receptors, lacking this domain, might use the receiver domain of other proteins through the formation of heterodimers (72).

Despite these structural differences among the different ET receptors, various genetic and biochemical studies revealed a remarkable functional redundancy. They all appear to be inhibited by ET binding and to act as negative regulators of ET responses (71, 127, 131). As such, the observed induction of their genes by ET should be seen as a means of resensitization of the plant to ET. Additional posttranscriptional regulation has been proposed for ETR1, although the underlying mechanism is not yet known (29).

There is solid evidence that ET perception occurs at the ER based, among others, on the observation that ET receptors are localized in the ER membrane (29, 30). This is consistent with the fact that (*i*) the ER is a point of junction for diverse cellular processes including several stress responses (reviewed in 65), and (*ii*) ET is a key regulator in several of these physiological processes (reviewed in 12, 25, 78, 162).

Downstream Intracellular Ethylene Signaling

Studies of the Arabidopsis mutant *ctr1* (constitutive triple response) resulted in the identification of CTR1 as a negative regulator of ET responses (82). Cloning of the CTR1 gene revealed that it is composed of an N-terminal domain of unknown function and a C-terminal kinase domain with highest homology to Raf-like serine-threonine kinases (82). Such Raf-kinases have been demonstrated in mammalians to act in mitogen-activated protein (MAPK) signaling cascades (116). Similarly to ET receptors, localization studies indicated that CTR1 is primarily associated with the ER (52), although CTR1 does not contain any predicted transmembrane domain or membrane attachment motifs. The ER-location of CTR1 has been explained through its association with the ET receptors (21, 33, 52, 71, 73). Such a direct interaction is also the basis for signal transmission between both types of components of the ET signaling pathway. It appears that binding to the ET receptors (through the N-terminal part of CTR1) and kinase activity (through its C-terminal kinase moiety) are both necessary for correct negative regulation of downstream ET responses (21, 52, 73). A model for the signaling through the ETR1-CTR1 complex is suggested by Gao et al. (52). In the absence of ET, binding of the receptors to CTR1 would maintain the latter's conformation such that it remains active and thus acts as a negative regulator able to repress downstream ET responses. Binding of ET would cause a conformational change of CTR1, resulting in release of its downstream inhibitory effect. Such a conformational change in the N terminus could autoinhibit its C-terminal Ser/Thr kinase activity, a model earlier proposed for the mammalian protein Raf kinase (68). In analogy with Raf kinase signaling, a MAPK kinase pathway has been proposed to function in ET signaling (104, 112). More recent studies, however, raised serious doubts about the validity of these conclusions (37, 88), and rather

EIL: EIN3-like

propose such a pathway to be positioned upstream of ET perception, as mentioned earlier. A MAPK kinase cascade may still function downstream of CTR1, but in the absence of conclusive experimental evidence, this MAPK module has again been removed in the most recent model for primary ET signaling (139).

Another key mutant resulting from screens for ET insensitivity was *ein2* (61). Cloning of the EIN2 gene led to the identification of a novel plant-specific protein consisting of two well-defined domains: a unique hydrophilic C terminus harboring motifs typically involved in protein-protein interactions, and a hydrophobic N-terminal part containing 12 predicted transmembrane helices showing similarity to Nramp-proteins (2). Nramp-related proteins are described as metal transporters in different organisms ranging from bacteria to humans (80, 140). However, no metal transport activity has been detected so far for EIN2 (2). Although EIN2 behaves as an integral membrane protein, attempts to localize it at the subcellular level have been unsuccessful (2). The complete lack of ET sensitivity in *ein2* loss-of-function mutants suggests an essential role of this protein as the first positive regulator of ET responses. Overexpression of the C-terminal part appeared sufficient to constitutively activate ET responses, suggesting that this part of EIN2 is responsible for further downstream signal transduction (2).

NUCLEAR TRANSMISSION OF THE ETHYLENE SIGNAL

Primary Transcriptional Regulation: EIN3/EIL Transcription Factors

The ET signal arrives at the nucleus through derepression of EIN2 by CTR1 and leads to the activation of EIN3 and EIN3-like transcription factors (26) (**Figure 2**). In Arabidopsis, there are six members of the EIN3 family (EIN3 and EIL1–5) among which EIN3 and EIL1 are the most closely related proteins and apparently the most important for ET sensitivity (4). The other family members (EIL2–5) seem to play a marginal role in the ET response or, alternatively, may participate in specific tissues or may even function in different ET-independent signaling pathways (27, 59, 76, 126, 151). Recent data support a tissue-specific role for the EIL2–5-like transcription factors during growth and development (76, 174).

Modulation of EIN3/EIL1 activity by ET is not primarily achieved by transcriptional regulation but through proteolytic control of EIN3 protein levels by a SCF (SKP1/Cullin/F-box protein) E3 ubiquitin ligase complex (26, 49, 58, 124, 126, 151, 172). The Arabidopsis F-box proteins AtEBF1 and AtEBF2 were shown to physically interact with EIN3 and analysis of *atebf1/2* mutant plants and *AtEBF*-overexpressing lines confirms their role in destabilization of EIN3 (49, 58, 124). Therefore, it seems that, in the absence of ET, EIN3/EIL1 proteins are continuously degraded through the AtEBF1/2-directed and proteasome-mediated pathway, thereby preventing activation of their transcriptional targets. In the presence of ET, degradation of EIN3 is suppressed, thereby allowing EIN3 protein levels to increase and promote downstream events. At least for AtEBF2, up-regulation of expression by ET has been demonstrated (49, 124), suggesting that a negative feed-back mechanism might equilibrate EIN3/EIL protein levels. Since multiple protein kinases have been placed upstream of EIN3/EIL (59), it is also possible that phosphorylation of EIN3 inhibits its association with AtEBF1/2, thereby preventing its degradation (49, 58, 124). Still undetermined is if and how this modification would take place through action of EIN2. However, EIN3/EIL proteins are clearly essential "ethylene switch" molecules in the sense that slight changes in their level controls the signal flux to downstream nuclear events.

The EIN3 transcription factor recognizes its DNA target, the so-called EIN3-binding site (EBS) or primary ET response element (PERE) in the promoters of ET response element binding proteins (EREBP) genes, and

does this in the form of a homodimer protein complex (59, 84, 137). Recently, determination of the DNA-binding region of AtEIL3 by NMR spectroscopy revealed that it consists of five α-helices, possessing a novel fold unlike known DNA-binding domain structures (171).

Secondary Transcriptional Regulation: AP2/ERF Transcription Factors

Biochemical and genetic studies in Arabidopsis have identified the transcription factor AtERF1 as an immediate target for the primary ET-responsive transcription factor EIN3 (3, 59, 137). Overexpression of ERF1 genes from Arabidopsis, tomato, tobacco, and rice was shown to rescue the loss of EIN2 and EIN3 functions (20, 59, 89, 92, 137), providing evidence that ERF1 acts downstream of EIN3.

AtERF1 belongs to a family of so-called Ethylene Response Factors (ERFs), also termed Ethylene Responsive Element Binding Proteins (EREBPs) (59, 60, 108, 137, 176). ERFs have been identified in several plant species as proteins that bind to the so-called GCC-box present in promoters of several ET-inducible genes, for instance those encoding pathogenesis-related (PR) proteins. The GCC box is a *cis*-acting ET response element, consisting of an 11-bp conserved sequence (TAAGAGCCGCC), shown to be necessary and sufficient for ET regulation of ET-responsive effector genes in a variety of plant species (66, 93, 108). ERFs interact in vitro with the GCC-box through a domain homologous to that previously observed in the floral homeotic protein APETALA2 (AP2) from Arabidopsis (reviewed in 60). ERFs also show homology to transcription factors that bind to the dehydration-responsive element binding (DREB) in the promoters of genes that are responsive to abiotic stress (60, 92). Together, the AP/ERF superfamily of transcription factors comprises at least 145 members in Arabidopsis (60).

Recently, the Arabidopsis HDA19, one of the histone deacetylases essential for eukaryotic gene expression regulation, has been implicated in EIN3-ERF1–mediated ET signaling (175). How the interplay between EIN3 and HDA19 precisely enables transcriptional activation of ERF1 and/or downstream nuclear events is not clear thus far. In addition to transcriptional regulation of ERF proteins through action of EIN3/EIL factors, posttranscriptional regulation through phosphorylation may be a common theme in the secondary ET-dependent level of transcriptional regulation: AtERF5 contains a potential MAPK phosphorylation site (48) and phosphorylation of the rice OsEREBP1 protein was shown to enhance its binding to the GCC-box (31).

Most of the ERF proteins identified so far have been shown to function as transcriptional activators (48, 110, 111, 137, 170, 176). For example, AtERF1, AtERF2, and AtERF5 transcriptionally activate GCC-box containing genes (48). However, a second class of ERF proteins act as transcriptional repressors. Arabidopsis AtERF3, AtERF4, AtERF7, AtERF10–12, and tobacco ERF3 repress the expression of a GCC-box-containing reporter gene (48, 109, 110). The gene repression motif (L/F)DLN(L/F)(x)P, also termed ERF-associated amphiphilic repression (EAR) motif, was found to be present in all these ERF proteins (109). At least eight Arabidopsis AP2/ERF protein family members contain this motif (60). Recently, overexpression of *AtERF4* was shown to confer an ET-insensitive phenotype and to repress the expression of the GCC-box containing genes encoding basic chitinase and β-glucanase (173). Remarkably, and illustrating the complexity and specificity of the ET-responsive network of secondary transcriptional regulation, overexpression of *AtERF2* and *AtERF4* results in opposite disease-resistance phenotypes toward infection with the necrotrophic pathogen *Fusarium oxysporum* (92).

Specific actions of the different ERF transcription factors that can either activate or

ERF: ethylene responsive factor

PR: pathogenesis-related

AP2: Apetala2

DREB: dehydration-responsive element binding

EREBP: ethylene response element binding protein

repress particular defense response genes likely provide for a level of fine-tuning according to the kind of biotic stress perceived. Such fine-tuning of the defense response would avoid unnecessary action by the plant.

ETHYLENE IN PLANT DISEASE RESISTANCE

As described above, ET biosynthesis is activated in many plants challenged by pathogens, and increased ET production induces defense-related effector genes through a cascade of events of which the penultimate step is the activation of ERF-type transcription factors. In this section we focus first on different types of effector genes that are induced upon pathogen challenge. Moreover, we briefly highlight the cross-talk between ET-signaling and other mechanisms of induced resistance, including those mediated by SA and JA. Finally, we discuss the role of ET and ET signaling in determining the modulation of resistance and susceptibility of host-pathogen interactions.

Ethylene-Dependent Induction of Effector Molecules

Pathogen-induced defense responses ultimately result in the expression of numerous defense-related genes. The corresponding proteins include (*i*) proteins that participate in the build-up of physical barriers and as such in the physical confinement of the pathogen; (*ii*) enzymes of secondary metabolism, for instance, those functioning in biosynthesis of antimicrobial secondary metabolites; and (*iii*) pathogenesis-related (PR) proteins, the latter representing the largest quantitative changes in soluble protein during defense responses.

The specific role of ET signaling in the formation of induced structural barriers has so far received relatively little attention. Cell-wall strengthening hydroxyproline-rich proteins accumulate in plants upon treatment with ET (38, 41, 141). Such hydroxyproline-rich proteins are structural components deposited in the cell wall and their presence has been associated with cell wall fortification, especially after oxidative cross-linking of such proteins (15). Furthermore, VanderMolen and coworkers (157) demonstrated that ET is required for the xylem occlusion response that occurs in plants to counter the further spread of wilt pathogens such as *Fusarium oxysporum* f.sp. *lycopersici*, through the plant's vascular system. On the other hand, the local deposition of callose in the cell wall at sites of attempted penetration by pathogens appears not to be mediated by ethylene but instead involves synthesis and perception of abscisic acid (153).

The role of ET in the pathogen-induced production of antimicrobial secondary metabolites (phytoalexins; 115) appears to be dependent on the type of phytoalexin and the metabolic pathway involved. In rice leaves, for example, ET induces the production of the phenylpropanoid-derived phytoalexin sakuranetin, but not of the terpenoid phytoalexin momilactone A (99). In general, phytoalexins derived from the phenylpropanoid pathway are inducible by ET in different plant species (32, 77, 79). In Arabidopsis, pathogen-induced production of camalexin, an indole alkaloid phytoalexin, is controlled by a reactive oxygen species-mediated pathway that exhibits little or no cross-talk with ET- and JA-dependent signaling, and mutants affected in ethylene signaling, such as *ein2*, are still able to synthesize camalexin in response to pathogen attack (43, 54, 149).

By far the most extensively documented ET-induced defense-related effector molecules are the so-called pathogenesis-related (PR) proteins. Currently, 17 PR-classes have been identified (160), of which the majority have been shown to exert direct antimicrobial activity against fungal species and occasionally against bacterial species (reviewed in 16; see Van Loon, this issue). Distinct PR gene classes were shown to be ET responsive through the GCC-box element in their promoter regions (see above), including

vacuolar β-1,3-glucanases (PR-2), vacuolar basic-chitinases (PR-3), acidic hevein-like proteins (PR-4), and plant defensins (PDFs; PR-12) (17, 23, 42, 89, 107, 117, 130, 148). Induction of these PR-genes occurs via a pathway in which ET and JA operate synergistically (118). Other types of PR genes, including PR-1 proteins, and extracellular β-1,3-glucanases and chitinases, are induced through an SA-dependent pathway, at least in Arabidopsis and tobacco (14, 47, 156). Both the ET/JA and SA-induced PR genes are induced in the infected zone as well as systemically (reviewed in 16; see Van Loon, this issue).

Recent high-throughput transcript profiling studies have shown that a range of PR-genes are induced in Arabidopsis plants in which either *AtERF1* or its tomato homolog *Pti4* is overexpressed, confirming the ET-responsiveness of these *PR*-genes and adding several novel potential ET regulated signaling and effector molecules to the list (23, 89). Remarkably, a recent proteomics study on proteins secreted by Arabidopsis upon pathogen challenge has revealed that GLIP1, a novel secreted lipase, acts as an ET-responsive antimicrobial effector molecule critical for disease resistance to the incompatible fungal pathogen *A. brassicicola* (106).

The plant defensin gene *AtPDF1.2* (group PR-12) is widely used as a marker for ET/JA-induced signaling in Arabidopsis defense responses (117, 118). The gene contains GCC box promoter elements and is inducible by both ET and JA through activation of AtERF1 (see below; 26, 89, 117, 137). Plant defensins in general are small, basic peptides that have a characteristic three-dimensional folding pattern that is stabilized by eight disulfide-linked cysteines, and typically inhibit the growth of a broad range of fungi after specific binding to membrane targets (144–146). Recent advances in gene annotation revealed the presence of 317 novel defensin-like (DEFL) genes in the Arabidopsis genome (136), including the 13 previously annotated plant defensin (*AtPDF*) genes (145). It is an intriguing question whether all or only a subset of these genes are responsive to ET. Our own observations on the originally identified *AtPDF* genes indicate a largely differential expression pattern in response to ET, SA, JA, and different marker pathogens. For example, the genes encoding AtPDF1.2a/b/c are induced by ET and JA and repressed by SA, whereas *AtPDF1.4* is not responsive to ET or JA but is induced by SA. Consistently, analysis of their promoter regions indicated a GCC-box domain present in all three *AtPDF1.2* genes, but not in *AtPDF1.4*. In contrast, *AtPDF1.5* appears unresponsive to any of these treatments, although also harboring a GCC-box (B.C., unpublished results). This suggests that a more complex network exists in which different groups of DEFLs respond to one or more signaling pathways.

PDF: plant defensin

DEFL: defensin-like protein

Interactions between Primary ET Signaling and Other Mechanisms of Induced Resistance

The primary ET signaling pathway components described earlier (ETR/ERS/EIN4, CTR, EIN2, EIN3/EIL) are required for all known ET responses and, to date, none has been found to respond to signals other than ET (3, 59, 143). Branch points in the ET response pathway therefore must lie downstream of EIN3/EIL. In fact, differential regulation by disease-related stimuli such as ET, JA, SA, and infection by virulent or avirulent pathogens has been shown for several ERF genes (20, 23, 28, 48, 57, 59, 89, 92, 111). As such, significant cross-talk between different signaling pathways appears to occur at this level. In the present review we do not specifically aim to present an overview of all reported points of cross-communication between disease-related signaling pathways but refer therefore to various excellent schematic presentations in recent reviews (5, 143, 162). In the section below we rather concentrate on some illustrative overlaps between ET-dependent disease responses and other mechanisms of induced

resistance in plants including gene-for-gene resistance, JA- and SA- dependent resistance, and rhizobacteria-induced resistance.

Gene-for-gene resistance: resistance based on the genetic interaction between a dominant plant resistance (R) gene and a complementary dominant pathogen avirulence (Avr) gene

Gene-for-gene resistance. In gene-for-gene resistance, the plant-pathogen interaction and subsequent plant signal transduction upon recognition of the pathogen avirulence products result in resistance against the pathogen in most cases through an HR. It is well documented that the HR involves and is potentiated by the SA-dependent signaling pathway (53, 63). However, in a number of cases gene-for-gene interactions were also linked to ET-dependent gene expression, as exemplified in the study of the protein kinase encoding resistance gene *Pto* from tomato. Recognition of the pathogen avirulence product AvrPto from *P. syringae* pv. *tomato* by Pto induces changes in the expression of over 400 genes, an oxidative burst and an HR (46, 96, 135). Remarkably, Pto interacts directly with the AP2/ERF transcriptional activators Pti4, Pti5, and Pti6 (56, 176). Furthermore, expression of the transcription factor Pti4 is rapidly induced by ET, and binding of Pti4 to the GCC box in defense response genes is regulated by its Pto kinase-mediated phosphorylation (57). However, consistent with its involvement in gene-for-gene resistance, Pti4 seems able to bind to non-GCC-box promoter elements as well (23), to induce SA-dependent PR-gene expression and to increase resistance to biotrophic pathogens when overexpressed in Arabidopsis (56). Therefore, it is unclear whether ET is causally involved in gene-for-gene resistance or whether gene-for-gene interactions rather trigger the induction of ET-regulated genes. In fact, the Arabidopsis ethylene signaling mutants *etr1* and *ein2* were shown not to be impaired in gene-for-gene–type resistance to biotrophic pathogens such as the Oomycete *Hyaloperonospora (Peronospora) parasitica* and the bacterium *P. syringae*, suggesting that ET is not required for gene-for-gene resistance per se (85, 86, 121).

Ja-dependent induced resistance. The ET- and JA-mediated signaling pathways act synergistically in defense responses (40, 117, 121). Such synergism has been supported by microarray analyses indicating clusters of genes that are commonly induced by ET or JA (55, 134). Furthermore, the GCC-box required for AtERF1 binding in the systemically pathogen-induced gene *AtPDF1.2* from Arabidopsis has also been identified as a JA-responsive element, indicating that AtERF1 is a point of integration for ET and JA acting downstream of the intersection between both signaling pathways (20, 89). It has been suggested that an unknown JA-induced transcription factor interacts cooperatively with EIN3 in the promoter of *AtERF1* (59). The presence of basal levels of either ET or JA signaling molecules would then be sufficient to allow *AtERF1* expression. A recent transcript profiling study identified at least ten different Arabidopsis *AP2/ERF* family members to be transcriptionally induced upon treatment with both methyl-JA and inoculation with the incompatible pathogen *A. brassicicola*, including the positive regulators AtERF1/2 and negative regulator AtERF4 (92).

Sa-dependent induced resistance. Numerous studies have shown that, whereas ET and JA interact synergistically to activate certain disease response, the ET and JA pathways act at least independently or even antagonistically with respect to the SA-dependent pathway. Arabidopsis mutants affected in ET or JA perception are still fully capable of mounting SA-dependent responses (118, 147, 148) or of inducing the expression of SA-controlled genes (55). On the other hand, transgenic Arabidopsis plants that are unable to accumulate SA (e.g., *nahG*-expressing plants) and mutants impaired in SA-synthesis (e.g., *sid2* and *eds5*) or SA signaling (e.g., *npr1/nim1*) are blocked in the induction of SA-dependent PR-genes but show an equal or even stronger induction of ET/JA-dependent PR-genes (55, 150). The negative regulation

between ET- and SA-mediated signaling is also reflected by their final disease resistance response. For example, overexpression of *AtERF1* in Arabidopsis results in increased ET-mediated resistance to *B. cinerea* but reduces SA-mediated resistance to *P. syringae* pv. *tomato* (10).

Rhizobacteria-induced resistance. A specific kind of cross-talk between systemic defense responses in plants occurs upon root colonization by nonpathogenic *Pseudomonas* spp., leading to the development of enhanced defensive capacities against a broad spectrum of pathogens. In contrast to pathogen-induced systemic acquired resistance (SAR), associated with an increase of SA and induction of a subset of PR-genes in distant uninfected tissues, this rhizobacteria-induced systemic resistance (ISR) is not associated with SA or systemic changes in the expression of PR genes (75, 121, 152). However, NPR1, a component of the SA pathway, has been shown to be required for ISR (120, 121) as well as the ET receptor ETR1, the root ethylene insensitivity locus *ISR*, and components of JA-dependent signaling (75, 121, 152). Remarkably, however, dependent on the rhizobacterial strain, the plant seems to activate different subsets of signaling branches to induce resistance (75, 152). A recent transcriptome analysis on *P. fluorescens* ISR-induced Arabidopsis plants showed considerable expression changes in several JA- and ET-responsive genes (161), suggesting that these genes are primed to respond strongly and consequently more effectively to pathogen attack. Very recently it was found that ISR-induced expression of a root-specific PR-5 gene (*AtTLP1*) can be mimicked by application of the ET precursor ACC, but not by JA or SA (87), confirming a specific involvement of ET in rhizobacteria-mediated resistance.

Apart from cross-talk with other defense response signaling pathways, ET-responsive ERF transcription factors also seem to act as connectors with general stress-related signal transduction pathways. Antagonistic interactions between multiple components of the abiotic stress hormone abscisic acid (ABA) and the JA or ET signaling pathways seem to modulate gene expression in response to biotic and abiotic stresses (5, 28, 48, 173). In addition to exhibiting enhanced disease susceptibility (92), *AtERF4* overexpressing Arabidopsis plants show increased resistance to abiotic stress accompanied by repression of GCC-box–containing genes (173), suggesting that the negative transcriptional regulator AtERF4 is capable of modulating both ET and ABA responses. Possibly, AtERF4 is responsible for regulating the antagonism observed between ET and ABA responses (5, 92). Regulation of general stress responses may very well be orchestrated at the level of the ET-responsive transcription factors of the AP2/ERF family, which would modulate gene expression to ensure that the most appropriate defense response is activated for the specific type of threat.

ISR: induced systemic resistance

SAR: systemic acquired resistance

ABA: abscisic acid

The Role of Ethylene in Determining the Outcome of Plant-Pathogen Interactions

Treatment of plants with ethylene has long been known to increase either susceptibility or resistance, depending on the plant-pathogen interaction. For instance, treatment of plants with ethylene enhances resistance to the fungus *B. cinerea* (*36a*), whereas in other cases exposure of plants to ethylene had either no effect or reduced the resistance level to pathogens (19, 39, 159).

The use of characterized mutants and transgenic plants has provided more compelling evidence as to the crucial role of ET in resistance to particular pathogens. The Arabidopsis mutant *ein2*, impaired in ET signal transmission, exhibits increased susceptibility to the necrotrophic fungus *B. cinerea* (18, 148) and the necrotrophic bacterium *Erwinia carotovora* (103) but shows no alteration in susceptibility to the biotrophic Oomycete

H. parasitica and the biotrophic bacterium *P. syringae* pv *tomato* (85, 86, 121). Similarly, a transgenic tobacco line transformed with a dominant negative allele of the Arabidopsis ET receptor, *etr1*, suffered badly from challenge by a normally nonpathogenic soilborne Oomycete *Pythium* sp., whereas its level of resistance to biotrophic Tobacco mosaic virus (TMV) was unaffected (83). Conversely, superactivation of ET responses by *AtERF1* overexpression in Arabidopsis plants increased resistance to *B. cinerea*, *Plectosphaerella cucumerina*, and different *F. oxysporum* species but reduced SA-mediated tolerance against *P. syringae* pv. *tomato* (9, 10, 92). On the other hand, overexpression of *NtERF5* from *Nicotiana tabacum* resulted in an enhanced resistance of the transgenic tobacco plants to TMV (44). Similarly, overexpression in Arabidopsis of the ERF genes *Pti4* and *Pti5* from tomato provided resistance to *P. syringae* and the biotrophic fungus *Erysiphe orontii* (56, 67). In general, these results indicate that ERF-like transcription factors are involved in defense responses of various plant species, but that their effect on disease resistance depends on the specific plant-pathogen interaction.

Next to its involvement in disease resistance, ET also appears to affect disease symptom development. For instance, when infected with the pathogenic bacteria *Xanthomonas campestris* or *P. syringae*, the ET-insensitive Arabidopsis mutant *ein2* showed less chlorosis symptoms as compared with wild-type plants, despite an equal amount of infecting bacteria (8). Similarly, ethylene-insensitive soybean plants were less chlorotic than wild-type plants upon inoculation with virulent *P. syringae* pv *glycinea* strains (69). This discrepancy might be explained by the fact that, apart from its role in defense responses, ET is involved in many other aspects of plant physiology including mechanisms of chlorosis, senescence, and cell death (1).

As a final remark, different phytopathogens have been demonstrated to autonomously produce ET in vitro and in planta. Examples of ET-producing pathogens include the fungus *B. cinerea* (35) and the bacterium *P. syringae* (97, 165). The role of ET production for the pathogen is presently unclear as is the significance in host-pathogen interactions; further investigations are thus warranted. Moreover, in view of the documented ET-production by pathogens one should not preclude the possible perception of ET and particular responses to ET (produced by either the pathogen or the plant) in these pathogens when unraveling host-pathogen interactions.

SUMMARY POINTS

1. ET biosynthesis and downstream signaling in plants occurs through a well-conserved linear pathway leading to a cascade of transcription factors that differentially regulate ET-mediated responses, including various mechanisms of the plant's defense against pathogens.
2. Control of this pathway occurs at both the transcriptional and posttranscriptional levels.
3. A significant cross-talk appears between ET-dependent disease responses and other mechanisms of induced resistance including gene-for-gene resistance, JA- and SA-dependent resistance, and rhizobacteria-induced resistance, allowing the plant to further differentiate its defense response.

FUTURE DIRECTIONS

1. In the whole process from pathogen attack to the plant's final ET-mediated defense responses, many gaps in our understanding remain, not the least regarding the early steps that occur between pathogen perception and the resulting induction of ET biosynthesis.
2. For a complete picture of the role of ET-mediated mechanisms in the final plant's defense response, more investigations are needed on the complex cross-communication with other signaling pathways, specifically the conditions that determine either a positive or negative cross-talk.
3. Based on the increasing evidence for the importance of posttransciptional regulation present at all levels of the ET pathway, continued efforts should be made to further unravel this level of control of ET-dependent responses.
4. The observed production of ET by pathogens should be further investigated to get a complete picture of the role of ET in host-pathogen interactions.

This paper describes the cloning of *EIN2*, encoding the first positive regulator and an essential central component in the ET signaling pathway. Overexpression of the hydrophilic C terminus was sufficient to constitutively activate ET responses, suggesting that this part of EIN2 is responsible for further downstream signal transduction.

Of the five mutated loci identified in this study two were allelic to previously identified mutations (*ers1* and *transport inhibitor response 1* [*tir1*]), two represented new mutations in previously unidentified ET pathway genes, and one was the *eil1* mutation. *ein3 eil1* double mutants showed a complete ET insensitivity in many known ET responses, including the triple response and pathogen resistance, demonstrating the essential role of EIN3/EIL2 in myriad ET responses.

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The recessive ctr1 mutant constitutively expresses ET-regulated genes and exhibits phenotypes observed in wild-type plants treated with exogenous ET. CTR1 has high homology with serine/threonine protein kinases closely related to the Raf protein kinase family. CTR1 was shown to be the first negative regulator of downstream ET responses.

AtMPK6 phosphorylates two ACS isozymes, which leads to the stabilization of ACS and results in increased ET biosynthesis. This is the first paper to provide in vivo data for the phosphorylation of a plant MAPK substrate and sheds light on both plant defense and stress-related ET biosynthesis.

ET and JA induce several plant defense genes synergistically. ERF1 is a common downstream component of two hormone response pathways, and its expression is induced by both hormones. Overexpression of *ERF1* suppresses the defense response defects observed in JA-insensitive mutants. ERF1 is demonstrated to be a key factor in the integration of 2 hormone signals.

Expression of several AP2/ERF family members is altered by treatment with either methyl JA or inoculation with the incompatible pathogen *A. brassicicola*. AtERF2 and

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***ETO1* encodes a novel plant-specific protein family. ETO1 regulates ethylene biosynthesis by a proposed dual mechanism.**

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