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PROGRESS REPORT

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Progress report: ethylene signaling and responses

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The pathway for ethylene signal transduction

Ethylene regulates many cellular and developmental processes in plants through a signaling pathway conserved in monocots and dicots (reviewed in Chen et al. 2005). The current model for the ethylene-signaling pathway, based on genetic analysis in *Arabidopsis thaliana*, is shown in Fig. 1a. Ethylene is perceived by a family of receptors related to bacterial histidine kinases (O'Malley et al. 2005) that modulate the activity of the Raf-like kinase CTR1, a negative regulator of the pathway. Functioning downstream of CTR1 is EIN2, a protein with similarities to Nramp metal-ion transporters, followed by members of the EIN3/EIN3-like (EIL) family of transcription factors, which regulate the transcription of primary ethylene-response genes.

Additional components of the signal transduction pathway have been proposed, but their contribution to signaling is not yet resolved. Due to the similarity of CTR1 to Raf, a mitogen-activated protein kinase kinase kinase (MAPKKK), a MAP-kinase (MAPK) cascade has been proposed to act downstream of CTR1. In 2003, a putative MAPK module was implicated in ethylene signal transduction (Ouaked et al. 2003), but additional research indicates that the primary role of the MAPK in question (MPK6 in *A. thaliana*) is in the regulation of ethylene biosynthesis, not signaling (Ecker 2004; Liu and Zhang 2004). Because the ethylene receptors are related to histidine kinases, researchers have also wondered if the receptors might participate in phosphorelays involving other two-component signaling elements. Consistent with this possibility, the response regulator ARR2 was proposed to transduce the ethylene signal as part of a CTR1-independent pathway (Hass et al. 2004). However, additional research suggests a primary role for ARR2 in signaling by cytokinin, rather than ethylene (Mason et al. 2005).

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Signaling by the ethylene receptors

Although the signal input (ethylene binding) domain for the ethylene receptors has been defined (Chen et al. 2005; O'Malley et al. 2005), we know relatively little about the mechanism for signal output from the receptors. Recent studies have shed light on how protein–protein interactions and enzymatic activity may modulate signal output. Recently, CTR1 was shown to localize to the endoplasmic reticulum of *Arabidopsis* due to interactions with the ethylene receptors (Gao et al. 2003). Direct evidence that CTR1 associates with ethylene receptors in planta was obtained by co-purification of the ethylene receptor ETR1 with a tagged version of CTR1 from an *Arabidopsis* membrane extract (Gao et al. 2003). These data support the existence of ethylene receptor signaling complexes (signalosomes), which would facilitate the transfer of information from receptors to downstream signaling components such as CTR1.

An area of debate for some years now has been the role of enzymatic activity in signaling by the ethylene receptors (Mason and Schaller 2005). Phylogenetic and sequence analyses divide the ethylene receptors into two subfamilies (Fig. 1b). Members of subfamily I have histidine kinase (HK) activity, but members of subfamily II lack HK activity and potentially have serine kinase activity (Moussatche and Klee 2004). Recent experiments indicate that the HK activity found in subfamily I modulates the ethylene response, even if it is not absolutely required for establishing the ethylene re-

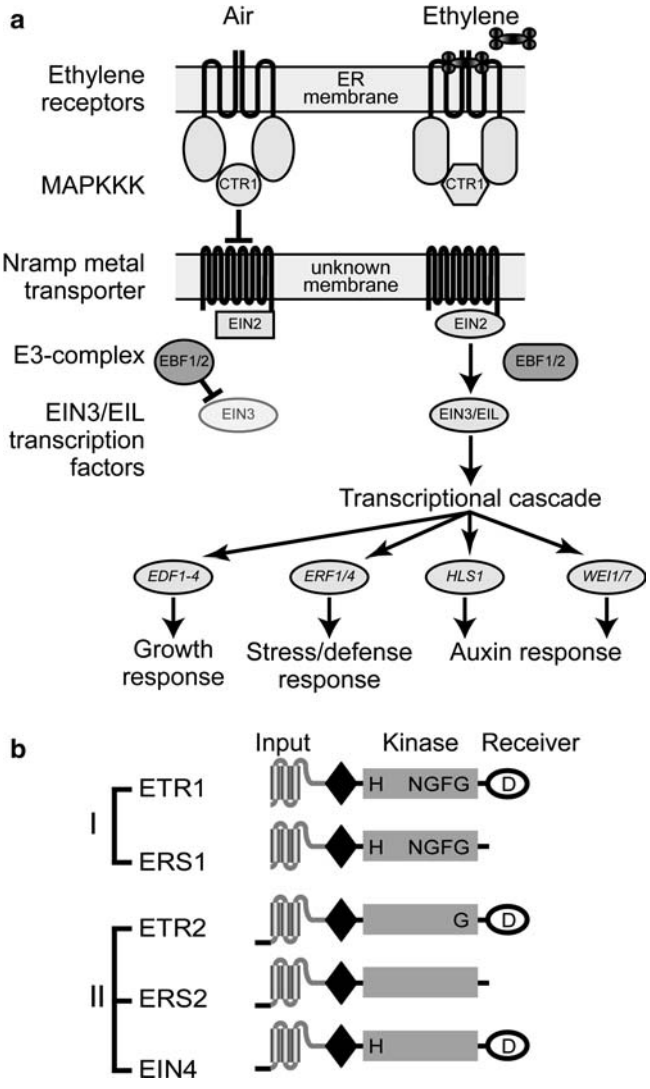

Fig. 1 **a** Ethylene signal transduction. In the absence of ethylene, the receptors activate CTR1, a negative regulator that suppresses downstream signaling. CTR1 is related to the MAPKKKs, but it is unclear whether CTR1 functions as the first step in a MAP kinase cascade. Downstream of CTR1, EIN3 levels are reduced by proteasome-mediated degradation, involving action of the E3 complex components EBF1 and EBF2. Perception of ethylene results in the inactivation of CTR1 and prevents EIN3 degradation, thus activating the ethylene-signaling pathway. Activation of the EIN3/EIL family of transcription factors induces a transcriptional cascade to establish ethylene responses. **b** Ethylene receptors. The family is divided into two subfamilies (I and II) based on phylogenetic analysis and the presence of conserved sequences (H, N, G, F, and G) in the histidine kinase domain (*gray rectangle*). The ethylene-binding domain (input) consists of three transmembrane domains (*shaded bars*). Subfamily II receptors have an additional putative signal sequence (*black bar*) preceding the transmembrane domains. All five members of the ethylene receptor family have a GAF domain (*black diamond*) of unknown function. The receiver domain is indicated by an *oval*. Conserved histidine (H) and aspartate (D) phosphorylation sites are indicated

sponse. As a first step for defining the role of the HK domain, it was shown through truncation analysis that the HK domain is required for signal output from the receptor ETR1 (Qu and Schaller 2004). A site-directed mutation that eliminates HK activity, but otherwise leaves the HK domain intact, had a modest effect upon the ability of the receptor to repress ethylene responses in air (Qu and Schaller 2004); the mutation also slowed the recovery of seedlings to normal growth rates after ethylene-induced growth inhibition (Binder et al. 2004b). Some members of the ethylene receptor family (ETR1, ETR2, and EIN4) also contain a C-terminal receiver domain, which may be the recipient of phosphate transfer from the histidine kinase domain. Lack of a receiver domain increased the sensitivity of seedlings to ethylene-mediated growth inhibition (Qu and Schaller 2004). A site-directed mutation that eliminated the receiver domain's site of phosphorylation slowed the recovery of seedlings to normal growth rates after ethylene-induced growth inhibition (Binder et al. 2004b).

Thus, while the precise roles that the HK and receiver domains play in ethylene signaling remain enigmatic, some basic conclusions can be made. First, the HK domain is essential for signal output and its enzymatic activity plays a role in modulating the ethylene signal. Second, the receiver domain is not essential for signaling output, but is likewise involved in modulating the ethylene signal. These domains now appear to be established as players in the ethylene response, although much remains to be determined about their mechanism of action.

Transcriptional regulation by ethylene

The EIN3/EIL family of transcription factors regulates gene expression, including that of other transcription factors such as ERF1, indicating that the ethylene response involves a transcriptional cascade (Fig. 1a). The breadth of ethylene-regulated gene expression is now becoming clear through microarray analysis: 3–7% of the genes examined are ethylene-regulated and these genes are involved in many biological processes (Alonso et al. 2003; De Paepe et al. 2004; Van Zhong and Burns 2003). The levels of ethylene-responsive gene expression increase and decrease, indicating the importance of both activators and repressors in the establishment of the ethylene response. Significant differences were found in the genes regulated during short (10 min) or medium (6 h) term ethylene exposure (De Paepe et al. 2004). In addition, substantial differences were found between the genes regulated in a constitutive ethylene-response mutant (*ctr1-1*) to those regulated in wild-type plants treated with ethylene for 24 h (Van Zhong and Burns 2003). Thus the length of exposure to ethylene, the ethylene dosage, the plant cell and/or tissue, and the plant developmental state are all likely to modify the transcriptional response to ethylene.

Post-translational regulation of ethylene signaling

Post-translational regulation, particularly protein turnover mediated by the ubiquitin-proteasome pathway, plays a significant role in regulating ethylene signaling. Ubiquitin is attached to its targets via the activity of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzyme complexes, specificity being conferred by the E3 complex through direct interaction of its components (such as F-box proteins) with the substrate (Moon et al. 2004). The poly-ubiquitinated proteins are then targeted for degradation by the proteasome.

Ethylene biosynthesis is regulated at the post-translational level by controlling turnover of several members of the ACC synthase (ACS) family, which converts *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC), before further conversion to ethylene (Yang and Hoffman 1984). ACS5 levels are post-translationally regulated by ETO1 (Ethylene overproducing 1), a component of an E3 complex (Chae et al. 2003; Wang et al. 2004). Thus, ETO1 may be modulating ACS5 activity by recruiting it for poly-ubiquitination and subsequent degradation through the proteasome (Wang et al. 2004). Accumulation of another ACS family member, ACS6, is controlled by phosphorylation via a MAP kinase, MPK6 (Liu and Zhang 2004), suggesting that ACS6 degradation may also occur in a phosphorylation-dependent manner through a proteasome-mediated pathway.

Two independent studies identify a RUB-dependent pathway as a regulator of ethylene production in *Arabidopsis* (Bostick et al. 2004; Larsen and Cancel 2004). RUB (related to ubiquitin) is a ubiquitin-like protein that covalently attaches to an SCF (Scp1 Cdc 53 F-box) ubiquitin-ligase complex to modify its activity. In one study, both *RUB1* and *RUB2* were shown to regulate a variety of plant processes including ethylene production, increased ethylene biosynthesis being found in plants with reduced levels of RUB1/2 (Bostick et al. 2004). In a second study, a mutation in *RCE1*, which is responsible for the covalent attachment of RUB to SCF, was shown to result in increased levels of ethylene biosynthesis (Larsen and Cancel 2004). The *rce1* mutant plant had increased ACC oxidase activity, but whether ACC oxidase levels are directly regulated by the RUB-dependent pathway is not yet known.

A major control point for ethylene signal transduction is the transcription factor EIN3. In the absence of ethylene, the EIN3 protein is continuously degraded (Fig. 1a) (Gagne et al. 2004; Guo and Ecker 2003; Potuschak et al. 2003; Yanagisawa et al. 2003). Degradation of EIN3 is mediated by two F-box proteins, EIN3 Binding factor (EBF) 1 and 2, which recognize EIN3 and recruit it to an E3 complex for poly-ubiquitination. Activation of ethylene signaling prevents this degradation, thus allowing EIN3 to accumulate and activate its transcriptional targets (Fig. 1a). EBF1 and EBF2 work together to regulate EIN3 accumulation; however, the ethylene-stimulated expression of *EBF2* (Alonso et al. 2003; Gagne et al. 2004) suggests distinct roles during the ethylene response.

Kinetics of the seedling growth response to ethylene

Ethylene inhibits seedling growth but, until recently, this response had only been analyzed in *Arabidopsis* after ethylene treatment for several days. An elegant kinetic analysis of this inhibition by time-lapse imaging has now revealed an extremely rapid and biphasic ethylene response (Binder et al. 2004a, b). Within 15 min of ethylene application, seedlings respond with a rapid decrease in their growth rate (phase I) to reach a new steady-state growth rate. This is followed by a slower and sustained inhibition of growth (phase II), with the final steady-state growth rate reached at approximately 75 min after ethylene application (Binder et al. 2004b).

The phase I growth response is sensitive to very low levels of ethylene (0.2 nl l^{-1}), independent of dose, and does not require EIN3 and EIL1, the two major transcription factors involved in ethylene signaling (Binder et al. 2004a). These observations suggest either that phase I inhibition occurs through a post-transcriptional mechanism or that other transcription factors are involved (Binder et al. 2004a). In contrast, phase II is dose dependent and appears to be transcriptionally mediated by EIN3 and EIL1 (Binder et al. 2004a).

Crosstalk between ethylene and other signaling pathways

From the recent explosion of research into hormone crosstalk, it is becoming increasingly apparent that there is significant interconnectivity between the signaling pathways of ethylene and other hormones. Several recent studies point out the complex interplay between signaling by ethylene and auxin. Part of the seedling growth response to ethylene is dependent on auxin biosynthesis as revealed by the study of mutations in *Weak ethylene insensitive2* and *7* (Stepanova et al. 2005). Auxin response factor 2 (ARF2) protein levels are regulated by ethylene-induced *Hookless 1* (*HLS1*) to coordinate auxin-mediated cell elongation during apical hook formation in the dark (Li et al. 2004), a process which is also dependent on gibberellins (Vriezen et al. 2004) and brassinosteroids (De Grauwe et al. 2005). Hypocotyl elongation in the light also involves these three hormones, along with cytokinin, in what appears to be a complex interaction between the different hormone biosynthesis and signaling pathways (De Grauwe et al. 2005; Smets et al. 2005). Ethylene also plays a significant role in modulating production of auxins, gibberellins, cytokinins, and abscisic acid during seed

maturation as a means to control dormancy (Chiwocha et al. 2005).

The interplay between ethylene signaling and the pathways for defense and stress responses is also becoming clearer. Defense and stress responses are regulated by several members of the EIN3/EIL-regulated *Ethylene-response factor (ERF)* transcription-factor family, often in conjunction with other hormones such as jasmonate (*ERF1*) (Berrocal-Lobo and Molina 2004; Lorenzo et al. 2003) or abscisic acid (*ERF4*) (Yang et al. 2005). Similarly, the ERF1 homolog, *Ethylene-responsive element binding protein* (*AtEBP*), regulates defense responses in an ethylene-regulated manner (Ogawa et al. 2005).

Anthony B. Bleecker (1950–2005)

It would be negligent, even while describing the recent advances in our understanding of ethylene signal transduction, if we did not acknowledge the severe loss that this field suffered with the death of Tony Bleecker. Tony began his studies on ethylene while a Ph.D. candidate in the laboratory of Hans Kende (Michigan State University) and continued these as a post-doctoral research associate in the laboratory of Elliot Meyerowitz (Caltech). Starting in 1989, he established his own laboratory at the University of Wisconsin-Madison, where he served as mentor to numerous undergraduate, graduate, and post-doctoral researchers, including an author of this review (G.E.S). As a Ph.D. student, Tony was responsible for the isolation of the first *Arabidopsis* mutant compromised in its ethylene response (*etr1-1*). The *etr1-1* mutation that he and colleagues described turned out to be a lesion in the ethylene receptor itself, the ethylene-insensitive phenotype of *etr1-1* resulting from a single amino acid change that abolished the receptor's ability to bind ethylene. Tony continued expanding the boundaries of the field up until his death, as exemplified in the kinetic analysis on ethylene growth responses, described earlier in this review, that was undertaken with his colleagues Brad Binder and Edgar Spalding. Significantly, the technology developed for these studies now opens up the short-term ethylene response of *Arabidopsis* seedlings to a physiological analysis, where previously only long-term responses could be so assayed. The shape of the field as we know it today would not exist without Tony's work, and he will be sorely missed.

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