

Molecular mechanisms of ethylene signaling in *Arabidopsis*

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Ethylene is a gaseous plant hormone involved in several important physiological processes throughout a plant's life cycle. Decades of scientific research devoted to deciphering how plants are able to sense and respond to this key molecule have culminated in the establishment of one of the best characterized signal transduction pathways in plants. The ethylene signaling pathway starts with the perception of this gaseous hormone by a family of membrane-anchored receptors followed by a Raf-like kinase CTR1 that is physically associated with the receptors and actively inhibits downstream components of the pathway. A major gap is represented by the mysterious plant protein EIN2 that genetically works downstream of CTR1 and upstream of the key transcription factor EIN3. Transcriptional regulation by EIN3 and EIN3-family members has emerged as a key aspect of ethylene responses. The major components of this transcriptional cascade have been characterized and the involvement of post-transcriptional control by ubiquitination has been determined. Nevertheless, many aspects of this pathway still remain unknown. Recent genomic studies aiming to provide a more comprehensive view of modulation of gene expression have further emphasized the ample role of ethylene in a myriad of cellular processes and particularly in its crosstalk with other important plant hormones. This review aims to serve as a guide to the main scientific discoveries that have shaped the field of ethylene biology in the recent years.

1. Introduction

Hormones act as chemical messengers in the control of the molecular, biochemical, and physiological events underlying growth and development. In plants and other sessile organisms, whose survival heavily relies on properly adjusting internal biochemical and physiological processes in accordance with environmental cues, such as light, water, and nutrient

availability, hormones also serve as essential integrators of developmental programs with the environmental signals. A basic challenge in biology is, therefore, to understand the molecular mechanisms that underlie hormone action, in other words, how those chemical signals are sensed by and communicated within the cells to trigger the relevant responses. This process can be divided into three main steps: signal perception, signal transduction or a cascade of biochemical events that ultimately leads to the induction of the final step, the response. The identification and characterization of the individual elements that constitute each module is

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investigating the ethylene signal transduction pathway.

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therefore essential to obtaining a comprehensive view of plant hormone biology.

Among the plant hormones, ethylene distinguishes itself by its simple hydrocarbon chemical structure (C₂H₄) and its gaseous nature. This simple molecule, however, plays a major role in plant growth and development by influencing a wide range of complex physiological processes throughout the entire plant life cycle, from seed germination to flowering, fruit ripening, and senescence.^{1,2} One of the most dramatic effects of ethylene on plant morphogenesis is the classical “triple response” exhibited by dark-grown seedlings exposed to ethylene. The triple response in *Arabidopsis* is characterized by exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and inhibition of hypocotyl and root growth (Fig. 1). The reproducibility and specificity of this response along with the use of the powerful genetic model system *Arabidopsis thaliana* has provided the tools essential for approaching the analysis of ethylene signaling from a molecular genetics perspective. A series of elegant genetic, molecular, and biochemical studies are uncovering a largely linear pathway that transduces the ethylene signal from the endoplasmic reticulum membrane to the nucleus (Fig. 2). This signaling pathway culminates in triggering the appropriate changes at the gene expression level, which in turn translate into a wide array of morphological and physiological responses. The wealth of knowledge accumulated both at the physiological and molecular level has made the ethylene signaling and response pathway an ideal system to study the role of hormones in signal integration. This review aims to summarize the current state of knowledge in the ethylene field,

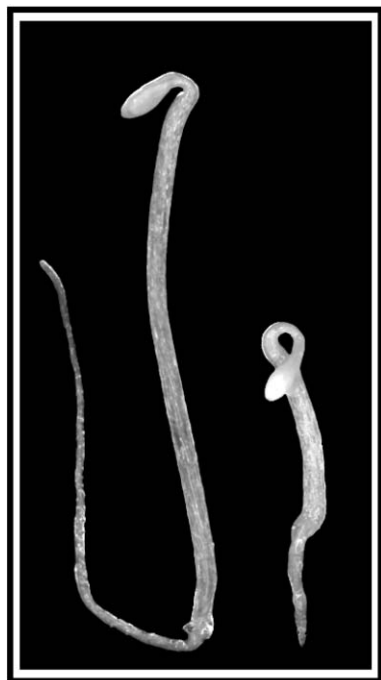


Fig. 1 Phenotypes of dark-grown three-day-old seedlings of *Arabidopsis thaliana*. The plant on the left was grown without hormonal supplementation, whereas the plant on the right was exposed to 10 μM ethylene precursor ACC and thus shows a typical triple response.

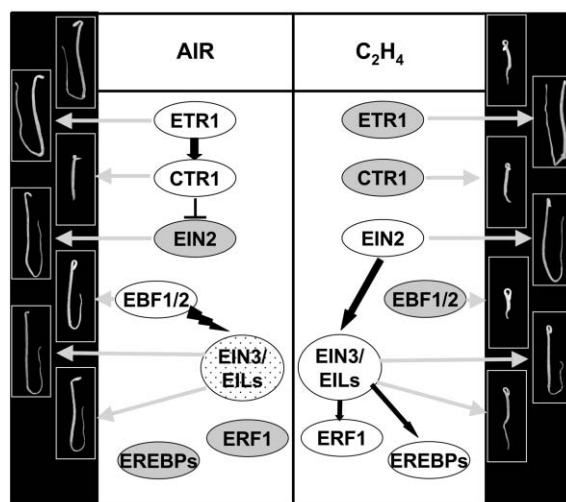


Fig. 2 The ethylene signaling pathway and its genetically characterized components. The signaling pathway components are shown in their sequential order of action. Components drawn in white represent active forms, whereas gray ovals represent their inactive versions. Binding of ethylene to the receptors, represented by ETR1, leads to activation of ethylene responses. Dotted oval represents EIN3 degradation by the 26S proteasome pathway due to action of EBF1 and EBF2. Arrows indicate activation steps, whereas a blocked arrow depicts repression of downstream elements by CTR1. Illustrations of the classical ethylene mutants and their respective phenotypes both in “air” and “ethylene” (as observed in the triple response assay) are also provided. *etr1* is a dominant ethylene insensitive GOF allele; all other mutants shown are LOF. Images on the top correspond to wild-type Columbia responses and are shown for comparison.

to expose the current challenges in hormone biology, and to provide a list of major references for the interested reader searching for more detailed information. We will start with the description of the main experimental breakthroughs that resulted in the discovery of the known ethylene signaling components to then describe the genomic approaches employed to characterize the molecular aspects of the ethylene response.

2. Ethylene perception is mediated by a small family of receptors

One of the earliest and most intriguing questions in ethylene biology originates from the structural simplicity of this molecule, an unusual feature for chemicals with hormonal activities that makes specific recognition of the ligand by the receptors challenging. Not only do such receptors need to possess high affinity for their ligand with a very limited number of possible interaction points, but should also provide the required specificity of a sensor molecule. A possible solution to this problem was suggested in the 60’s when Burg and Burg³ speculated that a metal cofactor could provide the required chemical properties to a proteinaceous molecule to function as an ethylene receptor. These speculations were based on the known properties of olefins to form complexes with transition metals,³ but the confirmation of this hypothesis had to wait over 30 years, encountering a long list of unsuccessful attempts to identify the ethylene receptors (reviewed in ref. 2). The development of modern molecular

genetic approaches and the selection of *Arabidopsis* as a plant model system opened a new door not only for the identification of the ethylene receptors, but for the elucidation of the entire signal transduction pathway.^{4,5}

The new quest for the molecular components of the ethylene signal transduction machinery first led to the isolation of *ethylene response1*, *etr1*, a dominant mutation that confers ethylene insensitivity.⁴ Further studies led to cloning of the corresponding gene and characterization of the biochemical properties of the gene product. *ETR1* encodes a histidine kinase with similarity to the classical bacterial two-component histidine kinases.⁶ The novel hydrophobic amino-terminal domain of *ETR1* heterologously expressed in yeast was shown to possess high-affinity binding properties to the gas ethylene.⁷ *ETR1* was found to act as a dimer that localizes to a cellular membrane system.⁸ A decade later, the ability of *ETR1* and four other ethylene receptors of *Arabidopsis* (see below) to bind ethylene was demonstrated *in planta*.⁹

A series of elegant studies performed in the nineties revealed that ethylene receptors are encoded by a small gene family that in *Arabidopsis* consists of five members: *ETR1*, *ethylene response2* (*etr2*), *ethylene insensitive4* (*ein4*), *ethylene resistant1* (*ers1*), and *ethylene resistant2* (*ers2*).^{6,10,11} All of the receptors share the highest degree of similarity in the amino-terminal domain, consistent with their ability to bind ethylene with similar affinity.⁹ Besides ethylene-binding properties, the *ETR1* amino-terminal portion that includes the transmembrane regions and a GAF domain of unknown function are sufficient for targeting to and retention of this receptor at the endoplasmic reticulum (ER) membrane system.¹² Such intracellular localization is believed to be energetically efficient, allowing for the rapid delivery of the receptors to their site of action.¹² Furthermore, the solubility of ethylene in aqueous as well as lipid environments is thought to make the ER-localized receptors easily and rapidly accessible to ethylene, without the need for an active transport mechanism.

Based on their sequence similarity and structural organization, the five receptors are categorized into two subfamilies¹⁰ (Fig. 3). Subfamily I members (*ETR1* and *ERS1*) harbor three hydrophobic transmembrane domains in the amino-terminus followed by a conserved histidine kinase domain. Subfamily II members (*ETR2*, *ERS2*, and *EIN4*) possess four predicted amino-terminal hydrophobic transmembrane regions followed by a less conserved kinase domain that lacks several of the canonical features required for histidine-kinase activity.¹⁰ Furthermore, three of the five receptors, *ETR1*, *ETR2*, and *EIN4*, also possess a carboxyl-terminal receiver domain,¹⁰ whose role in ethylene signaling remains unknown.

One of the most significant contributions to comprehending the mechanism of action of the ethylene receptors comes from the genetic studies that employed various mutant receptor alleles to generate double and triple mutant combinations. The original ethylene insensitive mutant alleles of the receptors were all genetically dominant^{6,10,11} and harbored mutations in the ethylene binding amino-terminal region of the protein. It was, however, unclear whether ethylene insensitivity arose from a gain of function (GOF) or a dominant-negative effect. A major breakthrough came with the isolation of loss-of-function (LOF) alleles of the receptors.¹³ Each of the single

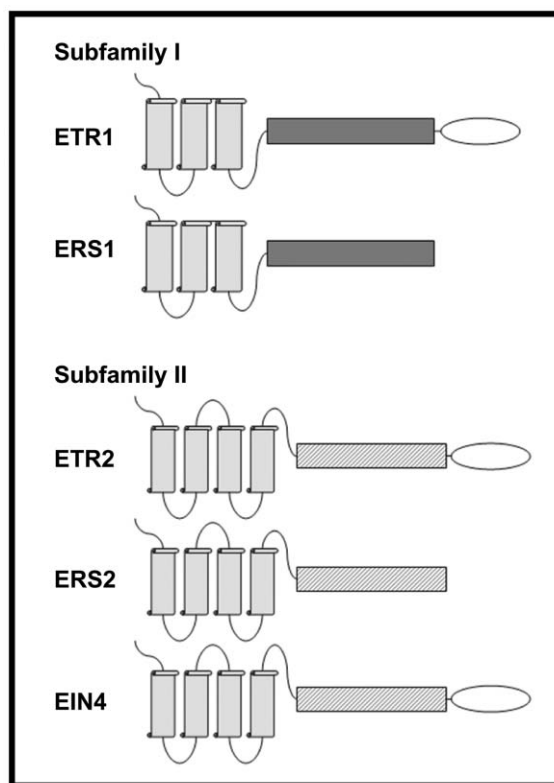


Fig. 3 Schematic representation of the ethylene receptor subfamilies I and II. The N-terminal ethylene-binding transmembrane domains are shown in light gray. Horizontal dark gray boxes represent the conserved histidine kinase domain in subfamily I members, while the degenerate kinase domain in subfamily II is shown as a dashed rectangle. The C-terminal receiver domain of *ETR1*, *ETR2*, and *EIN4* is shown as an oval.

LOF alleles was still able to respond to ethylene, indicating a high degree of functional redundancy among the receptors. Furthermore, triple and quadruple LOF mutants displayed a constitutive ethylene response in the absence of the hormone.¹³ This, together with the finding that several of the original dominant insensitive alleles that harbored mutations in the hydrophobic amino-terminus were impaired in their hormone binding capacity,^{7,14} suggested that the receptors are actively inhibiting ethylene responses in air and are turned “off” upon ethylene binding.¹³ A detailed study conducted with *etr1-7*, a LOF allele, revealed an enhanced sensitivity of these plants to the ethylene gas,¹⁵ further corroborating the proposed model. Mutations in other regions of the receptors that conferred dominant ethylene insensitivity were also identified,^{14,16} presumably by locking the receptors in a constitutively active form. Both receptor subfamilies appear to be able to sense ethylene, as double *etr1;ers1* and triple *etr2;ers2;ein4* LOF mutants are still able to respond to ethylene in the triple response assay.¹⁷ However, a particular role for subfamily I receptors in light-grown plants has been recently suggested.^{17,18} Double *etr1;ers1* LOF mutants displayed severe phenotypes, including miniature rosettes, fertility defects, and altered flower morphology. All of these effects were dependent on a functional ethylene signaling pathway, implying that the

observed growth defects arose from a misregulation of ethylene responses.^{17,18} Overexpression of the subfamily II members was unable to rescue the observed phenotypes, whereas ectopic expression of either wild-type *ETR1* or *ERS1* restored normal development,¹⁸ further supporting the notion of a unique role for the subfamily I receptors.

As anticipated by Burg and Burg,³ ethylene was found to bind to the receptors through a transition metal cofactor, copper.¹⁹ The interaction of recombinant ETR1 with copper ions was demonstrated in yeast using atomic absorption spectroscopy.¹⁹ Moreover, the copper cofactor was shown to be essential for ethylene binding, and thus, proper receptor function.¹⁹ The current structural model for the ethylene-binding domain suggests that the copper(I) cofactor is located in the electron-rich hydrophobic pocket formed by the N-terminal transmembrane domains of the receptors. In particular, residues Cys65 and His69 are thought to play a fundamental role in this protein-metal-hormone interaction. Ethylene binding results in an alteration of the coordination chemistry of the copper, triggering a conformational change in the binding site that is then transmitted to the C-terminal domains, initiating the signaling cascade.¹⁹ *In planta*, the relevance of this interaction was further confirmed with the identification of *RESPONSIVE TO ANTAGONIST1 (RANI)*.²⁰ *RANI* was isolated using a screening for mutants with altered specificity in hormone binding by employing the ethylene antagonist trans-cyclooctene (TCO).²⁰ *ran1* plants are defective in a copper transporter similar to P-type ATPases. The lack of the metal cofactor impairs receptor function in *ran1* mutants by causing altered ligand specificity and thus rendering the plants responsive to the antagonist TCO.²⁰ Furthermore, a strong LOF *ran1* allele results in a constitutive ethylene response phenotype in the absence of the gaseous hormone.²¹

Autophosphorylation activity has been demonstrated for ETR1²² and all other members of the receptor family.²³ However, while ETR1 autophosphorylates in the predicted conserved histidine residue,^{22,23} ERS1 and all of the subfamily II members display a serine-kinase activity *in vitro*.²³ ERS1 also possesses a histidine-kinase activity, whereas its serine autophosphorylation is thought not to be significant *in vivo*.²³

Although the data discussed above provided valuable insights into the receptor function, the exact molecular mechanism of action of the ethylene receptors is not clearly understood. In particular, the role of the kinase domain has long been questioned. Due to the similarity of the receptors to two-component histidine kinases, which act through phosphorylation of a response regulator protein,²⁴ receptor function has also been hypothesized to involve a similar mechanism. In fact, it has been proposed that a response regulator, ARR2, a typical target of the two-component histidine kinases, was involved in the ethylene response.²⁵ Recent reports, however, have questioned these findings, although the involvement of ARR2 in some ethylene responses has not been completely ruled out.²⁶ While the ability of the receptors to undergo autophosphorylation^{22,23} has opened the possibility that ethylene signaling involves a phosphorelay event, recent experimental evidence still upholds the controversy. For example, the ability of an “inactive kinase domain”

version of ETR1 to rescue the double LOF mutant *etr1ers1*¹⁸ and the ethylene insensitivity conferred to plants by a truncated version of the dominant *etr1-1* form that lacks the entire kinase domain²⁷ imply that this domain does not have an important role in the ethylene response. On the other hand, it has been previously suggested that receptors belonging to distinct subfamilies may interact *in planta*.^{15,27} Even though this interfamily interaction has not been demonstrated experimentally, the interaction with the remaining intact receptors could explain how kinase-inactive versions of ETR1 could still be functional.²⁷

Another interesting study addressed the roles of the kinase activity and of the carboxyl-terminal receiver domain of ETR1, ETR2, and EIN4 in ethylene signaling by looking at the effect of expressing truncated versions of ETR1 in a triple LOF *etr1;etr2;ein4* mutant background.²⁸ Transformation of the triple mutant with a truncated version of ETR1 that lacks both the histidine-kinase and the receiver domain failed to restore ethylene responsiveness to the triple mutant. Conversely, a second truncated ETR1 construct in which only the receiver domain was missing did rescue the partial constitutive triple response phenotype of the triple knockout line. Moreover, transgenic plants harboring this particular construct displayed hypersensitivity to ethylene.²⁸ These observations implied that the kinase domain was necessary for signal transmission by the receptors and that the receiver domain was not essential for restoring ethylene responsiveness. However, the observed hypersensitivity of the triple LOF *etr1;etr2;ein4* mutant expressing the ETR1 version lacking the receiver domain implicated this region in regulating signal output.²⁸ Modulation of the ethylene responses by the receiver domain could be achieved, for instance, through its reported interaction with another negative regulator of the pathway, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1)²⁹ (see below), assuming that in the absence of the receiver domain the function of CTR1 is impaired.²⁸ Interestingly, a detailed kinetic analysis of seedling growth response in the presence of exogenous ethylene and of the consecutive recovery after ethylene withdrawal also indicated the importance of both the kinase and receiver domains in specific aspects of the ethylene response.³⁰ While the inhibitory effect of ethylene on hypocotyl elongation appeared to be identical in wild-type and several receptor LOF mutant seedlings, a delay in growth recovery after a short 2 h ethylene treatment was detected in *etr2-3*, *ein4-4*, and the triple LOF mutant *etr1-6;etr2-3;ein4-4*, with the latter showing the slowest recovery kinetics. Conversely, the double LOF mutant *ers1-2;ers2-3* behaved similarly to wild-type seedlings.³⁰ Remarkably, mutant versions of ETR1 harboring mutations either in the kinase or the receiver domain failed to rescue or only partially rescued, respectively, the growth recovery defect of the triple *etr1-6;etr2-3;ein4-4* LOF line.³⁰ Altogether, these results suggest that both the kinase and the receiver domain are important for the hypocotyl recovery from short ethylene treatment and indicate that, despite the large functional overlap,¹³ some specificity can be attributed to the different receptors.³⁰

Taken together, the accumulated data have provided a model for the role of the receptors in ethylene signaling. In the absence of the hormone, the receptors actively repress

downstream components of the pathway and inhibit ethylene responses. Ethylene gas binds to all five receptors, causing the receptors to become inactive and releasing the pathway from their repression. Although still controversial, receptor kinase activity possibly plays an important role in signaling. While the exact mechanism of action of the receptors remains unknown, further investigation should lead to a better understanding of how the ethylene signal is transmitted by the receptors and what the distinct roles of the kinase and receiver domains are in this process.

3. A Raf-like kinase acts downstream of the receptors repressing ethylene responses

Genetic screens designed to identify components of the ethylene signal transduction machinery also uncovered a class of mutants that display a constitutive activation of ethylene responses in the absence of exogenously supplied hormone. A subset of those was further characterized as defective in ethylene biosynthesis, namely *ethylene overproducer (eto)*.^{31,32} Conversely, the *ctr1* mutant did not respond to inhibitors of ethylene biosynthesis indicative of an alteration in signal transduction.³¹ *CTR1* encodes a serine/threonine kinase whose carboxyl-terminus shares sequence similarity with the Raf family of protein kinases. The constitutive response phenotype exhibited by LOF mutants (Fig. 2) indicates that *CTR1* negatively regulates the ethylene signaling pathway. Furthermore, epistatic analysis positioned *CTR1* downstream of the receptors.³¹

Interestingly, *CTR1* was shown to physically interact with the receptors. This association was first demonstrated using yeast two-hybrid assays.²⁹ Subsequently, *CTR1* was shown to co-localize with the receptors at the ER membranes. Moreover, co-purification of an affinity-tagged *CTR1* and endogenous *ETR1* in transgenic *Arabidopsis* lines strongly supported the *in vivo* interaction of these proteins.³³ The significance of this interaction in signal transmission is corroborated by the observation that double and triple LOF receptor mutants result in a loss of ER-localization of *CTR1*.³³ Similarly, mutations in the *CTR1* protein that disrupt its interaction with *ETR1* also result in mislocalization of *CTR1* and, consequently, constitutive activation of ethylene responses. Taken together, these data suggested that the receptors and *CTR1* function as part of an ER-localized complex that actively represses ethylene responses.^{33,34}

The serine/threonine kinase activity of *CTR1* was demonstrated *in vitro* and shown to be essential for proper functioning of the receptors/*CTR1* signaling complex, as kinase-inactive alleles of *CTR1* also resulted in a constitutive response phenotype.³⁴ Despite these findings, it remains unclear how the kinase activity of *CTR1* participates in the regulation of the downstream components of the pathway. It has been established that ethylene binding to the receptors affects neither the interaction between the receptors and *CTR1* nor their sub-cellular localization.³³ The current proposed model predicts that upon ethylene binding, the receptors/*CTR1* signaling complexes are turned “off”, presumably by adopting an inactive conformation, thus releasing the repression of the downstream signaling pathway.³³

The characterization of *CTR1* and the requirement of its kinase activity for proper signaling raised once again the possibility that a phosphorylation event participates in ethylene signal transduction, with *CTR1* acting as a mitogen-activated protein kinase kinase kinase (MAPKKK). Indeed, a MAP kinase cascade involving the protein *MPK6* has been proposed to operate downstream of *CTR1*.³⁵ However, analyses of *MPK6* knockouts revealed no ethylene phenotypes in the mutants.³⁶ Moreover, a careful genetic and biochemical study further concluded that *MPK6* regulates ethylene production rather than signaling.³⁷ Overall, beyond the kinase activity of *CTR1* and its similarity to MAPKKKs, to date, there are no conclusive data to implicate a MAPK cascade in the ethylene signaling pathway.

4. A unique plant protein is a central component of the signaling pathway and positively regulates ethylene responses

Downstream of the receptors/*CTR1* complexes there acts a positive regulator of the pathway, ETHYLENE INSENSITIVE2 (*EIN2*) (Fig. 2). *EIN2* is required for all ethylene responses studied and constitutes a critical step in the signal transduction.³⁸ This plant-specific protein displays a bifunctional structure. The hydrophobic amino-terminus is predicted to form twelve transmembrane domains and shares sequence similarity with the family of *NRAMP* metal ion transporters. The functional relevance of this similarity may have little or no significance, as *EIN2* itself has no demonstrated metal transporting activity.³⁸ Moreover, in addition to *EIN2*, the *Arabidopsis* genome encodes six *NRAMP*-like proteins³⁹ and the involvement of at least one family member in metal homeostasis has been proven experimentally.⁴⁰

The long *EIN2* carboxyl-terminus contains a coiled-coil structure (a motif typically involved in protein–protein interactions) but otherwise displays no similarity to known protein domains.³⁸ Transgenic lines overexpressing the *EIN2* carboxyl-terminal domain exhibit constitutive activation of some of the ethylene responses, implying that this part of the protein is responsible for transducing the ethylene signal to the downstream targets. Conversely, the *NRAMP*-like amino-terminal domain of *EIN2* is believed to “sense” the upstream signaling events.³⁸ To date, it is still unclear how this protein receives and transmits the signal from and to other components of the pathway nor is it established what its interactors are. The mechanism of action and subcellular localization of *EIN2* remain unknown and constitute a major gap in the pathway.

5. A transcriptional cascade mediates ethylene responses at the gene expression level

ETHYLENE INSENSITIVE3, *EIN3*, is a nuclear-localized protein required for ethylene signaling that genetically works downstream of *EIN2*.⁴¹ LOF *ein3* alleles result in ethylene insensitivity in both seedlings (Fig. 2) and adults.⁴¹ *EIN3* belongs to a small gene family that in *Arabidopsis* also includes five *EIN3-LIKE* (*EIL*) proteins. All six *EIN3* family members display structural features characteristic of transcription

factors.^{41,42} These observations suggested that EIN3 and at least some of the EILs may act as transcriptional regulators of ethylene responses.⁴¹ Further studies confirmed this hypothesis and established the involvement of a transcriptional cascade regulating ethylene-mediated gene expression.⁴³ Previous work in tobacco revealed a family of proteins named ETHYLENE-RESPONSE-ELEMENT-BINDING-PROTEINS (EREBPs), which bind to the *cis*-element GCC box in the promoters of ethylene-regulated genes and mediate ethylene responses to pathogen attack.⁴⁴ In *Arabidopsis*, EIN3, EIL1, and EIL2 were demonstrated to bind to a short palindromic region, known as the EIN3-binding site, or EBS, in the promoter of the *EREBP* family member *ETHYLENE RESPONSE FACTOR1* (*ERF1*). *ERF1*, in turn, is a GCC box-binding transcription factor that acts downstream of EIN3 and EILs and is responsible for the modulation of a set of secondary ethylene responsive genes.⁴³ Consistent with these results, transgenic lines overexpressing *EIN3* show a constitutive triple response, whereas in *ERF1*-overexpressing plants only some of the ethylene responses are activated.^{41,43}

Curiously, *ERF1* is also required for the activation of defense-related responses by the plant hormone jasmonic acid.⁴⁵ Moreover, both ethylene and jasmonate pathways must be intact for proper *ERF1* expression, *i.e.* mutations disrupting signaling in either hormone pathway result in the inability of the two hormones to induce *ERF1* expression. Ethylene and jasmonate interact synergistically to achieve maximal expression of *ERF1*.⁴⁵ This transcription factor, therefore, represents a critical element in the interaction between these two important hormones. It also exemplifies how specific subsets of EIN3-mediated ethylene responses can be modulated independently by downstream switches such as *ERF1* that are regulated not only by ethylene but also by other factors.

6. The ubiquitin/26S proteasome pathway regulates EIN3 activity

The ubiquitin/26S proteasome pathway is known to be responsible for the selective degradation and turnover of several key regulatory proteins, being crucial for a range of cellular functions.⁴⁶ This pathway operates through multi-protein assemblies commonly referred to as SCF complexes (named after the yeast proteins SKP1, Cullin, and F-box). Target selectivity is conferred by the F-box containing E3-ubiquitin ligases, which then deliver the targeted proteins to the 26S proteasome.⁴⁶ A recently emerging picture positions the SCF-E3 ligases in the center of many plant hormone signaling pathways, including those of auxin,⁴⁷ gibberellins,⁴⁸ and jasmonic acid.⁴⁹

The ethylene signaling pathway also employs the SCF/26S proteasome to regulate the levels of at least one of its components, EIN3 (Fig. 2). As ethylene produces no changes in the *EIN3* transcript levels, a post-transcriptional mechanism for the regulation of *EIN3* has been hypothesized early on.⁴¹ Recently, two F-box proteins, EIN3 BINDING FACTOR1 (EBF1) and EBF2, were shown to act as part of an E3-ligase and to bind and target the constitutively produced EIN3 for degradation in the absence of ethylene.^{50,51} Conversely, in the

presence of exogenous ethylene, EIN3 protein levels were found to increase.^{50,51}

Although it is clear that EIN3 function is regulated by the ubiquitination pathway *via* EBF1 and EBF2, the exact mechanism by which ethylene prevents EIN3 degradation is not understood. Two main scenarios can be hypothesized. Ethylene may act by inducing EIN3 modifications so that it is no longer recognized by EBF1 and EBF2, or alternatively, ethylene may down-regulate the levels/activities of the F-box proteins EBF1 and EBF2 or other components of the ubiquitination complex. Both types of regulatory mechanisms are employed in plants and animals. For example, phosphorylation of the mammalian c-MOS protein kinase (MAPKKK) inhibits its ubiquitination and consequent degradation.⁵² In contrast, the plant hormone auxin regulates its signaling pathway by directly interacting with the F-box protein TIR1 and activating the SCF^{TIR1} ubiquitination complex that recruits the corresponding target proteins for degradation.⁴⁷

In addition to the negative regulation by EBF1 and EBF2, a stimulatory mechanism that activates the stabilized EIN3 protein is also expected to participate in the regulation of the activity of this critical transcription factor.^{41,51} This observation is supported by the ethylene responsiveness of EIN3-overexpression lines and *ebf1;ebf2* double LOF mutants. These plants respond to exogenous ethylene in spite of having elevated levels of EIN3, suggesting that regulation of the protein levels is not the only mechanism that governs the EIN3 activity.^{41,51}

7. Ethylene triggers genome-wide changes in gene expression

EIN3 is the initial factor in the transcriptional cascade responsible for inducing ethylene responses at the molecular level. The importance of this transcriptional regulation in ethylene responses is emphasized by the observation that *ein3;eil1* double mutants display almost complete insensitivity to the ethylene gas.¹⁶ In addition, regulation of EIN3 by the SCF/26S proteasome pathway serves as an efficient mechanism for the prompt and fine-tuned control of this transcriptional cascade. Taken together, these observations point towards the regulation of gene expression as a key aspect of ethylene responses. At the whole plant level, coordinated changes in expression levels of different groups of ethylene responsive genes ultimately lead to the variety of physiological responses triggered by this hormone.

In order to gain a better understanding of the ethylene effects in transcription, several research groups have performed genome-scale studies using microarrays and other high-throughput techniques. Early studies relied on cDNA microarrays covering only a fraction of the genome, and thus Schenk and coworkers analyzed the expression of 2 375 ESTs enriched in putative defense-related genes.⁵³ Interestingly, the authors evaluated the pattern of gene expression in response not only to ethylene, but also to a fungal pathogen, salicylic acid, and jasmonate. A high level (50%) of coordinated gene expression was observed for the ethylene and jasmonate treatments.⁵³ This finding is in agreement with the role of

ERF1 as a point of interaction between these two hormones in the regulation of plant defenses,⁴⁵ as discussed in section 5.

This initial study was followed by a broader analysis of ethylene responses relying on an EST microarray representing 6 000 unique genes.⁵⁴ The authors also took advantage of the ethylene mutants *etr1-1* and *ctr1-1* as controls. The comparative analysis of the profiles identified approximately 7% of genes as ethylene-regulated. Genes affected belonged to different functional categories, from primary metabolism to defense responses and regulatory proteins, such as transcription factors, kinases, and phosphatases. Ethylene was also found to change expression levels of known jasmonate- and auxin-regulated genes, indicative of the interaction between ethylene and these hormones. A possible interaction at the biosynthesis level was also suggested, as ethylene treatment was shown to influence expression of an *allene oxide synthase* gene that encodes an important enzyme in jasmonic acid biosynthesis, as well as genes for enzymes participating in the biosynthesis of the auxin precursor tryptophan.⁵⁴ Ethylene gas was also found to regulate expression of its own biosynthetic (*ACO2*, encoding an ACC oxidase) and signal transduction genes (for the receptors *ERS1* and *ERS2*).⁵⁴

In contrast with the two studies above, which employed a prolonged (24 h) exposure to the hormone, De Paepe and coworkers⁵⁵ performed a kinetic analysis of early responses to ethylene (ranging from 10 min to 6 h). Transcriptional profiles were analyzed by both cDNA-AFLP and a cDNA microarray containing approximately 6 000 unique ESTs. The authors also employed two signaling mutants, the ethylene insensitive *ein2-1* and constitutive triple response *ctr1-1*, as reference points in the analysis of exogenously supplied gas treatments. A hierarchical cluster analysis of the data revealed distinct subsets of genes with correlated patterns of expression. These included: (1) a group of very early, transiently inducible genes (after 10 to 30 min of the beginning of the treatment), (2) an early-inducible subset (30 min to 1 h), (3) a group of genes up-regulated at the intermediate (between 1–6 h of ethylene exposure) time-points, and (4) a group consisting of late-inducible genes (at 6 h of treatment). A large number of clones also displayed higher expression levels in the *ein2-1* ethylene insensitive background and were down-regulated in wild-type ethylene-treated plants. Consistent with the previous studies, ethylene was shown to be involved in a diversity of cellular processes, from metabolism to cell rescue and defense, among others.⁵⁵

One of the novelties in the study by De Paepe and coworkers⁵⁵ was the discovery of an ethylene-mediated regulation of the genes involved in the ubiquitin/26S proteasome pathway. These results were further confirmed by RT-PCR analysis, which revealed three *ubiquitin-specific proteases* and an *ubiquitin-conjugating enzyme* as being up-regulated by the ethylene treatment.⁵⁵ It is possible that these genes work in coordination with the well-characterized *EBF1* and *EBF2*,^{50,51} and therefore participate in the regulation of the ethylene signaling. Alternatively, they could be involved in any of the myriad of processes that compose the ethylene response.

While the aforementioned studies were limited to a relatively small subset of genes, a whole-genome assessment of the ethylene effects on gene expression was accomplished by

Alonso and coworkers⁵⁶ employing the *Arabidopsis* ATH1 Genome array from Affymetrix[®]. This study resulted in the identification of 628 genes with significantly altered transcription levels in response to ethylene. Among those, 244 and 384 genes were found to be induced and repressed by the treatment, respectively. The ample involvement of ethylene in diverse processes was once again supported by the finding that ethylene-regulated genes revealed in this study were involved in processes ranging from metabolism to signal transduction.⁵⁶ Importantly, a small subfamily of AP2-domain containing transcription factors was found to be coordinately induced by ethylene. Mutant analysis indicated that four of these genes, named *ETHYLENE DNA BINDING FACTOR1* to *4* (*EDFs*), had overlapping roles in the ethylene response and represented a branch in the ethylene response involved in the regulation of cell elongation.⁵⁶

An interaction between ethylene and the carbohydrate glucose was revealed in several genomic studies.^{54,55} Albeit not considered a primary plant hormone, glucose displays a variety of hormone-like activities, modulating plant gene expression, metabolism, growth, and development.⁵⁷ Evaluation of ethylene effects in gene expression has uncovered the role of this gas in carbohydrate metabolism and photosynthesis,⁵⁴ processes ultimately related to carbon homeostasis in plants. Likewise, a genome-wide study focused on glucose effects in transcription provided additional clues on the interaction between ethylene and glucose. Curiously, an *ACC synthase* and an *ACC oxidase*, two genes involved in ethylene biosynthesis, and the signaling components *EIN3*, *EIL1*, and *CTR1* were down-regulated by the exogenous supply of the carbohydrate.⁵⁸ This antagonistic relation between ethylene and glucose has been previously documented, as ethylene insensitive mutants were found to exhibit glucose hypersensitive phenotypes, whereas the constitutively responsive mutant *ctr1* was shown to possess glucose insensitivity.⁵⁹ *EIN3* was further suggested as a point of interaction between the two signaling pathways.⁶⁰ While ethylene promoted *EIN3* stability and accumulation, glucose had a detrimental effect on the *EIN3* protein levels. This glucose-enhanced degradation of *EIN3* was mediated by the 26S proteasome.⁶⁰ Nonetheless, the physiological significance of the interaction between ethylene and glucose, as well as the *in planta* role of *EIN3* in the crosstalk of these two signals, awaits further experimental confirmation.

Overall, the genomic analysis of transcript profiles has provided novel insights into ethylene biology and shed new light onto interactions between ethylene and other hormones. The large number of genes found to be regulated by ethylene reflects the broad impact of this hormone on many plant biochemical and physiological processes, including plant defense, primary and secondary metabolism, and cell signaling among others.

Most importantly, hormone crosstalk has also emerged as a prominent theme in these analyses, particularly, the interaction between ethylene and jasmonate in plant defense responses leading to the identification of several common target genes. The molecular basis for this interaction is currently better understood upon the characterization of *ERF1* as a point of interaction between the two signaling pathways⁴⁵ (see above).

Besides jasmonate, auxin is another important plant hormone that was found to share common regulated genes with ethylene.⁵⁴ In addition to co-regulating a number of target genes, the ethylene-jasmonate and ethylene-auxin interactions were also suggested to take place at the level of hormone biosynthesis.⁵⁴ Interestingly, the recent characterization of two ethylene insensitive mutants with altered auxin biosynthesis not only supports the importance of the ethylene-auxin interaction, but provides a clear evidence of its physiological significance.⁶¹ Similar functional studies will need to be performed to confirm the significance of the interactions observed at the gene expression level in the microarray studies described above. Regardless, gene expression profiling is generating additional clues of how a single hormone can modulate different responses by interacting with other signals. Hormonal crosstalk is achieved by synergistic activation of common target genes, as in the case of the ethylene-jasmonate crosstalk controlling plant defenses,⁴⁵ or by altering the levels of other hormones in specific tissues, as in the case of ethylene enhancing auxin biosynthetic rates.⁶¹ For a more detailed review with emphasis on ethylene interactions with other plant hormones, the reader is referred to a recent review.⁶² A schematic diagram of our current view of hormonal crosstalk, as based on the discussion above, is presented in Fig. 4.

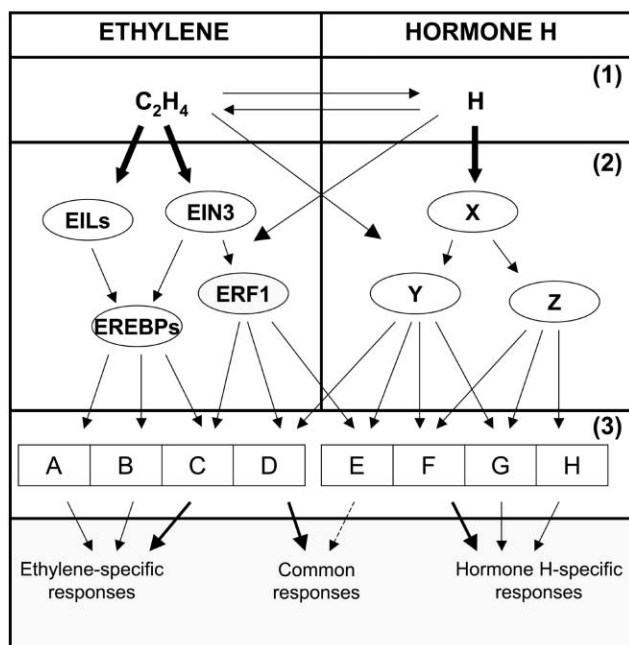


Fig. 4 Simplified diagram of hormonal crosstalk in plants, as based on the current knowledge of the interactions between ethylene and other plant hormones in *Arabidopsis*. Three basic levels of interactions are presently known. (1) Hormones may influence the synthesis of each other through the mutual regulation of expression or activity of key biosynthetic genes, as in the case of ethylene and auxin. (2) Crosstalk may be achieved through sharing of common components of the signal transduction machineries, as exemplified by ERF1 in the ethylene–jasmonate crosstalk. (3) Signals may converge on the regulation of expression of common target genes, an interaction mode exhibited by both ethylene–auxin and ethylene–jasmonate crosstalks.

The wealth of knowledge accumulated on ethylene signaling established this pathway as one of the best characterized pathways in plant hormone signaling. Nevertheless, many questions remain unanswered about the mode of action of some components of the machinery that transmit this important signal from the cellular membrane system to the nucleus and trigger the appropriate responses. While traditional genetic approaches aimed at the isolation of novel and unidentified components of this pathway are nearly exhausted, alternative and creative approaches combining genetic, molecular, pharmacological, and genomic analyses will need to be utilized to provide new insights in the field.

Pharmacological approaches are particularly well suited to the study of plant hormone signaling and response, as illustrated by the isolation of *ram1*, a mutant in which the ethylene response is activated by the ethylene antagonist TCO (see above).²⁰ A logical extension of such pharmacological approaches would be the utilization of chemical genetic strategies that combine the power of genetic and modern combinatorial chemistry. Although still in its infancy, chemical genetics has begun to penetrate into *Arabidopsis* research,^{63,64} and there are already a few examples of how this chemistry–genetics symbiosis can be applied in the study of plant signal transduction.^{65–70} In fact, there are a number of reasons to be optimistic about the future of this field in plants, in particular, in the area of hormone biology where phenotypic screening in response to chemicals has a long tradition.⁶³ Moreover, chemical genetics may provide, among others, new approaches to overcome the problem of studying gene function in organisms with extensive gene duplication such as *Arabidopsis*, in which more than two thirds of all genes belong to multigenic families.⁴²

Future ethylene signaling research will most likely focus on uncovering the remaining signaling components, as well as on dissecting the regulatory mechanisms that fine-tune this crucial signal transduction pathway in plants, from hormone perception by the receptors, to signal transmission through CTR1, EIN2, and the transcriptional cascade initiated by EIN3. Due to the key role of transcriptional control in mediating ethylene responses at the whole-plant level, genome-wide studies of gene expression profiles will certainly become critical for the in-depth understanding of this hormone action. Experimental refinement such as genetic and/or pharmacological manipulation of specific signaling steps/pathways and the analysis of ethylene effects in particular cell or tissue types will yield a more comprehensive view of the mechanisms involved in the crosstalk of this hormone with other signals. This complex web of hormonal interactions is an essential system employed by plants to integrate and properly respond to endogenous and environmental signals modulating their growth, development, and reproduction.

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References

- 1 F. Abeles, P. Morgan and M. Saltveit, in *Ethylene in Plant Biology*, vol. 2, Academic Press, San Diego, California, 1992.
- 2 A. B. Bleeker and H. Kende, *Annu. Rev. Cell Dev. Biol.*, 2000, **16**, 1–18.
- 3 S. P. Burg and E. A. Burg, *Plant Physiol.*, 1967, **42**, 144–152.
- 4 A. B. Bleeker, M. Estelle, C. Somerville and H. Kende, *Science*, 1988, **241**, 1086–1089.
- 5 P. Guzman and J. R. Ecker, *Plant Cell*, 1990, **2**, 513–523.
- 6 C. Chang, S. F. Kwok, A. B. Bleeker and E. M. Meyerowitz, *Science*, 1993, **262**, 539–544.
- 7 G. E. Schaller and A. B. Bleeker, *Science*, 1995, **270**, 1809–1811.
- 8 G. E. Schaller, A. N. Ladd, M. B. Lanahan, J. M. Spanbauer and A. B. Bleeker, *J. Biol. Chem.*, 1995, **270**, 12526–12530.
- 9 R. C. O'Malley, F. I. Rodriguez, J. J. Esch, B. M. Binder, P. O'Donnell, H. J. Klee and A. B. Bleeker, *Plant J.*, 2005, **41**, 651–659.
- 10 J. Hua, H. Sakai, S. Nourizadeh, Q. G. Chen, A. B. Bleeker, J. R. Ecker and E. M. Meyerowitz, *Plant Cell*, 1998, **10**, 1321–1332.
- 11 H. Sakai, J. Hua, Q. G. Chen, C. Chang, L. J. Medrano, A. B. Bleeker and E. M. Meyerowitz, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 5812–5817.
- 12 Y. F. Chen, M. D. Randlett, J. L. Findell and G. E. Schaller, *J. Biol. Chem.*, 2002, **277**, 19861–19866.
- 13 J. Hua and E. M. Meyerowitz, *Cell*, 1998, **94**, 261–271.
- 14 A. E. Hall, Q. G. Chen, J. L. Findell, G. E. Schaller and A. B. Bleeker, *Plant Physiol.*, 1999, **121**, 291–300.
- 15 J. D. Cancel and P. B. Larsen, *Plant Physiol.*, 2002, **129**, 1557–1567.
- 16 J. M. Alonso, A. N. Stepanova, R. Solano, E. Wisman, S. Ferrari, F. M. Ausubel and J. R. Ecker, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 2992–2997.
- 17 A. E. Hall and A. B. Bleeker, *Plant Cell*, 2003, **15**, 2032–2041.
- 18 W. Wang, A. E. Hall, R. O'Malley and A. B. Bleeker, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 352–357.
- 19 F. I. Rodriguez, J. J. Esch, A. E. Hall, B. M. Binder, G. E. Schaller and A. B. Bleeker, *Science*, 1999, **283**, 996–998.
- 20 T. Hirayama, J. J. Kieber, N. Hirayama, M. Kogan, P. Guzman, S. Nourizadeh, J. M. Alonso, W. P. Dailey, A. Dancis and J. R. Ecker, *Cell*, 1999, **97**, 383–393.
- 21 K. E. Woeste and J. J. Kieber, *Plant Cell*, 2000, **12**, 443–455.
- 22 R. L. Gamble, M. L. Coonfield and G. E. Schaller, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 7825–7829.
- 23 P. Moussatche and H. J. Klee, *J. Biol. Chem.*, 2004, **279**, 48734–48741.
- 24 P. M. Wolanin, P. A. Thomason and J. B. Stock, *Genome Biol.*, 2002, **3**, REVIEWS3013.
- 25 C. Hass, J. Lohrmann, V. Albrecht, U. Sweere, F. Hummel, S. D. Yoo, I. Hwang, T. Zhu, E. Schafer, J. Kudla and K. Harter, *Embo J.*, 2004, **23**, 3290–3302.
- 26 Y. F. Chen, N. Etheridge and G. E. Schaller, *Ann. Bot. (London)*, 2005, **95**, 901–915.
- 27 R. L. Gamble, X. Qu and G. E. Schaller, *Plant Physiol.*, 2002, **128**, 1428–1438.
- 28 X. Qu and G. E. Schaller, *Plant Physiol.*, 2004, **136**, 2961–2970.
- 29 K. L. Clark, P. B. Larsen, X. Wang and C. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 5401–5406.
- 30 B. M. Binder, R. C. O'Malley, W. Wang, J. M. Moore, B. M. Parks, E. P. Spalding and A. B. Bleeker, *Plant Physiol.*, 2004, **136**, 2913–2920.
- 31 J. J. Kieber, M. Rothenberg, G. Roman, K. A. Feldmann and J. R. Ecker, *Cell*, 1993, **72**, 427–441.
- 32 K. E. Woeste, C. Ye and J. J. Kieber, *Plant Physiol.*, 1999, **119**, 521–530.
- 33 Z. Gao, Y. F. Chen, M. D. Randlett, X. C. Zhao, J. L. Findell, J. J. Kieber and G. E. Schaller, *J. Biol. Chem.*, 2003, **278**, 34725–34732.
- 34 Y. Huang, H. Li, C. E. Hutchison, J. Laskey and J. J. Kieber, *Plant J.*, 2003, **33**, 221–233.
- 35 F. Ouaked, W. Rozhon, D. Lecourieux and H. Hirt, *Embo J.*, 2003, **22**, 1282–1288.
- 36 J. R. Ecker, *Plant Cell*, 2004, **16**, 3169–3173; F. L. Menke, J. A. Van Pelt, C. M. Pieterse and D. F. Klessig, *Plant Cell*, 2004, **16**, 897–907.
- 37 Y. Liu and S. Zhang, *Plant Cell*, 2004, **16**, 3386–3399.
- 38 J. M. Alonso, T. Hirayama, G. Roman, S. Nourizadeh and J. R. Ecker, *Science*, 1999, **284**, 2148–2152.
- 39 S. Thomine, R. Wang, J. M. Ward, N. M. Crawford and J. I. Schroeder, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 4991–4996.
- 40 S. Thomine, F. Lelievre, E. Debarbieux, J. I. Schroeder and H. Barbier-Brygoo, *Plant J.*, 2003, **34**, 685–695.
- 41 Q. Chao, M. Rothenberg, R. Solano, G. Roman, W. Terzaghi and J. R. Ecker, *Cell*, 1997, **89**, 1133–1144.
- 42 Arabidopsis Genome Initiative, *Nature*, 2000, **408**, 796–815.
- 43 R. Solano, A. Stepanova, Q. Chao and J. R. Ecker, *Genes Dev.*, 1998, **12**, 3703–3714.
- 44 M. Ohme-Takagi and H. Shinshi, *Plant Cell*, 1995, **7**, 173–182.
- 45 O. Lorenzo, R. Piqueras, J. J. Sanchez-Serrano and R. Solano, *Plant Cell*, 2003, **15**, 165–178.
- 46 R. D. Vierstra, *Trends Plant Sci.*, 2003, **8**, 135–142.
- 47 N. Dharmasiri, S. Dharmasiri and M. Estelle, *Nature*, 2005, **435**, 441–445; S. Kepinski and O. Leyser, *Nature*, 2005, **435**, 446–451.
- 48 A. Dill, S. G. Thomas, J. Hu, C. M. Steber and T. P. Sun, *Plant Cell*, 2004, **16**, 1392–1405.
- 49 L. Xu, F. Liu, E. Lechner, P. Genschik, W. L. Crosby, H. Ma, W. Peng, D. Huang and D. Xie, *Plant Cell*, 2002, **14**, 1919–1935.
- 50 H. Guo and J. R. Ecker, *Cell*, 2003, **115**, 667–677; J. M. Gagne, J. Smalle, D. J. Gingerich, J. M. Walker, S. D. Yoo, S. Yanagisawa and R. D. Vierstra, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 6803–6808.
- 51 T. Potuschak, E. Lechner, Y. Parmentier, S. Yanagisawa, S. Grava, C. Koncz and P. Genschik, *Cell*, 2003, **115**, 679–689.
- 52 J. Sheng, A. Kumagai, W. G. Dunphy and A. Varshavsky, *Embo J.*, 2002, **21**, 6061–6071.
- 53 P. M. Schenk, K. Kazan, I. Wilson, J. P. Anderson, T. Richmond, S. C. Somerville and J. M. Manners, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 11655–11660.
- 54 G. V. Zhong and J. K. Burns, *Plant Mol. Biol.*, 2003, **53**, 117–131.
- 55 A. De Paepe, M. Vuylsteke, P. Van Hummelen, M. Zabeau and D. Van Der Straeten, *Plant J.*, 2004, **39**, 537–559.
- 56 J. M. Alonso, A. N. Stepanova, T. J. Lisse, C. J. Kim, H. Chen, P. Shinn, D. K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C. C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M. Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D. E. Carter, T. Marchand, E. Risseu, D. Brogden, A. Zeko, W. L. Crosby, C. C. Berry and J. R. Ecker, *Science*, 2003, **301**, 653–657.
- 57 F. Rolland, B. Moore and J. Sheen, *Plant Cell*, 2002, **14**, S185–205.
- 58 J. Price, A. Laxmi, S. K. St Martin and J. C. Jang, *Plant Cell*, 2004, **16**, 2128–2150.
- 59 P. Leon and J. Sheen, *Trends Plant Sci.*, 2003, **8**, 110–116.
- 60 S. Yanagisawa, S. D. Yoo and J. Sheen, *Nature*, 2003, **425**, 521–525.
- 61 A. N. Stepanova, J. M. Hoyt, A. A. Hamilton and J. M. Alonso, *Plant Cell*, 2005, **17**, 2230–2242.
- 62 A. N. Stepanova and J. M. Alonso, *Physiologia Plantarum*, 2005, **123**, 195–206.
- 63 H. E. Blackwell and Y. Zhao, *Plant Physiol.*, 2003, **133**, 448–455.
- 64 N. Raikhel and M. Pirrung, *Plant Physiol.*, 2005, **138**, 563–564.
- 65 Y. K. Min, T. Asami, S. Fujioka, N. Murofushi, I. Yamaguchi and S. Yoshida, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 425–430.
- 66 K. Hayashi, K. Ogino, Y. Oono, H. Uchimiya and H. Nozaki, *J. Antibiot.*, 2001, **54**, 573–581.
- 67 D. R. Spring, S. Krishnan, H. E. Blackwell and S. L. Schreiber, *J. Am. Chem. Soc.*, 2002, **124**, 1354–1363.
- 68 J. I. Armstrong, S. Yuan, J. M. Dale, V. N. Tanner and A. Theologis, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 14978–14983.
- 69 J. Zouhar, G. R. Hicks and N. V. Raikhel, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 9497–9501.
- 70 M. Surpin, M. Rojas-Pierce, C. Carter, G. R. Hicks, J. Vasquez and N. V. Raikhel, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 4902–4907.