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Modulation of Plant Defenses by Ethylene

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Abstract

Ethylene (ET) plays a critical role in the activation of plant defenses against different biotic stresses through its participation in a complex signaling network that includes jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA). Pathogen attack, wounding, and herbivory trigger asymmetric activation of this defense signaling network, thereby affecting the final balance of interactions between its components and establishing a targeted response to the initial threat. Ethylene's contribution to the modulation of this defense network relies on the complexity of the regulation of multigene families involved in ET biosynthesis, signal transduction, and crosstalk and enables the plant to fine-tune its response. The function of the members of these multigene families is tightly regulated at transcriptional, post-transcriptional, and post-translational levels. It is generally accepted that ET cooperates with JA in the activation of defenses against necrotrophic pathogens and antagonizes SA-dependent resistance against biotrophic pathogens. However, this is likely an oversimplified view, because cooperative interactions between ET and SA pathways have been reported and ET has been implicated in the activation of defenses against some biotrophic and hemibiotrophic pathogens. Therefore, deciphering ET's place in this hormonal network is essential to understanding how the cell orchestrates an optimal response to a specific biotic stress.

Key words: Ethylene; Plant defense; Necrotroph; Jasmonic acid; Salicylic acid; Abscisic acid; Hormone crosstalk; PRs; ERFs; GCC box

Introduction

Plants as sessile organisms have evolved intricate hormonal networks to respond appropriately to external stimuli. These networks allow plants to react with exquisite precision to different biotic and abiotic stresses. Ethylene (ET) alone and in combination with other hormones has been implicated as one of the key players in the determination of the most suitable genetic defense response. Nevertheless, the convoluted network interactions between ET and other hormonal pathways, such as jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA), in relation to defense are only beginning to be fully appreciated (Xu and others 1998; Lorenzo and others 2003; Veselov and others 2003; Zhao and others 2004).

Exogenous treatments of ET and/or its precursors, as well as ET inhibitors, have demonstrated clear links between this volatile plant hormone and a

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plant's defense responses (Beckman 2000; Kamo and others 2000; Nakazato and others 2000). The availability of mutant and transgenic plants with an altered functionality of ET-signaling components, as well as phytoalexin detoxification knock-out mutants in pathogens, has allowed ET's effect on defense to be studied *in vivo* without the problems associated with exogenous chemical treatments (Rojo and others 2003; Guo and Ecker 2004; Glazebrook 2005; Lorenzo and Solano 2005).

At present, it is generally accepted that ET cooperates with JA in the activation of defenses against necrotrophic pathogens and that it antagonizes SA dependent resistance against biotrophic pathogens (Knoester and others 1998; Thomma and others 1998, 1999; Berrocal-Lobo and others 2002; Diaz and others 2002; Rojo and others 2003; Lorenzo and Solano 2005). However, this is likely an oversimplified view because cooperative interactions between ET and SA pathways have been reported and ET has been implicated in the activation of defenses against some biotrophic and hemibiotrophic pathogens. A comprehensive list of ET-associated mutant and transgenic plants and their susceptibility to pathogens has been presented recently by van Loon and others (2006).

Ethylene's contribution to the determination of the appropriate defense response to any given biotic stress relies on the complexity of the regulation of multigene families involved in ET biosynthesis, signal transduction, and crosstalk. Understanding ET's place in this hormonal network is all the more important because of its broad regulatory function in the plant's physiology.

Within this review we aim to highlight the most important aspects of the ethylene-dependent defense response, encompassing pathogen attack, wounding, and herbivory. This discussion will begin with defense-induced ET production, before consideration of ET-dependent plant responses and the ET-induced arsenal. The greatest portion of this review reflects the growing appreciation of the hormonal network's importance in plant defense. Thus, the developments in our understanding of ET-related *cis*- and *trans*-regulatory elements and their regulation are discussed before network interactions between ET and other phytohormones or defense systems are examined.

Regulation of ET Biosynthesis by Biotic Stress

The specific recognition of different wound-derived and pathogen elicitor molecules, such as plant cell wall oligosaccharides and bacterial virulence factors, constitutes the first stimulus leading to ET production (Avni and others 1994; Rojo and others 1999; Nimchuk and others 2003; Glazebrook 2005; Zhao and others 2005).

Ethylene biosynthesis is a very tightly regulated pathway, including overlapping transcriptional and post-transcriptional points of control for the enzymes involved. This redundant regulation suggests that ET modes of action are modulated by its concentration rather than purely by its presence or absence (Pierik and others 2006).

Methionine constitutes the essential ''fuel'' for the first step of ET production by S-AdoMet synthase (SAM synthase; Peleman and others 1989). However, conversion of S-AdoMet to 1-aminocyclopropane-1-carboxilic acid (ACC) by ACC synthase (ACS) is considered the rate-limiting step in ET production. Therefore, since the cloning of the first *ACS* gene from *Cucurbita pepo* (Sato and Theologis 1989), considerable efforts have been made to study this multigene family. Consequently, the *ACS* gene family is now known in *Arabidopsis* to include 12 members, only eight of which appear to be involved in ET biosynthesis (Yamagami and others 2003).

The impact of different abiotic stimuli and wounding on *ACS* gene expression has been thoroughly studied by means of traditional and whole genome analyses (Cheong and others 2002; Tsuchisaka and Theologis 2004a, 2004b). However, reports focusing on ACS behavior in relation to pathogen attack and herbivory are scarce. Nevertheless, Broekaert and others (2006) have recently offered a brief overview of the ACS transcriptional regulation following biotic stress by analysis of the Genevestigator database (Zimmermann and others 2004). This analysis shows that ACS2, ACS5, and ACS6 represent a major focus of transcriptional regulation after pathogen challenge. However, other members of the *ACS* gene family show redundant and overlapping expression patterns in response to different pathogens. The complexity of these patterns illustrates the importance of fine-tuning ET levels to determine the appropriate response against each particular threat.

An additional level of complexity is added to ACS function by post-transcriptional regulation via both phospho/dephosphorylation balance and homo-heterodimerization (Tsuchisaka and Theologis 2004a; Chae and Kieber 2005). ACS phosphorylation balance was first linked to the control of ET production rates in tomato (Spanu and others 1994). More recently, tomato CDPK and MAPK6 have been isolated as the main regulators of ACS stability that lead to the stimulation of ET production upon

wounding (Liu and Zhang 2004; Chae and Kieber 2005). Each kinase modulates specifically one of two different sets of ACS proteins, each set exhibiting specific phosphorylation target sequences within its C-terminal regions. Thus, although CDPK contributes to the stability of ACS4, 5, 8, and 9, a MAPK-dependent pathway is involved in preventing the turnover of ACS2 and 6. Moreover, a conserved domain in monocotyledonous and dicotyledonous plants that is specifically recognized by the ETO1, EOL1, and EOL2 proteins and known as TOE is located adjacent to the CDPK target sequence (Yoshida and others 2006). These proteins are members of the BTB family and have recently been demonstrated to be the variable part of the CUL3 based E3 ubiquitin–ligase complex (Gingerich and others 2005; Stogios and others 2005; Weber and others 2005). Thus, ETO proteins are involved in the turnover of ACS, targeting ACS4, 5, 8, and 9 to degradation by the 26S-proteasome pathway (Wang and others 2004; Chae and Kieber 2005). Interestingly, EOL2 is the only member of this multigene family transcriptionally regulated in response to biotic stimuli within the Genevestigator database, being slightly enhanced by *Pieris rapae*.

Homodimerization and heterodimerization among different members of the ACS protein family constitutes an additional level of regulation of their activity, further highlighting the complexity of ET biosynthesis. Different combinations give rise to dimers showing different substrate affinity and thus different efficiency toward ACC synthesis (Tsuchisaka and Theologis 2004a). This regulatory redundancy may contribute significantly to the fine-tuning mechanism that controls ACS activity and highlights the importance of ET levels in ensuring an accurate defense response against different stimuli.

The final conversion of ACC to ET, cyanide, and carbon dioxide is carried out by ACC oxidase (ACO). Like ACS, ACO proteins are encoded by multigene families in various plant species. Thus, in tomato and *Arabidopsis*, for instance, ACO gene families are composed of 4 and 6 members, respectively (Babula and others 2006).

Root colonization by *Pseudomonas fluorescens* bacteria has been shown to enhance ACO activity *in vivo*. In turn, this potentiates expression of *PDF1.2* and *HEL* after treatment of the leaves with 1 mM ACC and results in a significantly higher level of ethylene emission after infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000/avrRpt2 (Hase and others 2003). To date, transcriptional activation of *ACO* genes has been described in response to the potato A virus in potato (Nie and others 2002), tobacco mosaic virus (TMV) in tobacco, fungal elicitors in tobacco and ginseng (Kim and others 1998; Xu and others 2005), and, more recently, to *Pseudomonas syringae* infection in tomato, where AvrPto and AvrPtoB are involved in the induction of two *ACO* genes (Cohn and Martin 2005). Further, the differential expression of members of this gene family in response to several biotic stresses has been confirmed by analysis of the Genevestigator database (Zimmermann and others 2004).

The expression levels of *ACO1* and *ACO2* genes are upregulated by *Botrytis cinerea* infection. Additionally, *ACO4* and *ACO*5 are upregulated upon *P. rapae* attack. In contrast, both *Alternaria brassicicola* and *Erysiphe cichoracearum* downregulate the expression of *ACO3*. Interestingly, wounding of sunflower hypocotyls enhances transcription of *ACO* genes, but it has no effect on their protein levels (Liu and others 1997). Again, this suggests the existence of additional post-transcriptional controls yet to be identified, and it adds another level of complexity to the defense-related ET biosynthesis.

It is significant that several plant pathogens can produce ET themselves. Indeed, some *P. syringae* pathovars have shown an ability to synthesize this phytohormone both *in vitro* and *in planta* from methionine by means of the KMBA (2-keto-4-methylthiobutyric acid) pathway (Weingart and others 2001). This ability, together with the production of the jasmonate analog coronatine and auxins by the same microorganisms may contribute to hormonal saturation and circumvention of an appropriate defense response (Robinette and Matthysse 1990; Cui and others 2005; Sreedharan and others 2006). For instance, moderate ET concentrations stimulate the production of phytoalexin β-thujaplicin, whereas excessive ET has been shown to reduce its level below that of untreated plants (Zhao and others 2004). Moreover, activation of the ET pathway has a detrimental effect on SA-dependent defenses (Lorenzo and Solano 2005). More recently, *Ralstonia solanacearum* has been seen to produce ET and auxin by means of the HrpG regulon (Valls and others 2006). The increase of both phytohormones is simultaneous with *TTSS* (type three secretion system) gene expression and contributes to the plant defense imbalance that favors pathogen infection.

Paradoxically, therefore, the highly regulated ET biosynthesis, which allows plants to fine-tune defense responses to specific threats, may be used by ET-producing pathogens to circumvent defenses.

Spatial Patterns of ET-Dependent Defenses

The ability of a plant to express different defense genes in local and systemic tissues represents a modulation of defenses to maximize impact on the pathogen and minimize cost to the plant (Baldwin 1998). Ethylene has been revealed to be pivotal in the regulation of the local/systemic patterns of defense activation. For example, ET is known to repress JA-responsive genes in locally damaged tissue while having no effect on their expression systemically (Zhu-Salzman and others 1998; Rojo and others 1999). In agreement with the idea that ET functions locally rather than systemically after wounding, tobacco plants have been found to need ET to generate, but not receive, the SAR signal in response to TMV (Verberne and others 2003). Grafting experiments using wild-type and ET-insensitive transgenic tobacco plants (Tetr) indicated that Tetr rootstock was unable to produce, release, or transmit the mobile signal to wild-type scions, but conversely, Tetr scions exhibited SAR when grafted to wild-type infected rootstock. Intriguingly, SAR in other plant species is ET independent (Lawton and others 1995).

Fine-tuning of local versus systemic defense responses by ET has been highlighted in *Nicotiana attenuata* by Kahl and others (2000). They found that ET locally decreased nicotine accumulation in leaves following herbivory by larvae of the nicotine-intolerant *Manduca sexta*. However, local volatile terpenoids and endogenous JA pools remained unaffected. It was hypothesized that this adaptive tailoring of defenses would reduce nicotine uptake by the larvae, thereby making them more susceptible to their nicotine-sensitive parasitoids. Further to this tailoring of defenses, ET has been suggested to regulate, in a highly localized manner, the number and positioning of symbiotic infection events between *Rhizobium meliloti* and *Medicago truncatula* (Penmetsa and Cook 1997; Veereshlingam and others 2004).

Root colonization by certain other rhizosphere bacteria confers a form of systemic disease resistance called *induced systemic resistance* (ISR; Pieterse and others 1998; van Loon and others 1998). Induced systemic resistance requires responsiveness to ET and JA but is independent of SA accumulation despite its dependence on NPR1 (Pieterse and others 2000). Remarkably, rather than increasing levels of ET and JA or accumulation of *PR* genes, ISR primes ET and JA responses to pathogens following infection. *ISR1*, a locus required for ISR signaling, has been suggested to encode a component of the ET-response pathway because *isr1* accessions showed an impaired triple-response phenotype and a reduced expression of the ET-responsive genes *HEL* and *PDF1.2* after exogenous application of ACC (Ton and others 1999, 2001).

Finally, ET has been implicated in both local and systemic defense responses to *A. brassicicola* through its regulation of GLIP1. This secreted lipase has antifungal properties and is induced by ET but not by SA or JA (Oh and others 2005).

Inter-Organism Communication

It is noteworthy that ET activation of defenses may not be limited to local versus systemic within-plant signaling. Data are accumulating to indicate that ET may also function both in plant–herbivore and plant–plant communication. The release of volatile compounds by plants and the importance of ET within this process have been well studied in relation to insect herbivory, where ET's role appears synergistic to that of JA or even SA (O'Donnell and others 1996; Arimura and others 2000; Farmer 2001; Schmelz and others 2003).

There is also accumulating evidence that, like methyl jasmonate (Farmer and Ryan 1990; Karban and others 2000, 2003), ET operates as a plant–plant defense signaling molecule. Tscharntke and others (2001) following laboratory and field-based study of induced phenols and proteinase inhibitors proposed that ET transferred pathogen resistance to neighboring alder trees. Moreover, ET can synergize (Z)-3-hexen-1-ol's induction of herbivore-induced volatile organic compounds (HI-VOC) in intact maize plants (Ruther and Kleier 2005). HI-VOCs attract natural enemies of certain plant-eating insects, thereby reducing herbivory (Kessler and Baldwin 2001; Engelberth and others 2004). However, like methyl jasmonate plant–plant signaling, it is probable that ET plant–plant signaling will be limited to short distances (around 10 cm), and thus is only likely to affect other branches of the ET-producing plant or intertwined canopies (Karban and others 2003; Baldwin and others 2006). Accordingly, ET has been implicated in the shade-avoidance response (Knoester and others 1998), and it has been argued that measured neighbor-derived atmospheric ET levels are sufficiently high to influence surrounding plants (Pierik and others 2004).

In contrast, however, not all interorganism communication related to ET is beneficial to the plant. The EIN2- and EIN3-dependent hypersusceptibility

of the *Arabidopsis* mutant *rhd1-4* to the cyst nematode *Heterodera schachtii* has been attributed, at least in part, to juvenile nematodes' being more attracted to the roots in wild-type plants than in *ein2* or *ein3* mutants (Wubben and others 2001, 2004).

Ethylene-Mediated Defense Responses

Wounding, herbivory, and pathogen challenge ultimately lead the plant to accumulate defensive compounds directed toward reinforcing either structural or chemical barriers against the threat. Physical barriers often offer the first line of defense against pathogen attack. After all, the cell wall must be breached if the nutrients contained within are to be appropriated. Although ET has no effect on defense-related callose deposition (Ton and Mauch-Mani 2004), it certainly contributes to other wound- or pathogen-induced defenses, as described below.

Xylem Occlusions

After wounding, one of the highest priorities for the plant is to seal the site and thereby restrict opportunistic pathogen ingression (de Bruxelles and Roberts 2001). Ethylene has been discovered to help in this process. For example, stimulation of vascular gel production in explant castor bean leaves is ET dependent (VanderMolen and others 1983). Indeed, this gel, which blocks the xylem vessels in a manner similar to that after infection by the vascular pathogen *Fusarium oxysporum*, is produced in response to ET. VanderMolen and others (1986) went on to determine that this gel is rich in host cell wall components (neutral sugars and uronic acids), and Beckman (2000) suggested that it was later lignified, and thus reinforced, by the infusion of phenolics.

In an interesting twist, this ET-induced blocking of the xylem vessels, which has evolved to protect the plant from infection, has been commandeered by *Agrobacterium tumefaciens* to augment gall formation. Aloni and others (1998) described how wild-type tomato plants react to *Agrobacterium*-stimulated ET by restricting vessel diameter above the gall and producing a rough, unorganized callus surface. These adaptations, thought to ensure water-supply priority to the growing gall, were reduced or absent in the tomato ET mutant *Never ripe*.

Veselov and others (2003) have subsequently published that gall-derived and exogenously applied ET increased ABA concentrations in the host leaves, which in turn reduced water vapor conductance. Additionally, the development of these galls and the impact on the host's shoots is complex, with not only ET but many other plant hormones playing a role, including JA, auxin, cytokinin, and ABA.

Cell Wall-strengthening Hydroxyproline-rich Glycoproteins (HRGPs)

One of the most rapid responses to pathogen attack involves the insolubilization of pre-existing hydroxyproline-rich structural proteins. This can happen within two minutes of fungal elicitor treatment, and thus it precedes transcription-dependent defenses (Bradley and others 1992). More recently, a class III peroxidase (extensin peroxidise: ep) has been shown *in vitro* and *in situ* to specifically crosslink an 89.9-kD HRGP, extensin (GvP1), within less than 10 minutes (Jackson and others 2001). It has been proposed that defensive modes of action for HRGPs may include cell wall strengthening and ionic agglutination of certain plant pathogens (Showalter 1993).

As long ago as 1979, Esquerre-Tugaye and others revealed that ET-induced HRGP correlated with resistance to pathogen invasion (*Colletotrichum lagenarium*) and inversely, when inhibited, disease progression was enhanced. The first *in vivo* data showing the effect of ET on cell wall HRGP came from Toppan and others (1982). They reported that inhibitors of endogenous ET production, when applied in non-toxic quantities, lowered HRGP. Additionally, when ACC was applied to healthy plant tissue, ET and HRGP were concomitantly stimulated. These workers later established that an elicitor from *C. lagenarium* was sufficient to induce ET and HRGP, and, by using an inhibitor of ET synthesis (aminoethoxyvinylglycine), that HRGP production was dependent on the elicitor-induced ET (Roby and others 1985).

Phytoalexins

Phytoalexins are plant defensive compounds of low molecular weight produced *de novo* in response to pathogen attack (Morrissey and Osbourn 1999). An overwhelming amount of circumstantial evidence exists that these secondary metabolites have an antimicrobial role *in vivo*, but most concrete data has been derived recently, using molecular genetic approaches (Dixon 2001). For example, although camalexin (3-thiazole-2-ylindole) was found to inhibit the growth of *Cladosporium cucumerium* and

P. syringae in vitro and to be produced *in vivo*, direct evidence for *in vivo* efficacy was only presented when camalexin-deficient *Arabidopsis pad* mutants were generated (Tsuji and others 1992; Glazebrook and Ausubel 1994; Glazebrook and others 1997).

Although subsequent testing has shown that ET appears to play no role in the induction of camalexin (Thomma and others 1999), it does function in the production of other phytoalexins. Indeed, ET has even been implicated in the transcriptional induction of the phytoalexin elicitor-releasing factor, β-1,3-endoglucanase, in soybean (Takeuchi and others 1990). Examples of ET-dependent or ET-related phytoalexins include, isocoumarin in carrot roots (Fan and others 2000), sakuranetin in rice leaves (Nakazato and others 2000), and β-thujaplicin in Mexican cypress (Zhao and others 2004). The induction of this third example (β-thujaplicin) is interesting because it involves the interaction of ET and JA. While JA is deemed the ''main control,'' ET is considered a ''fine modulator'' because of its diminished capacity to induce β-thujaplicin and apparent JA dependency.

PR Proteins

Pathogenesis-related (PR) proteins are the most extensively studied set of defense molecules in relation to ET. They constitute a broad class of inducible defense-related proteins expressed either locally or systemically in response to biotic stress. Pathogenesis-related proteins have been described in many plant species and are classified into 17 families according to their structural and functional features (van Loon and van Strein 1999; van Loon and others 2006). Antimicrobial activities of different PRs have been described as acting through contact toxicity or hydrolytic activity. Extensive work in the last two decades has demonstrated the broad role of ET in the regulation of expression of different classes of *PR* genes, such as *PR–2* (*β-1,3-glucanases*), *PR-3* (*basic-chitinases*), *PR-4* (*hevein-like*), and *PR-12* (*plant defensins*, *PDFs*) (Broglie and others 1989; Samac and others 1990; Penninckx and others 1996, 1998; Thomma and others 1998, 1999, 2002; van Loon and others 2006). However, ET does not regulate the expression of these genes alone, but as a component of a complex network of signaling molecules that, in addition to ET, includes SA, JA, and ABA. Therefore, understanding the role of ET in the transcriptional regulation of *PR* genes (or other defense-related genes) requires the understanding of the composition and dynamics of this signaling network. In the following sections we focus on the different components of ET-mediated transcriptional regulation, and on the interactions of these components with other hormonal signaling pathways.

Transcriptional Regulation of Defense-Related Genes by ET

Current understanding of ET's transcriptional regulation of many *PR* genes has been accomplished by employing two different strategies. On one hand, the molecular analysis of *cis*-elements and *trans*-acting factors responsible for ET inducibility yielded several relevant promoter elements and transcription factors that interact with them. On the other hand, a genetic approach based on the *Arabidopsis* ''triple response'' has allowed the identification of several classes of mutants impaired in the response to the hormone (Wang and others 2002; Guo and Ecker 2004). Molecular analysis to elucidate the biochemical function of the proteins identified by these mutations has helped uncover the players that participate in the ET-mediated transcriptional regulation and to merge the two approaches.

The molecular approach: *cis*-elements and *trans*-acting factors

Analysis of the promoters of some of the abovementioned *PR* genes identified a common *cis*-element required for ET regulation. This common *cis*-element (11-bp sequence TAAGAGCCGCC), called the GCC box or ethylene response element (ERE), was shown to be necessary, and in some cases sufficient, for ET regulation of *PR* genes in different plant species (Broglie and others 1989; Ohme-Takagi and Shinshi 1990; Roby and others 1991; Eyal and others 1993; Hart and others 1993; Meller and others 1993; Ohme-Takagi and Shinshi 1995; Shinshi and others 1995; Penninckx and others 1996; Solano and others 1998; Fujimoto and others 2000; Gu and others 2000; Brown and others 2003; Chakravarthy and others 2003). The GCC box is also present in the promoter of ET-regulated genes that are not obviously involved in the pathogen response (that is, *Hookless1*; Lehman and others 1996), suggesting a broader role for this element in the transcriptional regulation by ET.

Several independent groups reported the existence of proteins that were able to bind the GCC box *in vitro* (Hart and others 1993; Alonso and others

1995; Ohme-Takagi and Shinshi 1995; Shinshi and others 1995; Zhou and others 1997; Fujimoto and others 2000). Using a DNA fragment containing this element as a probe in southwestern experiments, four members of a family of DNA-binding proteins termed ethylene-responsive-element-binding-proteins (EREBPs or ERFs) were identified in tobacco (Ohme-Takagi and Shinshi 1995). *In vitro* DNA-binding experiments using truncated versions of these proteins delineated their DNA-binding domain to a 59-amino acid region that is well conserved among them and is similar to the DNA-binding domain of the homeotic protein APETALA2 (AP2; Ohme-Takagi and Shinshi 1995; reviewed in Riechmann and Meyerowitz 1998; and in Gutterson and Reuber 2004).

The ERF/AP2 superfamily is today one of the largest families of transcription factors (TFs) in plants, comprising three different subfamilies characterized by the number of ERF domains and the presence of additional DNA-binding domains. The AP2 subfamily contains two repeated ERF domains. The ERF subfamily contains a single ERF domain, and the RAV subfamily proteins contain an additional DNA-binding motif, the B3 domain. In *Arabidopsis*, for instance, the ERF subfamily consists of over 120 members (Riechmann and Meyerowitz 1998; Riechmann and others 2000; Sakuma and others 2002; Nakano and others 2006a).

The AP2/ERF DNA-binding domain is exclusive to plant transcription factors, although proteins with other functions (endonucleases) containing this domain have been reported in both bacteria and viruses (Magnani and others 2004). A 3D solution structure of the ERF/AP2 domain of AtERF1 showed that it consists of a 3-stranded anti-parallel β-sheet and an α-helix packed approximately parallel to the β-sheet. Arginine and tryptophan residues in the β-sheet contact 8 of the 9 consecutive base pairs in the major groove, showing the importance of the β-sheet in the determination of the DNA target specificity (Allen and others 1998). Further characterization of the DNA-protein interactions has comprehensively shown the residues of the GCC box that are essential for the recognition of the ERF proteins (2nd G, 5th G, and 7th C of the GCC box; Hao and others 1998), and that GCC box flanking nucleotides influence binding affinity of ERFs (Gu and others 2002; Tournier and others 2003).

Although most of the ERF TFs described to date function as transcriptional activators, repressors of transcription from several plant species have also been reported (Ohta and others 2001; Kazan 2006). These repressors, which include 8 members in the *Arabidopsis* ERF gene subfamily, share a conserved domain (L/FDLNL/F(x)P) within the C-terminal region of the protein, designated the EAR motif (Ohta and others 2001; Yang and others 2005; Kazan 2006).

Insights into the functionality of ERF subfamily members in different species have suggested, in several instances, their involvement in ET signaling and/or ET-activated defenses, including ERF1, AtERF2, AtERF3, AtERF4, AtERF13, and AtEBP in *Arabidopsis*, Pti4 in tomato, Tsi1 and OPBP1 in tobacco, and CaERFLP1 in hot pepper (Buttner and Singh 1997; Zhou and others 1997; Solano and others 1998; Fujimoto and others 2000; Park and others 2001; Berrocal-Lobo and others 2002; Oñate-Sánchez and Singh 2002; Lorenzo and others 2003; Berrocal-Lobo and Molina 2004; Guo and Ecker 2004; Lee and others 2004; McGrath and others 2005; Yang and others 2005). Independent of their biochemical activity as transcriptional activators or repressors, ERF family members can function as activators or repressors of particular defense pathways, often with opposing effects, resulting in resistance or susceptibility to different pathogens. For instance, *AtERF2* or *AtERF4* overexpression results in opposing disease-resistance phenotypes after infection by *F. oxysporum* (McGrath and others 2005). Furthermore, transcriptional activation of *Arabidopsis ERF1* enhances resistance to several necrotrophic pathogens including *B. cinerea*, *Plectosphaerella cucumerina* and *F. oxysporum*, but increases susceptibility to the biotrophic bacteria *P. syringa*e (Solano and others 1998; Berrocal-Lobo and others 2002; Berrocal-Lobo and Molina 2004). Additionally, and with regards to hemi- and biotrophic challenge, *Arabidopsis* plants expressing *Pti4* from tomato display increased tolerance to the bacterial pathogen *P. syringae* pv. *tomato* and increased resistance to the fungal pathogen *Erysiphe orontii* (Gu and others 2002). *Tsi* overexpression induces enhanced levels of several PR proteins, in cooperation with TSIP, resulting in improved tolerance to pathogens, such as *P. syringae* pv. *tabaci* in tobacco (Park and others 2001; Ham and others 2006). Additional examples of ET-induced ERFs whose overexpression enhances pathogen resistance include CaERFLP1 and OPBP1 (Guo and Ecker 2004; Lee and others 2004).

Ethylene genetic reprogramming includes chromatin rearrangements that enable the transcriptional regulation of ERFs. Thus, the histone deacetylase HDA19 has recently been implicated in the regulation of *PR* gene expression through the activation of *ERF1* and possibly other *ERF*s (Zhou and others 2005). In addition, HDA19 has been

connected with *AtERF7*'s ability to inhibit their target genes (Song and others 2005). Another histone deacetylase (RPD3b/HDA6) has been shown to interact with COI1 (in yeast two-hybrid assays) and thus suggests a regulatory role for this enzyme in the crosstalk with the JA signaling pathway (Devoto and others 2002).

Finally, phosphorylation has been proposed as a mechanism of post-transcriptional regulation of *ERF* genes (Yamamoto and others 1999). The involvement of protein kinases has been reported in ET signal transduction and in the transactivation of GCC box-dependent transcription. Pti4 phosphorylation by Pto kinase enhances protein activity (Gu and others 2000). In rice, OsEREBP1 binds GCC box elements of several *PR* gene promoters, and a MAPK, BWMK1, phosphorylates OsEREBP1, enhancing its DNA-binding activity (Cheong and others 2003). Moreover, putative sites for MAP kinase-mediated phosphorylation (PXXSPXSP) have been found in the class III protein AtERF5, but not in classes I and II (Fujimoto and others 2000).

These examples illustrate, as in the case of the regulation of ET biosynthesis described above, the complexity of the regulation of repressor and activator-types of ERFs during pathogen challenge and their potential to fine-tune defense gene expression and disease resistance.

The Genetic Approach: the EIN3/EIL Family of TFs

Genetic analysis based on the ''triple response'' phenotype identified many mutants, including *ethylene-insensitive3*, that impair plant responses to ET. Cloning of the *EIN3* gene (Chao and others 1997) identified the first member of a new family of proteins exclusive to plants that now includes five additional EIN3-LIKE (EIL) proteins. EIN3 and EIL1 have partially redundant functions, and both are required for full activation of ET responses (Chao and others 1997; Alonso and others 2003). Moreover, overexpression of *EIN3* or *EIL1* in transgenic wild-type or *ethylene-insensitive2* mutant plants conferred constitutive ET response phenotypes at all stages of development, indicating their sufficiency for activation of the pathway in the absence of ET (Chao and others 1997). The implication of other members of the EIN3/EIL family in the activation of ET responses has been reported in *Arabidopsis* and other species (Chao and others 1997; Tieman and others 2001; Rieu and others 2003; Chen and others 2004). Subcellular localization of EIN3 in the nucleus has suggested its putative function as a transcription factor (Chao and others 1997). Although these proteins do not contain any previously described DNA-binding motif, *in vitro* DNA-binding assays demonstrated that, indeed, EIN3, EIL1, and EIL2 are sequence-specific DNA-binding proteins that interact with 5′ sequences in the *ERF1* promoter (Solano and others 1998).

Determination of the EIL3 solution structure by NMR spectroscopy has shown that this new DNA-binding domain consists of five α-helices, possessing a novel fold dissimilar to known DNA-binding domain structures (Yamasaki and others 2005).

Interestingly, the EIN3 binding target shares significant base identity with the sequences defined as ET response elements in the promoter regions of tomato *E4* and *LEACO1* genes and carnation *GST1* (Montgomery and others 1993; Itzhaki and others 1994; Blume and Grierson 1997). Thus, the EIN3 DNA target site is a primary ethylene response element (PERE) conserved among different species and involved in the regulation of primary ET-response genes, including *ERF1* (Figure 1). On the other hand, the GCC box represents a secondary ethylene response element (SERE) present only in a subset of the ET-regulated genes such as pathogenesis-related genes, *HOOKLESS1*, and some *ERF*s that may be regulated by a subgroup of the ERF family of proteins. Therefore, ET signaling in the nucleus occurs through a cascade of TFs involving at least two families, the EIN3/EILs and the ERFs.

EIN3/EIL protein stability is regulated by EBF1/2 proteins. EBF1 and EBF2 are F-box components of the CUL1-dependent E3-ligase complex. Thus, EBFs' interaction with EIN/EIL proteins leads to their ubiquitination and subsequent degradation by the 26S proteasome pathway (Potuschak and others 2003; Guo and Ecker 2003). Interestingly, neither EBF1 nor EBF2 is significantly upregulated or downregulated following biotic stress responses in the Genevestigator database.

Ethylene's Role in the Defense Response Network

A plant's resistance to attack is not the result of isolated defense pathways, but rather, is based on a complex network of interactions between different signals, including not only ET but also JA, SA, and ABA (Figure 2). The modulation of this network allows the plant to fine-tune its response to a specific threat. Thus, the dissection of ET's role in plant defense requires an understanding of its place within the network and the way this hormonal pathway

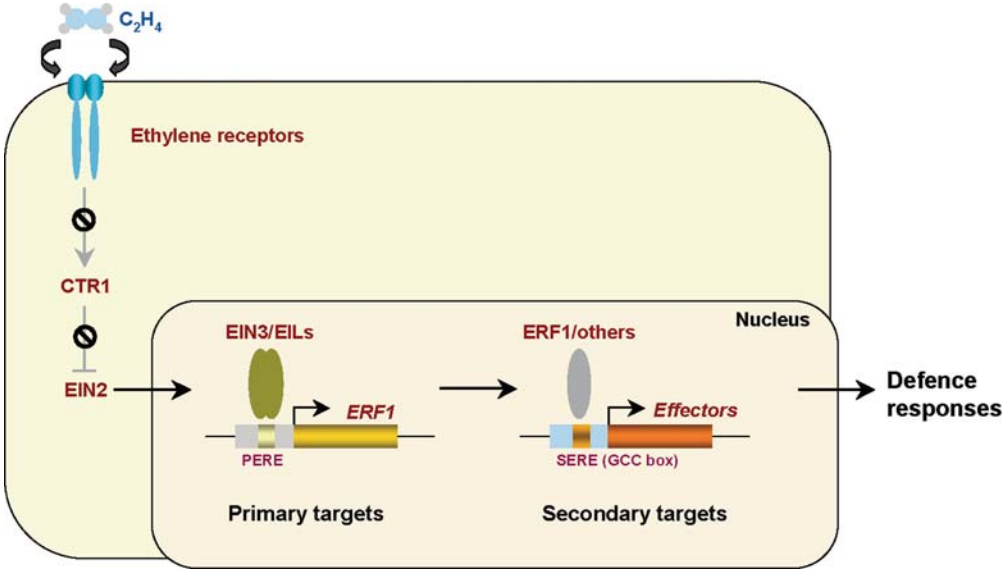

Figure 1. Schematic illustration of the ethylene gas signaling pathway. Binding of ethylene (C_2H_4) leads to inactivation of its receptors and in turn the deactivation of a Raf-like kinase CTR1. This allows EIN2 to function and signal positively downstream to the EIN3/EILs families of transcription factors located in the nucleus. EIN3 directs the expression of ERF1 and other primary target genes by binding directly, as a dimer, to the primary ethylene response element (PERE) present in their promoters. ERF1, and probably other ERFs, bind to the secondary ethylene response element (SERE/GCC box) and activate the expression of defense effector genes such as *PRs*.

interacts with others. Microarray analysis is beginning to unravel the intricacies of ET's interaction with different phytohormones (Maleck and others 2000; Schenk and others 2000; Van Zhong and Burns 2003; Nemhauser and others 2006). However, whole genome analysis is in its infancy and is only available for a limited number of species. Consequently, although these studies have confirmed many points of confluence between ET and some plant hormones (JA, SA, and ABA), they often pose more questions than they answer with regard to other plant hormones (auxin, brassinosteroid, cytokinins, and gibberellic acid). Nevertheless, in combination with complementary technologies, transcriptome analyses have the potential to greatly advance our understanding of integrated plant defense.

Ethylene and JA

The widely held belief is that ET acts cooperatively (or synergistically) with JA in the activation of responses to pathogens and antagonistically in response to wounding (Rojo and others 2003; Lorenzo and Solano 2005). Ethylene and JA have been demonstrated to act synergistically in the expression of several defense-related genes, including *PR1b*, *PR3* (chitinases), *PR4* (hevein-like proteins), *PR5* (osmotin), and *PDF1.2* (Xu and others 1994; Penninckx and others 1998; Thomma and others 1998, 1999; Lorenzo and others 2003). Additionally, mutants in either ET and/or JA defense pathways increase susceptibility to necrotrophic pathogens (Knoester and others 1998; Staswick and others 1998; Thomma and others 1999).

One of the most extensively studied ET- and JA-dependent genes is the fungicidal peptide PDF1.2. Induced after infection of necrotrophic fungi such as *A. brassicicola* or *B. cinerea*, it is already a ''classical'' marker to follow ET- and JA-dependent activation of defense responses after biotic stress (Penninckx and others 1996, 1998; Thomma and others 1998, 1999). *PDF1.2* gene induction requires simultaneous ET and JA signaling (Penninckx and others 1998), with these hormones operating through ERF1 (Figure 2). This TF is induced synergistically by ET and JA, and mutations that block either of these pathways are sufficient to prevent ERF1 induction and concomitantly its anti-pathogenic target genes (Solano and others 1998; Lorenzo and others 2003). In accordance, overexpression of *ERF1* triggers the activation of defense genes like *PDF1.2* and *PR*3 and enhances resistance against various necrotrophic pathogens (Berrocal-Lobo and others 2002; Berrocal-Lobo and Molina 2004). Furthermore, transcriptome analysis has shown that *ERF1* regulates a

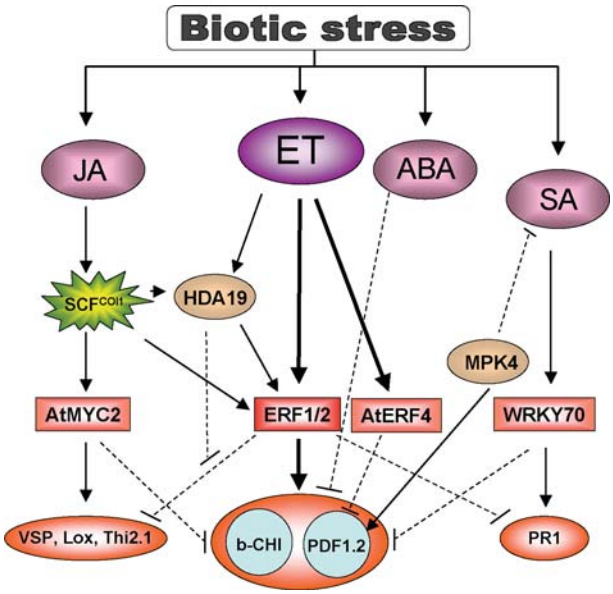

Figure 2. Plant defense response network involving ethylene (ET), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) hormonal pathways. Biotic stress triggers the synthesis of these hormones and should be considered to comprise pathogen attack, herbivory and wounding. Following stimulation, the asymmetric induction of these pathways and their interaction with one another allows the plant to fine-tune its defense response to a specific threat. In general ET and JA are considered to cooperate, through ERF1, in the induction of defenses against necrotrophic pathogens whilst repressing wounding and biotrophic pathogen responses. Conversely, SA induces defenses against biotrophic pathogens via the transcription factor WRKY70 and represses defenses against necrotrophic pathogens. Additionally, defense in response to wounding is JA dependent with AtMYC2 positively regulating genes such as *VSP*, *Lox* and *Thi2.1* and negatively regulating pathogen response genes such as *PDF1.2*, *b-CHI* and *HEL*. Nevertheless, whole genome microarray analyses are currently showing the complexity of hormonal interactions in the activation of defense responses, of which this model network represents only a simple view. Arrows indicate induction or positive interaction, whereas dashed lines indicate repression or negative interaction. Thicker arrows represent the main ET pathway.

high percentage of ET/JA-dependent responses, especially those related to defense (Lorenzo and others 2003). However, loss-of-function *erf1* mutants do not show enhanced susceptibility to pathogens or reduced defense gene expression, suggesting that other ERF genes may share redundant functions (O. Lorenzo and R. Solano, unpublished data). In line with this idea, several reports have shown ERF genes with patterns of expression and functional properties similar to those of ERF1 (Chen and others 2002; Onate-Sanchez and Singh 2002; Brown and others 2003; McGrath and others 2005). Indeed, Nakano and others (2006b) have recently described ET/JA-dependent expression of the *CEJ1* gene in *Arabidopsis*. Further analysis by means of Genevestigator (Zimmermann and others 2004) implicates *CEJ1* in the response against several pathogens and shows it to have an expression pattern similar to that of ERF1.

Other gain-of-function studies support the cooperation of ET and JA in the activation of defense responses to pathogens. The *Arabidopsis* mutant *cev1*, which constitutively activates both ET and JA signaling pathways, also shows constitutive expression of defense-related genes *PDF1.2*, *b-CHI*, *Thi2.1*, *VSP1*, and *VSP2*. Accordingly, this mutant also exhibits enhanced resistance to powdery mildew diseases (Ellis and Turner 2001; Ellis and others 2002).

Additionally, the simultaneous requirement of ET and JA for wound-induced *Pin2* expression in tomato has been reported (O'Donnell and others 1996). However, in contrast to the findings of O'Donnell and others (1996) in tomato, it has been widely demonstrated that ET prevents the JA-mediated induction of wound-response genes in damaged tissues of *Arabidopsis* and other species (Zhu-Salzman and others 1998; Rojo and others 1999; Lorenzo and others 2004). This antagonistic effect may be exerted through ERF1 and other ERFs, because activation of this TF prevents the induction of wound-responsive JA-regulated genes (Lorenzo and others 2004). Thus, ERF1 regulates differentially two sets of defense-response genes. On the one hand, it regulates positively the expression of pathogen-response genes, and on the other, it prevents JA-mediated induction of wound-response genes such as *VSP2* (Figure 2; Lorenzo and others 2004; Lorenzo and Solano 2005).

AtMYC2 is another important regulator of ET–JA interactions in plant defense; interestingly, however, it operates in the opposing manner ERF1. Identified as a JA-insensitive mutant (*jin1, jasmonate insensitive-1*), AtMYC2 is a key component of the JA-signaling pathway. Its expression is dependent entirely on COI1 and it has been shown to regulate the expression of the same two groups of ET/JA-responsive genes as ERF1. However, in contrast to ERF1, AtMYC2 induces the JA-mediated expression of wound-response genes while repressing the expression of pathogen-response genes (Figure 2). Consistently, *jin1* mutants show increased resistance to necrotrophic and hemibiotrophic pathogens (Anderson and others 2004; Lorenzo and others 2004; Nickstadt and others 2004). The AtMYC2

function seems to be conserved in dicotyledonous plants: two homologous proteins with a function similar to AtMYC2 (JAMYC2 and JAMYC10) have been described in tomato (Boter and others 2004). In addition to AtMYC2, AtERF4 is another player regulating this complex network. As already stated, AtERF4 negatively regulates expression of PDF1.2, and its overproduction in transgenic *Arabidopsis* renders the plants more susceptible to *F. oxysporum* (McGrath and others 2005; Yang and others 2005). The regulatory roles of these opposing TFs (AtMYC2, ERF1 and AtERF4) illustrate the complexity of the signaling network to fine-tune defenses to best suit a specific threat.

The ET–JA negative interaction in response to wounding (see above) is not the only example of antagonism between these phytohormones. In *M. truncatula*, ET controls nodule development by Rhizobium–legume symbiosis (Penmetsa and Cook 1997). Ethylene negatively regulates plant responses to the rhizobial bacterial signal Nod factor. This regulation occurs at an early step in the Nod factor signaling pathway, at or above Nod factor-induced calcium spiking. Jasmonic acid not only inhibits spiking but also suppresses frequency of calcium oscillations when applied at lower concentrations. This JA effect is amplified in the ET-insensitive mutant *skl*, indicating the antagonistic interaction between the two hormones for Nod factor signaling regulation (Sun and others 2006).

Two additional examples of negative ET–JA crosstalk come from plant–insect interactions. First, JA-mediated *Arabidopsis* resistance to *Spodoptera littoralis* is enhanced in ET-insensitive mutants and is decreased by treatment with ethephon (Stotz and others 2000). Second, the ET burst, seen in response to *M. sexta* larval feeding in *Nicotiana* plants, reduces JA-induced nicotine production (Winz and Baldwin 2001).

Ethylene and SA

It is generally accepted that SA plays a major role in activation of defenses against biotrophic pathogens, whereas ET and JA are more usually associated with defense against necrotrophic pathogen attack. Additionally, SA and JA/ET defense pathways are mutually antagonistic (Figure 2) (Thomma and others 2001; Kunkel and Brooks 2002; Turner and others 2002; Rojo and others 2003; Glazebrook 2005; Lorenzo and Solano 2005; van Loon and others 2006). To reiterate, however, this is likely to be an oversimplified model because cooperative interactions between ET and SA have also been reported.

Analyses of mutant and transgenic *Arabidopsis* plants have clearly demonstrated the existence of negative crosstalk between ET and SA in relation to defense. Ethylene-sensitivity mutants overactivate SA-dependent defenses (Thomma and others 1998; Clarke and others 2000), and transgenic plants affected in SA accumulation or signaling overexpress ET-dependent *PR* genes. For example, when tomato *nahG* plants (with depleted SA) were challenged with *Xanthomonas campestris*, they showed an increase in ET accumulation (O'Donnell and others 2001). Conversely, SA has been observed to block the synthesis of ET and JA in tomato, thereby inhibiting *Pin* accumulation (Peña-cortés and others 1993).

Although mechanistic explanations of this antagonistic crosstalk are scarce, several examples have been reported. Thus, antagonism between ET/JA and SA pathways requires the activation of proteins such as NPR1 and WRKY70, which activate expression of SA-responsive genes while repressing ET/JA-responsive genes (Spoel and others 2003). Further, MAPK4, probably working independently of ERF1, has been found to be a positive regulator of ET/JA signaling while negatively regulating SAR (Figure 2). It has been revealed that both ET/JA defense induction and SAR repression involve EDS1 and PAD4 proteins, functioning downstream of MAPK4 (Brodersen and others 2006).

Constitutive ERF1 overexpression that, as stated above, promotes the activation of JA/ET-dependent defenses, reduces tolerance against *P. syringae*, further supporting its role in the regulation of the negative crosstalk between the JA/ET and the SA signaling pathways (Berrocal-Lobo and others 2002).

The final example of an antagonistic interaction between ET and SA-dependent pathways relates to EDR1, a MAPKKK similar to CTR1. In the *edr1* mutant, ET potentiates SA-mediated *PR1* gene expression. EDR1, therefore, negatively regulates this process. *PR1* expression is highly induced by ET treatment in *edr1* mutant plants, whereas it is almost undetectable in wild-type plants. Consequently, *edr1* plants show enhanced resistance to *P. syringae* and *E. cichoracearum* (Frye and Innes 1998; Frye and others 2001).

Several examples of positive network connection between ET and SA have also been reported (Schenk and others 2000; Verberne and others 2003). A previously unmentioned example is that of the *hrl1* (*hypersensitive response-like lesions1*) in *Arabidopsis*. This mutant shows constitutive expression of SA and ET/JA defense genes, increased accumulation of SA and ET, and enhanced resistance against *P. syringae* and *Peronospora parasitica*

(Devadas and others 2002). It has also been demonstrated that ET induction following *P. rapae* feeding primes SA-dependent *PR1* expression and consequently improves defenses against Turnip Crinkle virus (TCV) (De Vos and others 2006).

Tsi and *Pti* genes may constitute a confluence point of ET and SA pathways. *Pti4*, *Pti5*, and *Pti6* genes encode related transcription factors that belong to the ERF family (Zhou and others 1997). Interestingly, Pti4 protein induction in tomato is involved in gene-for-gene interaction. The *R* gene *Pto* encodes a protein kinase that confers resistance to *P. syringa*e pv. *tomato* strains by specific recognition of its *avrPto* gene. Pto kinase is able to phosphorylate Pti4 *in vitro* and thereby enhance its GCC box binding activity (Gu and others 2000). In addition, *Pti4* is induced by SA, ethephon, and JA treatments. *Pti4* has been identified as a transcriptional activator of *PR* genes containing both GCC and non-GCC elements (Gu and others 2000). As mentioned above, *Pti4* overexpressors display increased resistance to the fungal pathogen *E. orontii* and increased tolerance to the bacterial pathogen *P. syringa*e pv. *tomato* (Gu and others 2002).

Additionally, in tobacco a *Tsi* ERF gene has been found to be induced by ethephon and SA treatments. Furthermore, *Tsi* overexpression induces the induction of several *PR* genes, resulting in improved tolerance to pathogens such as *Pseudomonas* (Park and others 2001).

Ethylene and Abscisic Acid

Abscisic acid regulatory function has been extensively studied in relation to plant abiotic stress responses, such as drought, salt, and cold (Finkelstein and Gibson 2002). Most examples of ET–ABA interactions have been described in sugar signaling (Leon and Sheen 2003), with interactions related to defense being less well documented. However, the existence of an antagonistic interaction between ABA and ET/JA signaling pathways that affects defense gene expression and disease resistance in *Arabidopsis* has been described (Figure 2) (Anderson and others 2004). Exogenous ABA suppresses ET/JA-responsive defense genes such as *PDF1.2*, *b-CHI*, and *HEL* while mutations in the ABA biosynthesis pathway have the opposite effect. Accordingly, *aba2-1* mutants with enhanced levels of these PR proteins exhibited improved resistance against *F. oxysporum*. AtERF4 has been recently shown to modulate the antagonistic ABA–ET/JA crosstalk. Thus, AtERF4 expression is induced by ABA, ET, or JA exogenous treatment, but its overexpression leads to the inhibition of GCC box-containing defense genes, ethylene insensitivity, and decreased ABA sensitivity (Yang and others 2005).

Concluding Remarks

Ethylene has been implicated in several structural and biochemical plant defense responses. Its function is modulated on several different levels.

First, following recognition of a specific attack, ET biosynthesis is tightly controlled through complex transcriptional and post-transcriptional biosynthesis mechanisms. These primary regulatory systems facilitate a discrete and targeted response to disparate threats from the moment a threat is recognized. However, the molecular mechanisms for the initial pathogen recognition that subsequently activate ET-mediated responses are still poorly understood.

Furthermore, ET's modulation of defense is not ''all or nothing,'' but rather is gradational. Like plant growth (Pierik and others 2006), it appears that the level of endogenous ET is pivotal in the establishment and fine-tuning of suitable defense responses. Thus, the spatial and temporal variation in endogenous ET concentration dictates how individual plant parts respond to the signal at any given time. For example, ET concentration not only determines local/systemic wound response (Rojo and others 1999), but it has also been demonstrated that ET concentration can affect the level of phytoalexin accumulation (Zhao and others 2004). The importance of ET's concentration to the plant's defense response may have led to the evolution of ethylene-producing pathogens. By interfering with the plant's endogenous ET status, these pathogens are able to prevent or alter the defense response to their advantage (Aloni and others 1998).

Finally, ET works within a phytohormone network. Thus, disease resistance is regulated by multiple signal transduction pathways in which ET, JA, SA, and ABA function as key signaling molecules. Wounding, pathogen attack, and herbivory trigger asymmetric activation of these signaling pathways, thereby affecting the final balance of interactions and determining the specific reaction to the initial stimulus. Deciphering this crosstalk between ET-, JA-, SA-, and ABA-dependent pathways in plant cells is a major challenge facing us as we seek to understand how the cell orchestrates this optimal response to a specific stress. Meeting this challenge will require identification of the molecular components involved in each signal transduction pathway and the characterization of their contribution to the regulation of the network. Post-transcriptional regulatory mechanisms such as protein

stability, protein-protein interactions, or covalent protein modifications may be key for this regulation. Technological advances, such as whole transcriptome analysis and proteomics will be critical to improving our comprehension of this complex signaling network.

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