Chapter 6
Ethylene Signaling from the Endoplasmic Reticulum Membrane to the Nucleus

Bram Van de Poel and Caren Chang

Abstract The molecular genetic dissection of the ethylene-signaling pathway that began 25 years ago has elucidated the framework of the ethylene-signaling pathway, from ethylene perception at the endoplasmic reticulum (ER) membrane to changes in gene expression in the nucleus. Recent discoveries have uncovered how the ethylene signal is transmitted from the ethylene receptor complex at the ER membrane to the downstream nuclear transcription factors, further connecting the signaling components and filling in long-standing gaps in our understanding of the pathway. These findings raise intriguing new questions for the future.

Keywords Ethylene · Signaling · Perception · Receptors · Protein complex · EIN2 · CTR1 · Phosphorylation · Arabidopsis

6.1 Background

The gaseous molecule ethylene is a major phytohormone that regulates many aspects of plant development and physiology, including fruit ripening, senescence, abscission and cell elongation, as well as responses to biotic and abiotic stress (Abeles et al. 1992; McManus 2012). Accordingly, the production of ethylene by plants is regulated developmentally and environmentally in a tissue-specific manner (Bleecker and Kende 2000). Although the discovery of ethylene as an important plant growth regulator dates back to observations over a century ago (Neljubov 1901), the main components of ethylene perception and signaling have been uncovered in just the past 25 years. With the molecular mechanisms of ethylene signaling increasingly coming

B. Van de Poel · C. Chang (✉)
Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, USA
e-mail: carenc@umd.edu

B. Van de Poel
e-mail: bram.vandepoel@ugent.be

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into focus, it is clear that the ethylene-signaling pathway has many interesting and novel features. One of these is that ethylene signaling requires an ethylene receptor protein complex that resides at the endoplasmic reticulum (ER) membrane, interacting with other signaling components. Upon the perception of ethylene at the ER membrane, the signal is transmitted to the nucleus resulting in the accumulation of ethylene-responsive transcription factors that activate changes in gene expression that alter physiological responses. This chapter reviews our current understanding of the ethylene-signaling pathway from the perception of ethylene at the ER membrane to gene expression in the nucleus.

6.2 Elucidation of the Ethylene-Signaling Pathway

In the 1980s, the development of *Arabidopsis thaliana* as a genetic model system was a major advance that provided the critical tools needed to genetically dissect a full range of developmental processes and plant signaling pathways. In fact, the ethylene-signaling pathway was one of the first plant hormone pathways to be genetically dissected in *Arabidopsis*. Prior to this, there were essentially no studies that could definitively link ethylene responses with particular genes or protein activities. For example, specific ethylene-binding sites had been detected in plant tissue using feeding experiments with ^{14}C-labeled ethylene gas (Sisler 1979, 1980), but it could not be established whether bona fide ethylene receptors were responsible for the observed binding. The genetic dissection of ethylene signaling was greatly facilitated by the ability to isolate ethylene-specific mutants in *Arabidopsis*, based on the ethylene-response phenotype of dark-grown seedlings known as the "triple response" that was first observed in pea seedlings more than a century ago (Neljubov 1901) (Fig. 6.1). By screening for mutants that lack the triple response, Bleecker et al. (1988) isolated the first ethylene-insensitive mutant named *etr*. Soon after, additional ethylene-insensitive (*ein*) mutants were isolated, as well as ethylene overproducing (*eto*) and constitutive ethylene response (*ctr*) mutants (Guzman and Ecker 1990; Kieber et al. 1993; Roman et al. 1995). Generally, mutants that were isolated based on having an altered dark-grown seedling response to ethylene possessed similar defects in other tissues at other developmental stages, such as defects in leaf cell expansion, flowering time, organ abscission, and altered senescence response in mature plants. Using genetic epistasis analysis, it was determined that the corresponding genes for these mutants function in a linear signaling pathway. In the 1990s, given the development of molecular maps of the *Arabidopsis* genome and other molecular genetic resources, the corresponding genes were subsequently cloned based on their genetic map positions. The cloning of these genes provided the first glimpse of the molecular components of the ethylene-signaling pathway. Additional ethylene mutants that have been isolated

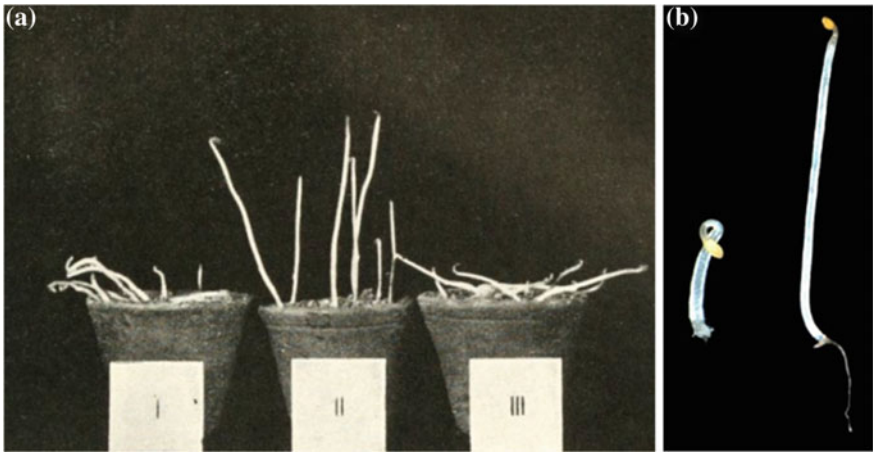

Fig. 6.1 The ethylene-signaling pathway was genetically dissected by exploiting the "triple response" in dark-grown seedlings treated with ethylene. **a** Discovery of the triple response phenotype of dark-grown pea seedlings by the Russian scientist Neljubov (1901). The pea seedlings showed *horizontal bending* when grown in laboratory air containing illumination gasses (*I*), but not if the air was filtered through KOH, $Ba(OH)_2$, $CaCl_2$, CuO, and water (*II*). If the CuO filter was removed, the seedlings showed bending again (*III*), which led to the conclusion that ethylene must be the gas causing this striking phenotype. **b** The triple response in dark-grown *Arabidopsis* seedlings, as first shown by Bleecker et al. (1988). When germinated in the presence of exogenous ethylene (*left*), dark-grown seedlings exhibit the triple response phenotype, which consists of radial hypocotyl swelling, hypocotyl shortening, and an exaggerated apical hook. The dark-grown seedling on the *right* has not been treated with ethylene and displays the wild-type etiolated phenotype

include enhanced ethylene response mutants (*eer*) (e.g., Larsen and Chang 2001), weak ethylene-insensitive mutants (*wei*) (e.g., Alonso et al. 2003), and suppressor/enhancer mutants, e.g., reversion-to-ethylene sensitivity (*rte*) (e.g., Resnick et al. 2006). Besides these *Arabidopsis* mutants, tomato fruit ripening mutants such as never-ripe (*nr*) and non-ripening (*nor*) have been valuable for studying ethylene responses in this commercially important vegetable crop. Further components in ethylene signaling have been subsequently identified based on protein–protein interactions using yeast two-hybrid library screens (e.g., Potuschak et al. 2003).

The subsequent subcellular localization of pathway components has advanced our understanding of the pathway at the cellular level, revealing that an ethylene receptor protein complex resides at the ER membrane and regulates the nuclear accumulation of transcription factors that activate ethylene-responsive gene expression. Figure 6.2 provides an overview of the key elements of the ethylene-signaling pathway from the ER membrane to the nucleus in *Arabidopsis*. In brief, ethylene is perceived at the ER membrane and the ethylene signal is transduced to the EIN3/EIL1 transcription factors, which control gene expression in the nucleus. Below we review these steps in more detail.

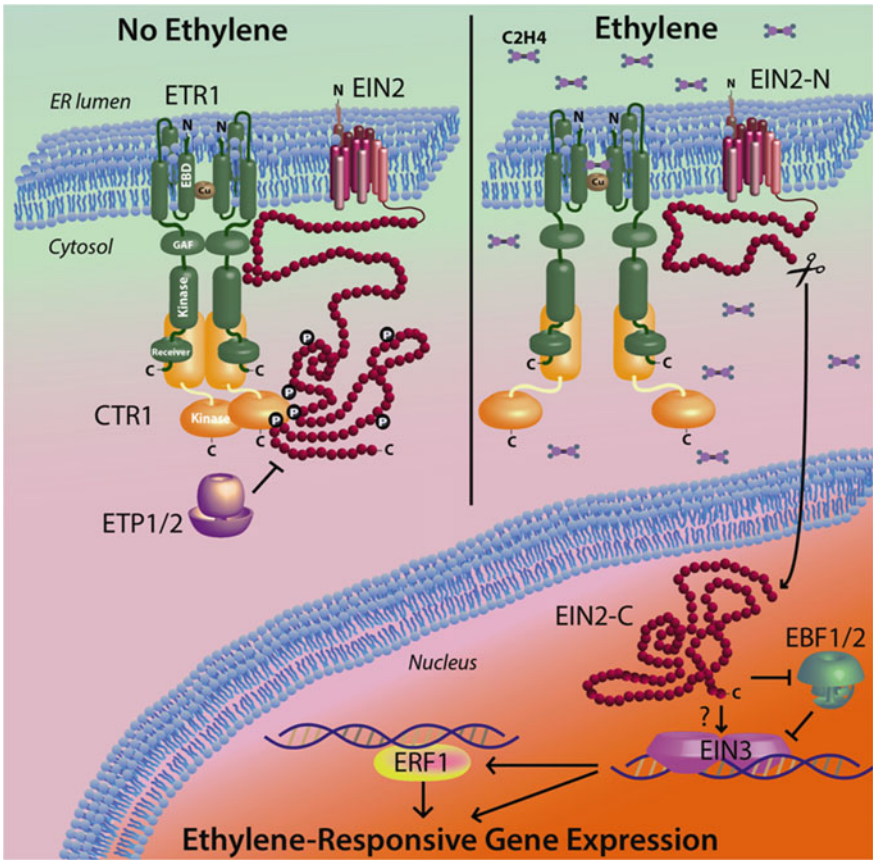

Fig. 6.2 Current model of the ethylene-signaling pathway in *Arabidopsis*. Without ethylene (*left side*), the ethylene receptor dimer (represented by ETR1) at the ER membrane activates the protein kinase CTR1, which is physically associated with the receptor's kinase and receiver domains. The CTR1 dimer phosphorylates the cytosolic C-terminal portion of EIN2, which has 12 N-terminal transmembrane domains spanning the ER membrane. The EIN2 C-terminal domain can physically associate with the ETR1 kinase domain as well, but the functional relevance of this interaction is unknown. When phosphorylated, EIN2 is inactive and possibly targeted for 26S proteasomal degradation via two F-box proteins ETP1/2. Transcription factors EIN3 and EIN3-LIKE1 (represented by EIN3) are synthesized but immediately targeted by two F-box proteins, EBF1/2, for degradation by the 26S proteasome. When ethylene is present (*right side*), the ethylene-binding domain (EBD) of the receptor binds ethylene with the aid of a copper (Cu) cofactor, deactivating receptor signaling and in turn deactivating CTR1. The unphosphorylated EIN2 C-terminal portion is now susceptible to proteolytic cleavage by an unidentified protease. The cleaved portion ("EIN2-C") migrates into the nucleus where it is involved in stabilizing the EIN3 and EIL1 transcription factors. EIN3 activates transcription of ethylene-responsive genes, one of which is *ERF1*, which encodes another transcription factor. This transcriptional cascade results in an array of physiological responses to ethylene (not shown)

6.3 Elements of the Ethylene-Signaling Pathway

6.3.1 The Ethylene Receptors

Ethylene is perceived by ethylene receptor complexes localized at the ER (Chen et al. 2002; Grefen et al. 2008). Given that ethylene gas is freely diffusible into cells and is more soluble in membranes than in aqueous environments, there is no requirement for ethylene perception to occur at the plasma membrane. Perception of ethylene at the ER membrane might allow for an energetically efficient and rapid response as proposed by Chen et al. (2002). It is also conceivable that some ethylene responses occur at the ER membrane and do not involve changes in gene expression.

The precise composition of the ethylene receptor complex is unclear. Ethylene is perceived by a family of receptors that has sequence similarity to the two-component histidine protein kinases of the two-component signaling system, which is widely known in bacteria (Chang et al. 1993). There are two subfamilies of ethylene receptors based on their structural similarity (for details, please see Chaps. 3 and 4). Receptors in both subfamilies consist of an N-terminal transmembrane ethylene-binding domain, followed by a cytosolic GAF domain and a cytosolic C-terminal histidine kinase (or histidine kinase-like) domain (Bleecker et al. 1988). Subfamily 2 has an additional N-terminal transmembrane domain, and some members in each subfamily carry a C-terminal receiver domain (of the two-component system) attached to the histidine kinase domain. The ethylene receptor unit is a homodimer, which is stabilized by two disulfide bonds at the N-terminus of the ethylene-binding domain (Schaller et al. 1995). The receiver domain, carried by some receptors, can dimerize as well (Müller-Dieckmann et al. 1999). The GAF domain is also thought to be involved in protein–protein interactions between the same or different ethylene receptor isoforms (Gao et al. 2008) resulting in higher order complexes of receptor dimers (Gao et al. 2008; Grefen et al. 2008; Chen et al. 2010). The receptor complex also contains the downstream protein kinase CTR1, which associates with the receptor kinase and receiver domains (Clark et al. 1998; Cancel and Larsen 2002; Gao et al. 2003). The receptor kinase domain also interacts with the downstream signaling component, EIN2, and the strength of this interaction appeared to be enhanced when histidine autophosphorylation of ETR1 was disrupted (Bisson and Groth 2010).

Despite their sequence similarity to prokaryotic receptor histidine protein kinases, the precise signaling mechanism of the ethylene receptors remains unclear. Genetic analyses have established that the ethylene receptors negatively regulate ethylene responses (Hua and Meyerowitz 1998). That is, in the absence of ethylene binding, the receptors signal to repress ethylene responses, whereas ethylene binding shuts off signaling and allows responses to proceed (Hua and Meyerowitz 1998; Wang et al. 2003; Binder et al. 2008). This is in contrast to the more straightforward concept of receptor signaling being activated by the signal. *Arabidopsis* has five ethylene receptors that are partially redundant, despite their

sequence differences and distinct enzymatic activities. Of the two subfamily 1 ethylene receptors in *Arabidopsis* (ETR1 and ERS1), ETR1 has histidine kinase activity while ERS1 has both histidine kinase activity and serine–threonine kinase activity. The *Arabidopsis* subfamily 2 ethylene receptors (ETR2, ERS2, and EIN4) exhibit only serine–threonine kinase activity (Binder 2008; Moussatche and Klee 2004). Interestingly, however, ETR1 histidine kinase activity is not essential for ethylene signaling (Wang et al. 2003; Gamble et al. 2002; Xie et al. 2006), although the presence of the ETR1 histidine kinase domain is required (Qu and Schaller 2004). Similarly, it remains unclear what role serine–threonine kinase activity plays in ethylene signaling. On the other hand, there is evidence indicating that the ethylene receptors can be differentially autophosphorylated, being less phosphorylated in the presence of ethylene (Voet-van-Vormizeele and Groth 2008; Kamiyoshihara et al. 2012). The receiver domain, which is present on some ethylene receptors, appears to be involved in the recovery from ethylene response (Binder et al. 2004; Kim et al. 2011).

The stability of certain ethylene receptors has been found to be ethylene-dependent, with the receptor being degraded via the proteasome upon ethylene treatment (Kevany et al. 2007; Chen et al. 2007). During tomato fruit ripening, an increased expression level of the receptor was not reflected at the protein level. This led to the conclusion that the level of receptor proteins is a negative measure of ethylene sensitivity (Kevany et al. 2007), i.e., having more receptors reduces ethylene sensitivity, which is in agreement with the negative mode of action of the receptors. Another factor that affects ethylene sensitivity is the potential non-redundant nature of the ethylene receptor family. Multiple reports point toward the receptors being non-redundant, leading to the conclusion that each receptor has a unique role in ethylene signaling, as well as overlapping roles (Shakeel et al. 2013).

Among the five ethylene receptors in *Arabidopsis*, ETR1 plays the largest role, as seen by the fact that loss-of-function mutations in *etr1* display an ethylene-hypersensitive phenotype not seen in the other ethylene receptor mutants (Hua and Meyerowitz 1998). ETR1 activity is specifically affected by a protein called RTE1 (REVERSION-TO-ETHYLENE SENSITIVITY1), which physically interacts with ETR1 (Dong et al. 2008, 2010) and promotes signaling by ETR1 but not the other ethylene receptors (Resnick et al. 2006, 2008; Rivarola et al. 2009) (Fig. 6.3). The exact molecular mechanism by which RTE1 promotes ETR1 signaling remains unclear, but it was shown that RTE1 acts through the N-terminus of ETR1 (Zhou et al. 2007). RTE1 can suppress some dominant missense alleles of *etr1* (RTE1-dependent) yet not others (RTE1-independent) (Resnick et al. 2008), raising the possibility that RTE1 plays a role in ETR1 folding. RTE1 was also shown to be involved in the recovery from ethylene (Kim et al. 2011). Although the *rte1-3* mutation did not affect growth inhibition kinetics when seedlings are exposed to ethylene, the typical growth overshoot after ethylene recovery was reduce when RTE1 function was inhibited, leading to a reduced hypocotyl growth when ethylene was removed (Kim et al. 2011). RTE1 also modulates nutational bending of hypocotyls exposed to ethylene (Kim et al. 2011). Another recent study showed that RTE1 in turn can interact with isoforms of cytochrome b5 (Cb5), a small

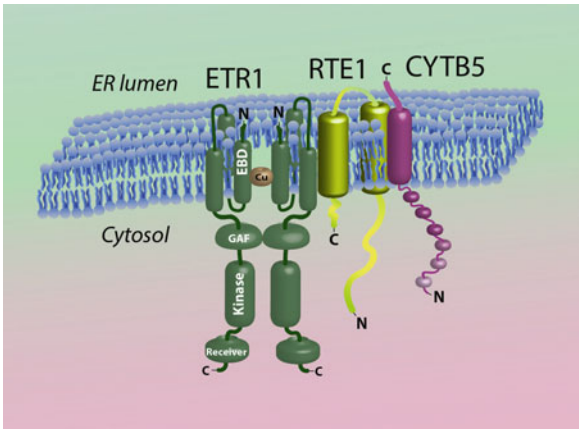

Fig. 6.3 Additional interactions of the ethylene-signaling complex at the ER membrane. Reversion-To-Ethylene Sensitivity1 (RTE1) interacts specifically with the *Arabidopsis* ETR1 ethylene receptor (Dong et al. 2008, 2010), promoting the active signaling form by an unknown mechanism (Resnick et al. 2008; Rivarola et al. 2009). RTE1 interacts with cytochrome b5 (CYTB5), a small hemoprotein, which appears to have a small effect on ethylene signaling (Chang et al. 2014)

hemoprotein (Chang et al. 2014). The exact role of Cb5 remains obscure, but it appears to act upstream of RTE1 and team up with RTE1 to activate the signaling of ETR1 (Chang et al. 2014).

Ethylene binding to the receptor requires a copper cofactor, which is supplied by the copper transporter RAN1 (Hirayama et al. 1999; Woeste and Kieber 2000). This copper molecule is not only essential for ethylene binding but for ethylene receptor biogenesis, since the *ran1* null mutant has a severe constitutive ethylene-response phenotype similar to that displayed by mutants lacking multiple ethylene receptors (Rodriguez et al. 1999; Woeste and Kieber 2000).

6.3.2 The Protein Kinase CTR1

As mentioned above, the ethylene receptor kinase domain physically interacts with the serine/threonine protein kinase CTR1. This protein–protein interaction is achieved between the kinase and receiver domains of the receptor and the N-terminal regulatory domain of CTR1, thus tethering the soluble CTR1 protein to the ethylene receptor complex at the ER membrane. The *Arabidopsis CTR1* gene was uncovered in a genetic screen for mutants that display the triple response phenotype in the absence of ethylene treatment, and like the ethylene receptors, CTR1 is a negative regulator of ethylene responses (Kieber et al. 1993). CTR1 has serine/threonine kinase activity in vitro (Huang et al. 2003). Recent 3-D structural analysis of the CTR1 kinase domain indicated that it forms a functional dimer in the

absence of ethylene (Mayerhofer et al. 2012). Together with the negative regulation of the ethylene receptors, the ETR1/CTR1 complex functions as a reverse agonist system to perceive and transduce the ethylene signal. In other words, in the absence of ethylene gas, the complex signals to repress ethylene responses, and this signaling is inactivated by ethylene perception.

CTR1 has been referred to as a "Raf-like kinase," because it is most similar in sequence to the family of Raf protein kinases, which are known to initiate mitogen-activated protein kinase (MAPK) cascades (Kieber et al. 1993). Thus, for many years, an unidentified MAPKK and MAPK were believed to be downstream of CTR1. To date, however, a MAPK cascade involving CTR1 has not been identified, and no MAPKK or MAPK has been conclusively placed in the ethylene-signaling pathway. Instead, it was recently shown that the CTR1 physically interacts with EIN2 and directly regulates EIN2 by phosphorylating the EIN2 C-terminal domain (Ju et al. 2012). This finding has finally resolved the long-standing question of the missing MAPKK and MAPK substrates downstream of CTR1.

While CTR1 directly phosphorylates EIN2, the possibility remains that there is a secondary ethylene-signaling pathway that involves a MAPK cascade. There is in fact some evidence for a secondary ethylene-response pathway that bypasses CTR1. For example, mutants lacking multiple ethylene receptors exhibit a slightly stronger constitutive ethylene-response phenotype than the *ctr1* null mutant (Hua and Meyerowitz 1998; Cancel and Larsen 2002; Liu et al. 2010), suggesting that the receptors can signal independently of CTR1. Bypass of CTR1 was also observed when expression of the N-terminal domain of the *Arabidopsis* ETR1 receptor (mainly consisting of the ethylene-binding domain) reduced the constitutive ethylene-response phenotype of *ctr1* mutants (Qiu et al. 2012; Xie et al. 2012). The underlying mechanism for this bypass is currently unknown.

6.3.3 The EIN2 Protein Bridges Ethylene Signaling from the ER to the Nucleus

The next downstream component in the pathway is EIN2, which remains mysterious in many respects. The *EIN2* gene was identified as an ethylene-insensitive mutant (Roman et al. 1995), and cloning of the gene in 1999 revealed a protein with two domains (Alonso 1999). The EIN2 N-terminal domain consisting of 12 predicted transmembrane domains has sequence similarity with the Nramp (natural resistance associated macrophage protein) family of metal ion transporters, which transport divalent metals across membranes (Fox and Guerinot 1998). The biochemical function of the EIN2 N-end remains obscure, as no metal transport has been observed for EIN2. The soluble C-terminal portion has no known protein domains, but contains a conserved nuclear localization signal (NLS). Overexpression of the EIN2 C-terminal domain alone results in constitutive ethylene responses (Alonso et al. 1999), suggesting that the C-terminal domain is important for ethylene responses.

For a decade, the subcellular localization of EIN2 was unknown. In fact, EIN2 was proposed to reside in the nuclear membrane so that it would be able to signal to the next known downstream components, which were transcription factors. EIN2 was finally localized to the ER membrane by Bisson et al. (2009). Moreover, the soluble C-terminal portion of EIN2 was shown to physically interact with the kinase domain of all five ethylene receptors, and the strength of the EIN2–ethylene receptor interaction was affected by the phosphorylation status of the ethylene receptor (Bisson and Groth 2010). This close interaction of the EIN2 C-terminal domain with the receptor kinase domain, together with the ETR1–CTR1 interaction, suggests that EIN2 is part of the ethylene-signaling complex at the ER membrane.

Once EIN2 was localized to the ER membrane in 2009, it was apparent that ethylene signal transduction needed to traverse a potential physical gap between the ER membrane and the nucleus. Filling in this gap might have involved a search for unidentified components, except for the clue that the EIN2 C-end contains an NLS. Indeed, three groups independently reported that a portion of the EIN2 C-terminus (C-end) is proteolytically cleaved in the presence of ethylene and migrates into the nucleus (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012; Fig. 6.4), while the N-end remains at the ER membrane (Ju et al. 2012). A mutation in the EIN2 NLS prevented nuclear translocation and downstream ethylene responses (Wen et al. 2012). This cleavage and translocation of EIN2 was a major discovery that resolved the question of how the ethylene signal is transduced from the ER to the nucleus.

Phosphorylation of the EIN2 C-end by CTR1 regulates whether or not the C-end is cleaved (Ju et al. 2012). Phosphorylation of EIN2 was first indicated by a proteomic study of the ethylene response that uncovered differential phosphorylation of the

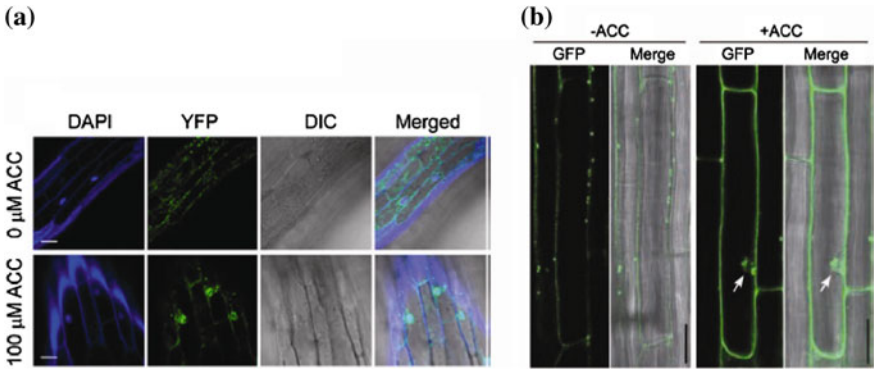

Fig. 6.4 Nuclear localization of the EIN2 C-end upon ethylene treatment **a** Confocal microscopy showing ethylene-responsive nuclear localization of EIN2 WT-YFP in *Arabidopsis* hypocotyl cells of four-day-old dark-grown wild-type seedlings transformed with *35S-EIN2 WT-YFP*. Seedlings were treated for 3 h with or without 100 μM ACC. Figure is from Ju et al. (2012); **b** GFP fluorescence in the roots of 3-day-old etiolated seedlings of *35S:EIN2-GFP/ein2-5* transgenic plants with or without ACC treatment. Arrows indicate the nuclei. Figure is reproduced from Wen et al. (2012) with permission from *Cell Research*

EIN2 C-end. EIN2 was found to be phosphorylated on at least six different sites, including Ser^{645} and Ser^{924} (Chen et al. 2011; Ju et al. 2012), but the phosphorylated peptides of EIN2 were observed only in the absence of ethylene (Chen et al. 2011). This was consistent with the fact that CTR1 kinase activity is a negative regulator of ethylene signaling, and thus led to the discovery that CTR1 is the protein kinase responsible for phosphorylating EIN2 (Ju et al. 2012). When EIN2 is phosphorylated by CTR1 in the absence of ethylene, EIN2 is inactive, and when CTR1 is inactive in the presence of ethylene, EIN2 is cleaved, resulting in translocation of the C-end to the nucleus. Protein degradation may play a role in blocking EIN2 signaling, since EIN2 appears to be degraded by the 26S proteasome via two F-box proteins ETP1 and ETP2 (Qiao et al. 2009).

This most recent breakthrough in our understanding of how the ethylene signal is transduced from the ER to the nucleus raises new mechanistic questions. A major question is how does the nuclear localization of the EIN2 C-end result in activation of downstream ethylene signaling? Is the C-end further processed and/or are other proteins involved in signaling to activate/stabilize the downstream transcription factors EIN3/EIL1? What is the identity of the protease that cleaves EIN2 and how is this protease regulated? The site(s) of EIN2 cleavage also remains to be resolved; Qiao et al. (2012) reported that EIN2 is cleaved at amino acid residue Ser^{645}, whereas Ju et al. (2012) found that an alanine substitution of Ser^{924} confers in strong constitutive ethylene-response phenotype, independent of an alanine substitution at Ser^{645} (Ju et al. 2012).

6.3.4 EIN3/EILs Activate Gene Expression in the Nucleus

The transcription factors EIN3 and EIN3-LIKE1 (EIL1) act downstream of EIN2 in the ethylene-signaling pathway. *EIN3* was discovered in a genetic screen for ethylene-insensitive mutants (Roman et al. 1995) and was subsequently cloned and characterized by Chao et al. (1997). Based on sequence homology with *EIN3*, three *EIL*'s (*EIL1-3*) were retrieved in the *Arabidopsis* genome (Chao et al. 1997). *EIL1* and *EIL2* rescued the *ein3-1* mutation showing functional redundancy of these transcription factors (Chao et al. 1997). EIN3 forms a dimer, which is not required for DNA binding (Solano et al. 1998), and there is some evidence that the dimerization of tomato EIL1 could involve phosphorylation (Li et al. 2012). Yamasaki et al. (2005) elucidated the DNA-binding domain (DBD) of *Arabidopsis EIL3* by determining its 3-D protein structure. There is a high structural similarity between EIN3 and the EILs, including the DBD. Upon ethylene treatment, this DBD specifically interacts with the primary ethylene response element in the promoter region of ethylene-responsive genes, initiating transcription. One of these genes is *ERF1*, encoding another transcription factor, which activates secondary target genes (Solano et al. 1998). Thus, downstream ethylene signaling involves a transcriptional cascade.

The critical regulatory mechanism of ethylene signaling in the nucleus is the control of EIN3/EIL1 protein levels. In the absence of the ethylene signal, EIN3/EIL1 are rapidly degraded by the 26S proteasome. This degradation is specifically mediated by two F-box proteins, EBF1 and EBF2 (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004; An et al. 2010). In the presence of the ethylene signal, the F-box proteins EBF1/EBF2 themselves are degraded, thus allowing for the accumulation of EIN3/EIL1 proteins and consequently the EIN3/EIL1 activation of ethylene-responsive gene expression (An et al. 2010). Wen et al. (2012) showed that the presence of the EIN2 C-end in the nucleus somehow leads to the stabilization of the EIN3 protein, thus resulting in ethylene responses. This is in accordance with the observation that EBF1/EBF2 turnover is dependent on *EIN2* (An et al. 2010). Whether EIN2 and EIN3 directly interact and exactly how EBF1/2 stability is regulated are not known. Differential phosphorylation of EIN3 has been suggested as a determinant for EIN3 protein stability (Yoo et al. 2008), although the responsible MKK9-MPK3/6 kinases identified by Yoo et al. (2008), have been found to play a role in ethylene biosynthesis and may not target EIN3 directly (Liu and Zhang 2004; Xu et al. 2008; An et al. 2010).

Finally, the expression of numerous genes is regulated in response to ethylene (e.g., De Paepe et al. 2004; Zhong and Burns 2003; Alonso et al. 2003; Nemhauser et al. 2006). A recent analysis using ChiP-seq and RNA-seq in a high-resolution ethylene time-course showed that EIN3 regulates transcription of ethylene-responsive genes in a four-wave manner, in which each wave is represented by a distinct set of EIN3 targets (Chang et al. 2013). This indicates that the nuclear events following ethylene treatment are discontinuous, and that several cascades or waves of responses are either activated or down-regulated. Further investigation into these transcriptional cascades is likely to provide insights into transcriptional networks involving crosstalk with other signaling pathways.

6.4 Conclusions and Future Challenges

The recent detailed characterization of EIN2 cleavage and the nuclear translocation of the C-end has finally bridged the physical gap between the ER-localized ETR1/CTR1/EIN2 signaling complex and the nuclear transcription factors EIN3 and EIL1. This discovery has precluded the necessity for a MAPK cascade in ethylene signaling. This advance raises questions concerning the mechanism by which the EIN2 C-end is cleaved and the biochemical function of the EIN2 C-end in the nucleus. Additionally, there are many questions concerning the function and dynamics of the ethylene receptor complexes at the ER membrane, how the ethylene receptors regulate CTR1 and the function of the EIN2 N-terminus. The mechanisms of a potential secondary pathway(s) that bypasses CTR1 also remain unknown. Another area requiring further investigation, but not discussed in this chapter, involves crosstalk between ethylene and other signaling pathways. What are the molecular elements and mechanisms by which ethylene crosstalk is achieved?

Future work will be required to answer the question of how the dose-responsiveness of hormone action is transmitted by a molecular mechanism that results in a differential response. How is the cell able to distinguish between a high concentration of ethylene versus a low concentration of ethylene, and how are these mixed signals perceived at the receptor level and how are the transcription events in the nucleus regulated to evoke the correct response? The problem with current applications is that one always looks at the tissue or even whole plant level. Single cell analysis of ethylene dose–response actions could uncover precise molecular mechanistic events on how pleiotropic signals are transduced, but not how a certain phenotype is achieved. Linking these two is a challenge for the future of ethylene-signaling research.

Molecular genetics has dramatically advanced our understanding of ethylene signaling in plants. While genetic screens have resulted in the discovery of major components of the ethylene signal transduction pathway, new methodologies, such as proteomics, bioinformatics, systems biology, and epigenetic studies can now be applied to expand our understanding of the ethylene-signaling network and its crosstalk with other pathways.

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