Chapter 5
Regulatory Components of Ethylene Signal Transduction

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Abstract Ethylene, the simple but vital gaseous hormone, affects an extensive array of developmental processes and responses to external and internal cues in plants. Extensive molecular genetic investigations during the past two decades have established a linear ethylene signaling pathway starting from endoplasmic reticulum (ER) membrane-spanning receptors to nuclear-localized transcription factors in the model plant *Arabidopsis thaliana*. The pathway involves negative regulation of ethylene signaling by ethylene receptor family members and Raf-like CONSTITUTIVE TRIPLE-RESPONSE1 (CTR1) and positive regulation by ER-associated ETHYLENE INSENSITIVE2 (EIN2) and nuclear-localized EIN3 and EIN3-LIKE1 (EIL1). Although ethylene is the signaling molecule that switches off the negative regulation by the receptors, several components fine-tune the signaling. In this chapter, we briefly summarize studies of ethylene signal transduction to give an overall picture of the ethylene signaling cascade. We also discuss regulatory components modifying the signaling components in the ethylene signaling pathway. Finally, we pose intriguing questions related to ethylene actions.

Keywords Plant hormone · Ethylene · Signal transduction · EIN2 · EIN3/EIL1 · EBF1 · EBF2 · ETP1 · ETP2 · ECR2 · Nuclear-cytoplasmic transport

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5.1 Overview

Ethylene is one of the earliest discovered plant growth regulators and the first gaseous hormone discovered (Burg 1973; Kepinski and Leyser 2003). Early in 1901, the Russian plant physiologist Dimitry K. Neljubov revealed an odd growth habit of dark-grown pea seedlings grown in laboratory air contaminated with illuminated gaseous ethylene. In 1934, the British scientist R. Gane reported that ethylene was synthesized by plants. Finally, in 1965, ethylene was established as a plant hormone regulating growth and development.

However, understanding ethylene signal transduction did not advance until the isolation of the involved signaling components by use of genetic and molecular approaches in the early 1990s. Results from those studies with the model plant *Arabidopsis* proposed a linear signal transduction pathway involving negative regulation by ethylene receptor family members and Raf-like CONSTITUTIVE TRIPLE-RESPONSE1 (CTR1) and positive regulation by ER-associated ETHYLENE INSENSITIVE2 (EIN2) and the nuclear transcription factors EIN3 and EIN3-LIKE1 (EIL1). Ethylene is the key that switches off negative regulation and switches on positive signaling. In addition, multilevel regulation of ethylene signaling by other components was later revealed in the 2000s with the isolation of components that regulate *EIN3*, and EIN3/EIL1 and EIN2 levels, facilitate signaling by the N-terminus of the ethylene receptor ETHYLENE RESPONSE1 (ETR1), are involved in CTR1 functions, and affect ethylene signaling with mechanisms to be addressed.

Among the components that regulate ethylene signaling components are the F-box proteins EIN3-BINDING F-BOX PROTEIN1 (EBF1)/EBF2 and EIN2-TARGETING PROTEIN1 (ETP1)/ETP2, which have major roles in negative regulation of EIN3/EIL1 and EIN2 to attenuate ethylene signaling; F-box protein level is attenuated by a negative feedback regulation of ethylene. EIN5 is a 5′ → 3′ exoribonuclease and its function is inversely associated with *EBF1/EBF2* level to facilitate EIN3 accumulation so that ethylene signaling proceeds. REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1) facilitates ETR1-receptor N-terminal signaling, which is mediated without involving the receptor histidine kinase (HK) and receiver domains and the downstream signaling component CTR1. Enhancer screening of the weak *ctr1-10* allele isolated mutations and components likely involved in CTR1 activity. Other components identified from mutations resulting in ethylene hypersensitivity could have a role in negative regulation of ethylene signaling by mechanisms yet to be determined.

5.2 A Model for Ethylene Signal Transduction

Ethylene signal transduction is described in Chap. 6, so here we only briefly describe the concept to better understand the regulation of ethylene signaling components. Ethylene signaling is negatively regulated by the ethylene receptor family members and CTR1. In the absence of ethylene, the receptor signal output,

despite its unclear biochemical nature, is mediated via the ethylene receptor C-terminal HK domain to CTR1 via protein–protein interaction with the N-terminal domain of CTR1. CTR1 is thus activated by receptor signaling and then can phosphorylate EIN2. EIN2 is a positive regulator of the ethylene response. Phosphorylated EIN2 stays at the ER, and ethylene signaling does not occur. With ethylene binding to the receptors, the receptor signaling is switched off, and CTR1 is prevented from activation; EIN2 is not phosphorylated and undergoes a proteolytic cleavage to produce a C-terminal fragment. With a nuclear localization signal (NLS), the EIN2 C-terminus enters the nucleus to mediate ethylene signaling, which is eventually transmitted to the transcription factors EIN3/EIL1 to switch on the expression of ethylene response genes.

The pathway involves four classes of signaling components: the ethylene receptors and CTR1 as negative regulators and EIN2 and EIN3/EIL1 as positive regulators. Little is known about the biochemical nature of CTR1 activation by the receptors and the underlying mechanisms of the proteolytic cleavage of EIN2. Whether the activation of EIN3 directly or indirectly involves the EIN2 C-terminus remains to be addressed. With the negative regulation of receptor signaling and CTR1 by ethylene, ethylene is the major player switching on ethylene signaling.

5.3 Components that Regulate Ethylene Signaling

Ethylene signaling involves negative regulation by ethylene receptors and CTR1 and positive regulation by EIN2 and EIN3/EIL1. Of the four classes of signaling components, the function of each is modulated by regulatory components to fine-tune ethylene signaling (Fig. 5.1).

5.3.1 RTE1 Facilitates ETR1 Receptor Signaling

RTE1 was isolated from a suppressor screening of the dominant ethylene-insensitive *etr1-2* allele. It encodes a membrane protein associated with the ER and Golgi apparatus. Sequence analysis of RTE1 did not identify domains of known function. The ethylene insensitivity conferred by the ethylene receptor etr1-2, which has the A102T substitution, requires RTE1, and the loss-of-function allele *rte1-2* confers increased ethylene sensitivity. *RTE1* overexpression confers ethylene insensitivity in an *ETR1*-dependent manner. Thus, RTE1 is involved in ETR1 receptor signaling (Resnick et al. 2006). In the presence of the two loss-of-function alleles of the ethylene receptor genes *ETR1* and *ETHYLENE RESPONSE SENSOR1* (*ERS1*), the *etr1-7 ers1-2* mutant shows a strong constitutive ethylene response phenotype, with severe growth inhibition and infertility, for largely prevented receptor signaling (Wang et al. 2003). If RTE1 is required for ETR1 receptor signaling, *rte1-2 ers1-2* would phenotypically resemble *etr1-7 ers1-2*; however, the double mutant is fertile

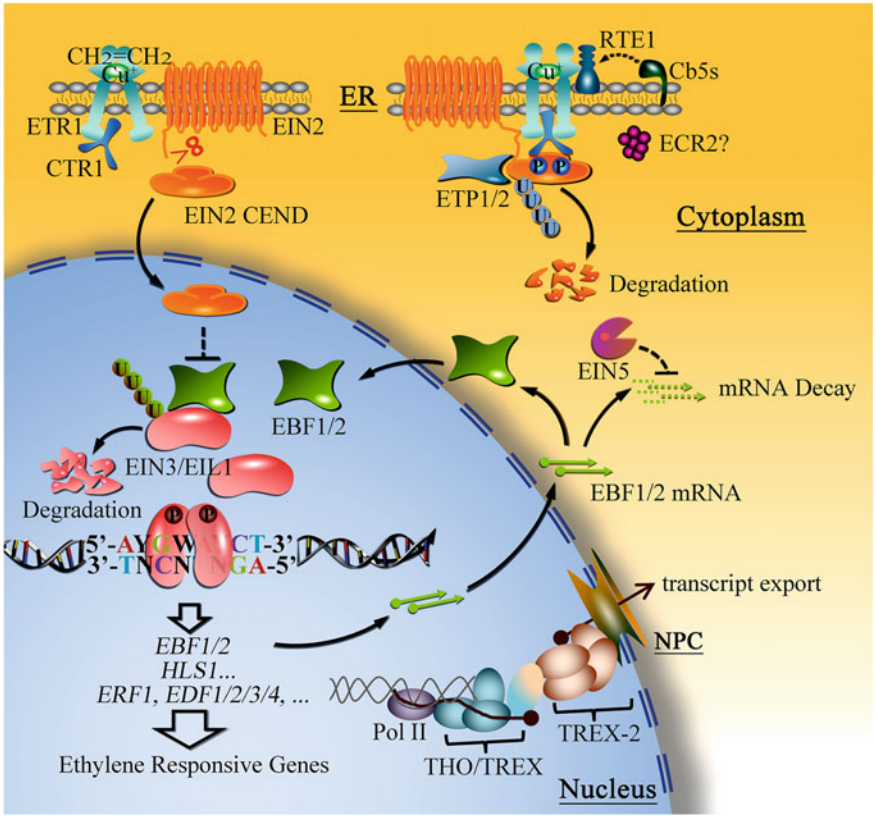

Fig. 5.1 Multilevel regulations of ethylene signaling. Involvement of RTE1 in ETR1 ethylene receptor signaling and ECR2 in CTR1 functions; ECR2 may act downstream of the ethylene receptors and upstream of EIN3/EIL1. ETP1/ETP2 are F-box proteins for EIN2 degradation, and EBF1/EBF2 for EIN3 degradation. EIN5, an exoribonuclease, acts as a negative regulator of EBF1/EBF2 functions. Ethylene signaling can be regulated at the level of RNA transcription export that involves THO/TREX and TREX-2 complexes, of which the latter is tethered with the nuclear pore complex (NPC)

and shows relatively normal growth. Therefore, RTE1 is involved in part but is not required for ETR1 receptor signaling (Zhou et al. 2007).

Several studies have advanced our knowledge of the role of RTE1 in ETR1 receptor signaling. ETR1 receptor signaling is mediated via its C-terminal HK domain to CTR1. Alternatively, the receptor signaling can be mediated by the truncated etr1$^{1-349}$ fragment (residues 1–349), which lacks the HK and receiver domains without involving CTR1 (Xie et al. 2012). Ethylene insensitivity conferred by *RTE1* overexpression is prevented with the *etr1-7* loss-of-function allele, which results from the early termination of the Trp74Stop; expression of the etr1$^{1-349}$ fragment restores the ethylene insensitivity conferred by *RTE1* overexpression in *etr1-7*. CTR1 prevents ethylene signaling, and the loss-of-function *ctr1-1* allele,

resulting from the D694E substitution that attenuates CTR1 Ser/Thr kinase activity, cannot suppress ethylene signaling, which produces a typical constitutive ethylene response phenotype with strong growth inhibition. Expression of etr1$^{1-349}$ rescues the *ctr1-1* mutant phenotype because ETR1 N-terminal signaling does not involve CTR1; however, ETR1 N-terminal signaling is prevented by the loss-of-function *rte1-2* allele. Thus, RTE1 is required for ETR1 N-terminal signaling to a pathway not involving CTR1 (Xie et al. 2012; Qiu et al. 2012).

The molecular mechanism for the involvement of RTE1 in ETR1 N-terminal signaling is revealed from studies that show co-localization and physical association of the two proteins at the ER and Golgi apparatus (Dong et al. 2008, 2010). With the Trp fluorescence spectroscopy technique (described in Chap. 12), the interaction between RTE1 and ETR1 occurs with high affinity (dissociation constant [*Kd*] 117 nM), whereas the *Kd* for the interaction between ETR1 and rte1-1 (from the C161Y substitution) is 1.38 μM, an increase of approximately of 12-fold. RTE1 may directly associate with ETR1 to promote ETR1 receptor signaling. The loss-of-function *rte1-1* impairs the interaction and thus cannot facilitate ETR1 receptor signaling. Yeast two-hybrid screening isolated RTE1-interacting partners: the ER-localized cytochrome *b5* (Cb5) isoforms B, C, D, and E all interact with RTE1. Genetic analyses revealed an association of Cb5 isoform functions and ethylene responses, so Cb5 isoforms and RTE1 may be functional partners involved in ETR1 receptor signaling (Chang et al. 2014).

5.3.2 Regulation of Ethylene Receptor Signaling by Receptor Cooperation

Ethylene receptor families sensing ethylene in plants are small in family member number. The *Arabidopsis* ethylene receptor family has five members and they may function in various clusters. Receptor clustering is believed to facilitate cooperative receptor signaling to respond to a wide range of ethylene concentration. Ethylene receptor clustering is described in Chap. 4.

Plant ethylene receptor family members have a redundant function in suppressing ethylene signaling. Redundancy could prevent lethality or severe impacts when some of the members are affected by mutations. However, duplicated genes may accumulate mutations during evolution to gain new functions that are important for survival so that the genes can remain stable in the genome. Duplicated genes without gaining new, vital functions may become lost on mutation accumulation during evolution.

Members of the *Arabidopsis* ethylene receptor family appear to have common and unique functions in ethylene signaling as well as in other aspects of plant growth and development. The unique functions of different receptor family members in receptor signaling facilitate differential receptor cooperation that may have a role in regulating ethylene signaling. From this perspective, different ethylene receptor members may function as regulators of ethylene signaling.

5.3.2.1 Negative Cooperation of the Ethylene Receptor ERS1

Hua and Meyerowitz showed that the constitutive ethylene-response phenotype was stronger in *Arabidopsis* mutants with increased rather than reduced number of ethylene receptor family members, which implies that the ethylene receptors negatively regulate the ethylene response (Hua and Meyerowitz 1998). Using various combinations of loss-of-function alleles of the ethylene receptor gene family, Liu et al. found that degrees of the ethylene response were alleviated in ethylene-receptor-defective mutants that contain *ETR1* on removal of the ethylene receptor gene *ERS1*. In contrast, the constitutive ethylene response was increased in *ERS1*-containing mutants on removal of *ETR1*. Overexpression of ERS1 greatly elevated the ethylene response in the mutant with ETR1 and ERS1 as the remaining wild-type ethylene receptors (Liu et al. 2010). Thus, ERS1 may negatively regulate the ETR1 receptor signal output, possibly via receptor clustering, in addition to suppressing ethylene signaling.

5.3.2.2 Differential Receptor Cooperation of ETR1 and ERS1 with Other Family Members

Arabidopsis mutants carrying ETR1 and ERS1 as the only wild-type ethylene receptor differ in degrees of ethylene response. ETR1 is the only remaining ethylene receptor in (*ETR1*) *ers1 etr2 ein4 ers2* and ERS1 is the only remaining receptor in (*ERS1*) *etr1 etr2 ein4 ers2*; the former displays relatively normal growth, whereas the latter features many aspects of strong constitutive ethylene response with severe growth inhibition throughout development (Liu and Wen 2012a; Liu et al. 2010). Therefore, ETR1 and ERS1 may function distinctly: ETR1 mediates a much stronger signal output than ERS1 in the absence of other ethylene receptor family members. Of note, ETR1 and ERS1 belong to qualitatively different protein complexes; one explanation for the difference in the signaling behavior is that each receptor protein complex could participate in unique, non-overlapping regulation of downstream responses (Chen et al. 2010).

Consistent with the respective receptor signal output behavior by the wild-type ETR1 and ERS1, each examined in mutants, the signal output for ethylene-insensitive receptors is much stronger for etr1-1 than ers1^{I62P} in the absence of other family members (Liu and Wen 2012a). Of the two receptors, etr1-1, with the C65Y substitution that prevents ethylene binding, is dominant and confers ethylene insensitivity (Rodriguez et al. 1999; Wang et al. 2006; Hall et al. 1999). The artificially created ers1^{I62P} receptor, with the I62P substitution, is also dominant and confers ethylene insensitivity (Hua et al. 1995). In a quintuple mutant defective in the five ethylene receptor genes, the expression of *ETR1p:etr1-1* but not *ERS1p:ers1^{I62P}* rescued the mutant phenotype and conferred ethylene insensitivity. However, the mutant phenotype was rescued with the expression of *ERS1p:ers1^{I62P}* in the quintuple mutant containing a single copy of *ETR1*, and the mutant became ethylene insensitive (Liu and Wen 2012a). Thus, ERS1 receptor signaling may depend in part

on ETR1. Given that ETR1 and ERS1 form heteromeric clusters via the GAF domain, ERS1 signaling is facilitated on clustering with ETR1 (Gao et al. 2008).

The effect of other family members on ERS1 receptor signaling is revealed by examining the ethylene response in quadruple mutants that contain one wild-type ethylene receptor gene and the *ERS1p:ers1*^{I62P} transgene. The ethylene response is strong throughout development in (*ERS1*) *etr1 etr2 ein4 ers2*, (*ETR2*) *etr1 ers1 ein4 ers2*, (*EIN4*) *etr1 ers1 etr2 ers2*, and (*ERS2*) *etr1 ers1 etr2 ein4*. The expression of the *ERS1p:ers1*^{I62P} transgene substantially alleviated many aspects of the ethylene response in (*EIN4*) *etr1 ers1 etr2 ers2* and (*ETR1*) *ers1 etr2 ein4 ers2*, and both transgenic quadruple mutants are ethylene insensitive. The ethylene response is weaker in *ERS1p:ers1*^{I62P} (*ERS2*) *etr1 ers1 etr2 ein4* than *ERS1p:ers1*^{I62P} (*ETR2*) *etr1 ers1 ein4 ers2* and stronger than in *ERS1p:ers1*^{I62P} (*EIN4*) *etr1 ers1 etr2 ers2*. Results from ethylene receptor gene expression analysis do not support that the difference in ethylene response between the quadruple mutants is associated with level of the receptor. Thus, ERS1 signaling is facilitated differentially by other family members, possibly via receptor cluster formation.

The ethylene receptors may function synergistically. With an identical mutation as *etr1-1*, which causes the C65Y substitution, the artificially created *ers1*^{C65Y} confers dominant ethylene insensitivity. The signaling of the ethylene-insensitive ers1^{C65Y} is synergistically facilitated by ETR1 and EIN4; in the absence of both ETR1 and EIN4, ers1^{C65Y} cannot mediate a signal output. The synergistic actions of these receptors indicate greater cooperation of different receptors; alternatively, ETR1 and EIN4 have redundant functions in ERS1 signaling (Liu and Wen 2012a, b). ETR1 and ERS1 activities show the synergistic actions of different ethylene receptors. Mutants defective in both *ETR1* and *ERS1* show extremely strong constitutive ethylene responses, and the mutant phenotype is not alleviated by ectopic expression of other family members. The two receptors play important roles in negative regulation of the ethylene response; ETR1 and ERS1 may synergistically mediate the signaling of the other receptors (Liu and Wen 2012a; Wang et al. 2003; Binder and Bleecker 2003).

The genetic and transformation studies suggest that the combination but not necessarily the number of receptor family members may determine the strength of the receptor signal output. Conceivably, the signal output strength may differ between ethylene receptor clusters differing in receptor composition.

5.3.2.3 Lateral Cooperative Ethylene Receptor Signaling via the GAF Domain

The GAF domain is responsible for non-covalent interaction between the ethylene receptors and, conceivably, the site where the ethylene receptor cooperation may occur (Gao et al. 2008; Xie et al. 2012). Evidence for cooperative ethylene receptor signaling via the GAF domain was strengthened by findings showing that expression of the ethylene-responsive etr1$^{1-349}$ fragment that lacks the HK and receiver domains restores the ethylene insensitivity conferred by ethylene-

insensitive receptor isoforms in *ctr1-1* (Qiu et al. 2012). The ethylene-insensitive receptors etr1-1, ers1-1, etr2-1, ein4-1, and ers2-1 confer ethylene insensitivity in the presence of CTR1. With the *ctr1-1* loss-of-function allele, these ethylene-insensitive alleles cannot confer ethylene insensitivity and the corresponding double mutants show constitutive ethylene responses throughout development. Expression of *ETR1p:etr1*$^{1-349}$ reverses the constitutive ethylene response phenotype in *ctr1-1*, and the transgenic plant is ethylene responsive. With any of those ethylene-insensitive receptor alleles present in *ETR1p:etr1*$^{1-349}$ *ctr1-1*, ethylene insensitivity conferred by the alleles is restored. GAF is involved in the heteromeric receptor interaction and possibly receptor signaling to a pathway not involving CTR1 (Gao et al. 2008; Xie et al. 2012). The heteromeric interaction of etr1$^{1-349}$ and a full-length, ethylene-insensitive ethylene receptor isoform in *ctr1-1* may facilitate the signal output of the full-length receptor via the GAF domain of etr1$^{1-349}$ to the alternative pathway not involving CTR1.

5.3.2.4 Perspectives of Cooperative Ethylene Receptor Signaling

Studies of differential signaling by ETR1 and ERS1 suggest that the composition of an ethylene receptor cluster is associated with its signaling strength so that degrees of the ethylene response are associated with the combination but not necessarily number of ethylene receptor family members. The ethylene receptor composition differs in various cell types (Sakai et al. 1998; Kevany et al. 2007; Lashbrook et al. 1998); conceivably, different cell types or tissues may show differences in ethylene sensitivity. A strong signal output can be mediated by clusters with strong positive receptor cooperativity and a weak signal output by that with weak cooperativity. Ethylene-binding results in the inactivation of the ethylene receptor: a low level of ethylene treatment may inactivate a portion of the receptors in a cell. When a portion of the receptor clusters is inactivated by the same level of ethylene, cell types with ethylene receptor clusters mediating a strong signal output will be less ethylene responsive than those with clusters mediating a weak signal output because the remaining active receptor clusters may suppress the ethylene response to a greater extent in the former than latter cell types. Conceivably, cell types with clusters mediating a wide range of signal output strengths can respond to a wide range of ethylene concentration.

The number of individual members of the ethylene receptor family may vary in response to stimuli, and the composition of the ethylene receptors and thus the receptor clusters in a cell type may vary over time. For instance, ETR2 level is reduced via protein degradation whereas ERS1 level is elevated on exposure to a high level of ethylene (Chen et al. 2007; Liu et al. 2010). Given that ERS1 has negative effects on ETR1 signaling (Liu et al. 2010), the signal output by ETR1 will be weakened in receptor clusters with elevated ERS1 level. The ethylene receptor cluster composition may be dynamic, in concert with changes in ethylene receptor composition, and such changes in a cell type may facilitate appropriate adaptation to corresponding stimuli.

5.3.3 Regulation of CTR1

CTR1 is a Raf-like protein and has Ser/Thr kinase activity that is required for its ability to transduce ethylene receptor signaling to suppress the ethylene signaling mediated by EIN2 and EIN3/EIL1. Mutations that attenuate CTR1 kinase activity relieve the suppression, and ethylene signaling occurs (Huang et al. 2003). Recent studies suggest that on CTR1 activation, EIN2 is phosphorylated and stays at the ER, and ethylene signaling does not proceed. Without CTR1 activation or with the lack of CTR1 kinase activity, unphosphorylated EIN2 undergoes proteolytic cleavage to produce a C-terminal fragment that enters the nucleus to mediate ethylene signaling to EIN3/EIL1 (Ju and Chang 2012). Although the docking of CTR1 on the HK domain of ethylene receptors leads to CTR1 activation, little is known about the underlying mechanism of the activation. The activation may involve a protein conformation change but not biochemical reactions.

Efforts to isolate components involved in CTR1 activity have involved genetic screening for mutations with enhanced constitutive ethylene response in the weak *ctr1-10* allele that results from a T-DNA insertion at the 5′-untranslated region (UTR) of *CTR1*. The *ctr1-10* allele produces a mild constitutive ethylene response throughout development and is hypersensitive to ethylene over a wide concentration range (Yu and Wen 2013; Xu et al. 2014). The T-DNA insertion does not impair *CTR1* transcription; our immunoassay data showed CTR1 protein in the wild type (Col-0) but not *ctr1-10* mutant (unpublished data). With an increase in ethylene sensitivity, CTR1 expression in *ctr1-10* may be not abolished but rather reduced to a level below the immunoassay detection limit.

Alleles of the loss-of-function mutations that enhance *ctr1-10* ethylene response to a degree comparable to the *ctr1-1* and ethylene-treated wild-type level are designated *ENHANCING CTR1-10 ETHYLENE RESPONSE*s (*ECR*s). *ECR2* was mapped to chromosome 2 and remains to be cloned (Xu et al. 2014). Genetic analyses suggest that ECR2 acts together with CTR1 downstream of the ethylene receptors and upstream of EIN3/EIL1. *CTR1* is tightly linked with *EIN2*; genetic analysis to determine whether EIN2 acts downstream or upstream of ECR2 is challenging and has not yet been performed. Identification of the relationship between *EIN2* and *ECR2* shall provide additional insight into functional modes and regulatory roles of ECR2. The kinase activity of ctr1-1 is substantially reduced, and the *ecr2-1 ctr1-10* and *ctr1-1* mutants are phenotypically similar to that of a typical constitutive ethylene-response phenotype. ECR2 is predicted to be involved in part in CTR1 activity. Of note, ETR1 receptor signaling can be alternatively mediated without involving CTR1 via the truncated N-terminal fragment $etr1^{1-349}$ (see Sect. 5.3.1), and N-terminal signaling by the full-length ETR1 but not truncated $etr1^{1-349}$ is prevented by the kinase-defective ctr1-1 (Qiu et al. 2012). Expression of $etr1^{1-349}$ rescues the *ecr2-1 ctr1-10* and *ctr1-1* mutant phenotype to a similar degree, which supports that CTR1 kinase activity is highly reduced in both mutants so that the truncated $etr1^{1-349}$ but not full-length ETR1 receptor signaling can be mediated to suppress ethylene signaling (Xu et al. 2014).

Loss-of-function *ctr1* mutations that lead to a typical constitutive ethylene-response phenotype result from defects in the kinase activity or a deletion of the kinase domain. However, the *ctr1-8* mutation, resulting from the G354E substitution, disrupts the interaction between the ethylene receptors and CTR1, and the mutant unexpectedly shows a relatively mild constitutive ethylene response phenotype (Huang et al. 2003; Xie et al. 2012). Immunoassay revealed ctr1-8 protein in the soluble fraction but CTR1 in the membrane fraction (Gao et al. 2003). The protein ctr1-8 does not dock at the ethylene receptors and thus cannot mediate receptor signaling to prevent the ethylene signaling mediated by the EIN2 C-terminus; conceivably the receptor signaling is predominantly mediated to an alternative pathway that does not involve CTR1 in the *ctr1-8* mutant. The alternative pathway may somehow suppress the ethylene signaling that is conveyed by the EIN2 C-terminus. In contrast, *ctr1* mutants with defects in kinase activity or domain show prevented signaling mediated to the alternative pathway by full-length ethylene receptors, for inability to suppress EIN2 C-terminus–mediated ethylene signaling.

An enhancer screening for *ctr1-10* isolated alleles that are involved in part in CTR1 activity. Future studies of these components will shed light on the possible regulation of CTR1 activity on perception of ethylene receptor signaling. The difference in ethylene response between *ctr1-8* and *ctr1* mutants with defective kinase activity or domain reveals a negative regulation of the N-terminal signaling of full-length ethylene receptors by kinase-defective ctr1 proteins.

5.3.4 Regulation of EIN2

Genetic analyses suggested that EIN2 acts downstream of CTR1 in the ethylene signal transduction pathway. *EIN2* encodes a polypeptide of 1,294 amino acid residues with a membrane-intrinsic amino-terminal domain (residues 1–461) and a membrane-extrinsic carboxyl-terminal domain (residues 462–1,294, designated CEND). EIN2 shares 21 % sequence identity at its N-terminus with the 12 predicted transmembrane domains of the NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP) family of metal ion transporters. Since the isolation of *EIN2*, in 1999, knowledge of how the ER-localized protein could be a signaling component to activate nuclear transcriptional events by EIN3/EIL1 has advanced little. Not until 2012 were possible mechanisms revealed for the mediation of ethylene signaling by the C-terminal portion of EIN2.

Alonso et al. (1999) found that, with the exception of *ein2-9*, all *ein2* alleles are completely insensitive to both exogenous and endogenous ethylene. Ectopic expression of EIN2-CEND in the *ein2-5* mutant conferred constitutive ethylene responses in both young seedlings and adult plants but not etiolated seedlings. EIN2 CEND is sufficient for activating downstream ethylene responses.

Qiao et al. (2009) used yeast two-hybrid screening to isolate proteins potentially interacting with EIN2-CEND and identified two novel F-box proteins, EIN2

TARGETING PROTEIN1 (ETP1) and ETP2. In the absence of ethylene, ETP1 and ETP2 physically interact with EIN2 and downregulate the protein level of EIN2 by the ubiquitin/26S proteasome proteolytic pathway. In the presence of ethylene, the protein levels of ETP1/2 are downregulated, which perturbs the interaction between ETP1/2 and EIN2, thus resulting in the accumulation of EIN2 protein and activation of the ethylene response. EIN2 accumulation was observed in ethylene-treated wild-type seedlings and constitutive ethylene-responsive *ctr1-1* seedlings but not ethylene-insensitive *etr1-1* seedlings with ethylene treatment. In contrast, in *ein3 eil1*, the accumulation of EIN2 was similar to that in the wild type.

These observations suggest that the accumulation of EIN2 is prevented by ethylene receptors and CTR1 while independent of downstream factor EIN3/EIL1. Therefore, ethylene that prevents ethylene receptor signaling and CTR1 functions can promote the stabilization and accumulation of EIN2 by impairing the proteasomal degradation of EIN2 and induce the ethylene response.

Chen et al. (2011) used mass spectrometry to examine microsomal membrane proteins from ethylene-treated and ethylene-untreated etiolated *Arabidopsis* seedlings and identified phosphorylation sites at the C-terminus of EIN2. The differential EIN2 phosphorylation led to uncovering the negative regulation of EIN2 functions by CTR1. In the absence of ethylene, CTR1 phosphorylates the cytosolic C-terminal region of EIN2 at Ser^{645} and Ser^{924}. One of the possible consequences of such modifications is that EIN2 is targeted to 26S proteasomal degradation by F-box proteins ETP1/2 (Qiao et al. 2009). In the presence of ethylene, ethylene binding to the receptors inhibits CTR1 activation, and inactive CTR1 no longer phosphorylates EIN2, so the cytosolic EIN2 undergoes proteolytic cleavage. With the NLS, EIN2 CEND is translocated to the nucleus to activate ethylene signaling that is mediated by the transcription factors EIN3/EIL1 (Ji and Guo 2013; Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012).

Of note, transient expression of a full-length form of EIN2 fused to the C-terminus of GREEN FLUORESCENT PROTEIN (GFP) in *N. benthamiana* produced fluorescence at the ER membrane (Bisson et al. 2009), and fluorescence resonance energy transfer (FRET) revealed interactions of EIN2-CEND with all five ethylene receptors at the ER membrane (Bisson et al. 2009; Bisson and Groth 2011). Those studies depicted a scheme whereby the ethylene receptors and CTR1 cooperatively inhibit EIN2-mediated ethylene signaling, leading to EIN2 degradation or inactivation, and consequently EIN3/EIL1 degradation via the 26S proteasome pathway. In contrast, ethylene prevents the inhibition, thus EIN2 and EIN3/EIL1 accumulate to facilitate the ethylene response.

With the expression of EIN2 in *Arabidopsis* or tobacco leaves, the EIN2 N-terminus is tethered at the ER, and EIN2-CEND is localized in the nucleus as well as packaged into discrete and prominent foci in the cytoplasm. These observations suggest that the EIN2 C-terminus could also modulate ethylene signaling in the cytoplasm as well as the nucleus (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012). It is speculated that the artificially created EIN2-CEND that is overexpressed by its transgene and the native endogenous EIN2 C-terminus that is released by proteolytic cleavage could have common and divergent biochemical and functional

functions. Thus, the artificially overexpressed EIN2-CEND may represent only a certain part of the function of the native EIN2.

5.3.5 EIN3/EIL1 Protein Accumulation Is Modulated by F-Box Proteins EBF1 and EBF2

EIN3 and *EIL1* are not altered at the mRNA level but are subjected to post-transcriptional regulation in response to ethylene (Chao et al. 1997). Western blot assay revealed that EIN3/EIL1 is degraded through the ubiquitin/26S proteasome pathway in the absence of ethylene but is rapidly stabilized and accumulates in the nucleus on ethylene treatment or application of proteasome inhibitors such as MG132. Mutations in the F-box gene *EIN3 BINDING F-BOX1* (*EBF1*) or *EBF2* enhanced the ethylene response in *Arabidopsis* by stabilizing EIN3 and EIL1. Weak alleles of *ebf1 ebf2* showed constitutive ethylene phenotypes, and strong alleles of *ebf1 ebf2* became lethal as early as the seedling stage. Interestingly, An et al. (2010) documented overaccumulated EIL1 protein in the *ein3 ebf1 ebf2* mutant. In contrast, overexpressing of *EBF1* or *EBF2* conferred ethylene insensitivity together with decreased protein accumulation of EIN3 and EIL1 (Guo and Ecker 2003; Potuschak et al. 2003). Therefore, EBF1 and EBF2 promote the degradation of EIN3 and EIL1, whereas ethylene stabilizes EIN3 and EIL1. Apparently, an important question is how EBF1/2-mediated EIN3/EIL1 proteolysis is repressed by ethylene. Two alternative models are proposed that the ethylene signal directly modulates EIN3/EIL1 or inhibits the function of EBF1/EBF2.

Although the *ein3 ebf1 ebf2* mutant overaccumulates EIL1 protein and shows a constitutive ethylene response phenotype, including an inhibited hypocotyl and a dwarf and bushy stature, it is almost completely insensitive to exogenous ethylene. Therefore, when EBF1 and EBF2 are functionally disrupted, EIL1 protein is unable to be further stabilized by the application of exogenous ethylene. Also, the *eil1 ebf1 ebf2* mutant was not responsive to exogenous ethylene as compared with the ethylene-induced stabilization of EIN3 protein in the wild type, which suggests that ethylene-promoted EIN3 accumulation relies on the presence of EBF1/2. Thus, EBF1 and EBF2 are required for the transmission of the ethylene signal to regulate EIN3/EIL1 accumulation. These data suggest that the ethylene signal may modulate EIN3/EIL1 by inhibiting the function of EBF1/EBF2. Indeed, immunoblot assay revealed that ethylene can promote the degradation of EBF1 and EBF2 protein. Meanwhile, several lines of genetic evidence revealed that ethylene-induced degradation of EBF1/2 requires EIN2 but not EIN3/EIL1. Thus, ethylene induces EIN3/EIL1 stabilization by promoting the proteasomal degradation of EBF1/2 proteins in an EIN2-dependent manner (An et al. 2010; Guo 2011; Zhao and Guo 2011). Further studies should focus on understanding the mechanism by which EIN2 facilitates the proteolysis of EBF1/2.

In light of the molecular genetic and genomic findings, a linear ethylene signal transduction pathway has been established and is generally accepted. Recently, EIN3/EIL1 level was found to be stabilized by auxin, cold stress and light, whereas glucose reduced the protein level (Yanagisawa et al. 2003; Zhou et al. 1998; He et al. 2011; Shi et al. 2012; Zhong et al. 2010). Dissections of EIN3 protein accumulation illustrated that EBF1/2 but not EIN2 appeared to be required for EIN3 stabilization under exogenous auxin (Yanagisawa et al. 2003; Zhou et al. 1998, 2010; He et al. 2011; Shi et al. 2012). These findings provide strong evidence that EIN3 and EIL1 are involved in other signaling and also mediate the interplay among ethylene and other signaling pathways. Increasing biochemical analysis has revealed that the stability of EIN3/EIL1 is tightly regulated.

The expression of *EBF1* and *EBF2* is induced by ethylene at the transcription level (Gagne et al. 2004; Guo and Ecker 2003; Konishi and Yanagisawa 2008a; Potuschak et al. 2003). The mRNA levels of *EBF1* and *EBF2* were significantly increased in *EIN3*-overexpressing plants but decreased in loss-of-function *ein3* mutants, so *EBF1/2* transcription may be controlled by ethylene in an EIN3-dependent fashion. Electrophoretic mobility shift assays in vitro revealed that EIN3 can directly interact with the sequence of 5′-TACAT-3′ (reverse complement sequence: 5′-ATGTA-3′) in the *EBF2* promoter and activate *EBF2* expression. Such a negative feedback mechanism may allow plants to fine-tune the abundance of EIN3/EIL1 by avoiding an overreaction to ethylene (Gagne et al. 2004; Guo and Ecker 2003; Konishi and Yanagisawa 2008a; Potuschak et al. 2003).

Because the level of the key transcription factor EIN3/EIL1 is significantly determined by the protein accumulation of EBF1/2, any regulation of *EBF1/2* may in turn affect the protein level of EIN3/EIL1. Importantly, the 5′ → 3′ exoribonuclease EXORIBUNUCLEASE4 (XRN4)/EIN5 was found to be a new component of the ethylene signaling pathway. Epistasis analysis placed EIN5 upstream of EBF1/2. Furthermore, RNA gel blot analysis and affymetrix *Arabidopsis* tiling array expression analysis showed that the ethylene insensitivity of *ein5* results from the overaccumulation of *EBF1/2* mRNA. Immunoblot assays clarified that EIN5 is required for ethylene-induced stabilization of EIN3 and ethylene-regulated gene expression. These results suggest that in regulating the ethylene signal cascade, XRN4/EIN5 antagonizes the negative feedback loop between EIN3/EIL1 and EBF1/2 by accelerating *EBF1/2* mRNA degradation, which allows for stabilizing EIN3/EIL1 protein level to induce the ethylene response (Olmedo et al. 2006; Potuschak et al. 2006).

However, when *ebf2* mutants expressed the *EBF2* transgene that lacks the sequence downstream of the stop codon, driven by the native *EBF2* promoter or by the *EBF2* promoter with mutations in the *EIN3 BINDING SITE* (*EBS*) sequence, all transgenic lines displayed an entirely ethylene-insensitive phenotype (Konishi and Yanagisawa 2008a, b). Thus, the sequence downstream of the *EBF2* coding region (3′-UTR) may be involved in modulating both the *EBF2* expression and sensitivity to ethylene. Because the 3′-UTR often affects the stability or translational activity of mRNA, the 3′-UTR of *EBF2* may be involved in modulating ethylene signaling.

Further investigation of the regulatory function of sequences downstream of *EBF2* coding sequence might provide new insights into the ethylene signaling cascade.

5.3.6 *EIN3/EIL1 Activity Controlled by JAZ/DELLA*

The ability of EIN3/EIL1 to regulate target gene expression is directly modulated by the transcription regulators JASMONATE ZIM DOMAIN (JAZ) and DELLA, referring to the GRAS (the GAI, RGA, and SCR proteins) protein family members with a conserved DELLA motif (Pan et al. 2012; Zhu et al. 2011). Multiple approaches, including yeast two-hybrid assay, GST-fusion pull-down, co-immunoprecipitation (Co-IP) assay and bimolecular fluorescence complementation (BiFC), provided steady evidence of the direct interaction between EIN3 (amino acid residues 200–500)/EIL1 (corresponding to residues 201–501) and C-terminus of JAZ1/3/9. Furthermore, HISTONE DEACETYLASE6 (HDA6) is an interacting partner of both EIN3/EIL1 and JAZ1. The findings established a triangular regulation circuit involving EIN3/EIL1-JAZs-HDA6. JAZs-HDA6 suppresses EIN3/EIL1 functions, which is eliminated by the plant hormone jasmonate. Additionally, expression of the luciferase reporter gene driven by the *ERF1* promoter was increased by EIN3 but suppressed by JAZ1 in *Arabidopsis* protoplasts. Binding of JAZs with EIN3/EIL1 may reduce the transcriptional activation of EIN3/EIL1. Yeast two-hybrid assays and Co-IP demonstrated that DELLA proteins physically interact with EIN3 and EIL1, the peptide fragment spanning residues 200–500 in the DNA-binding domain of EIN3 being responsible for the interaction. DELLA proteins may repress the transcription activity of EIN3 and EIL1 as well, thus suppressing the expression of the targeted gene.

Different pathways modulate EIN3/EIL1 by diverse mechanisms, including protein stability, transcriptional activity and choice of partners as well as target genes. These and yet-to-be identified diverse modulations of EIN3/EIL1 activity render multiple signal inputs through EIN3/EIL1 and diverse physiological outputs as a result of specific target gene expression. The multilevel regulation of EIN3/EIL1 activity by other hormones is addressed in Chap. 8.

5.3.7 *Regulation of Ethylene Signaling Involves Transcription-Coupled Export*

An array of *Arabidopsis* mutants show increased ethylene sensitivity and unexpectedly result in various defects in the RNA transcription export machinery. Ethylene signaling could be regulated by genes whose normal expression requires the RNA transcription export machinery.

The loss-of-function allele of *Arabidopsis ENHANCED ETHYLENE RESPONSE5* (*EER5*) increases ethylene sensitivity at the seedling stage. EER5 was predicted to be an uncharacterized protein with a PCI domain and the PCI-associated module (PAM) found in components of large protein complexes, such as the proteasome or COP9 signalosome (CSN). EER5 may bridge EIN2 and the CSN, serving as part of a resetting mechanism for ethylene signaling (Christians et al. 2008).

ECTOPIC EXPRESSION OF SEED STORAGE PROTEINS1 (*ESSP1*) was isolated from a genetic screening for mutants exhibiting ectopic expression of *βCGpro:GUS* in *Arabidopsis*; the gene was mapped to At2g19560 and allelic to *EER5*. With the isolation of the *ESSP1* allele in *Arabidopsis*, EER5 was predicted to be a yeast Thp1 homolog, with refined search programs (Lu et al. 2010). Yeast Thp1 is a component of the transcription-coupled export 2 (TREX-2) complex that comprises Sac3–Thp1–Sus1–Cdc31 for mRNA export and is tethered to the nuclear pore complex (NPC). Tagged with YELLOW FLUORESCENT PROTEIN (YFP), ESSP1/EER5/THP1-YFP produced fluorescence in the nucleus, and expression of the fusion protein complemented the *thp1-3* loss-of-function mutation. mRNA export assay, with hybridization of polyadenylated RNA with labeled oligo $d(T)_{50}$, showed an accumulation of nuclear mRNA of unidentified species in mutant but not wild-type cells. Yeast two-hybrid screening isolated SUPPRESSOR OF ACTIN (SAC3), which interacts with ESSP1/EER5/THP1, also supported by BiFC assay in tobacco (*Nicotiana benthamiana*) leaf cells. Yeast two-hybrid screening also isolated a putative nucleoporin (NUP1) protein, and BiFC assay supported the interaction. Consistently, cells of the *nup1* mutant but not the wild type showed nuclear mRNA accumulation. In the ethylene triple-response assay with etiolated *Arabidopsis* seedlings, both *thp1-3* and *sac3b* seedlings showed stronger growth inhibition than did wild-type seedlings in the presence of the ethylene biosynthesis precursor 1-aminocyclopropane-1-carboxylic acid at a high concentration (50 μM), which indicates increased ethylene sensitivity in the two alleles (Lu et al. 2010).

Another class of transcription export complex components is HYPER RECOMBINATION1 (HPR1) of the THO tetrameric protein complex (comprising Hpr1, Mft1, Tho2, and Thp2) of the TRanscription EXport (TREX) complex involved in RNA transcription export in various organisms (Yelina et al. 2010). The loss-of-function *hpr1-4* mutation leads to elevation in the ethylene response, with enhanced leaf senescence in response to ethylene (Pan et al. 2012).

Transcription involves various dynamic, coordinated processes, such as transcription elongation, 5′ capping, 3′ polyadenylation, splicing, and docking of various proteins to the nascent RNA to form the messenger ribonucleoprotein particles that are eventually exported to the cytoplasm though the NPC (Grunwald et al. 2011). The coupling of RNA transcription and export to the cytoplasm involves coordination of TREX, TREX-2, and the NPC. Expression of the genes requiring the transcription export machinery may be regulated by altering the complex components; the involved genes largely remain to be identified. The isolation of an array of mutants with an increase in ethylene sensitivity and defects in the transcription export machinery may indicate a role of the machinery in regulating

ethylene signaling. Defects in the THO/TREX complex components result in reduced amount of *trans*-acting small interfering (tasi) RNA species derived from *TAS1* and *TAS2* but not *TAS3* (Yelina et al. 2010; Jauvion et al. 2010). It is to be investigated whether small RNAs could be involved in regulation of ethylene signaling.

5.4 Concluding Remarks

In the ethylene signal transduction pathway, the ethylene receptor family members and CTR1 negatively regulate ethylene signaling mediated by the EIN2 C-terminus to the nuclear transcription factors EIN3/EIL1 induce genes elevating ethylene response. Ethylene is the key to "switch off" the receptors and ethylene signaling is "switched on." Except for the key that determines the "on" and "off" of ethylene signaling, the signaling components in the pathway are modulated by various components. The multiple levels of regulation may facilitate fine-tuning the ethylene signaling so that various degrees of the ethylene response, rather than an "on" and "off" response, can occur for corresponding responses to stimuli. The presence of multiple ethylene receptor family members may also have a role in the regulation of ethylene signaling by differential receptor cooperation and the negative cooperation of ERS1. The negative cooperativity by ERS1 in a receptor cluster could "buffer" the receptor signaling, which may facilitate plasticity of the receptor signaling. The presence of multiple, functionally redundant ethylene receptors may have biological significance beyond avoiding the impacts resulting from loss-of-function mutations. The modulation of EIN3/EIL1 activity by JAZ/DELLA indicates the presence of crosstalk of signaling pathways between ethylene and other plant hormones.

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