

## Chapter 2

# Isolation of Components Involved in Ethylene Signaling

Jian Hua

**Abstract** Ethylene is unique among all plant hormones in that it is an odorless and colorless gas, and yet like other hormones it has profound effect on many aspects of plant growth and development as well as the interaction of plants with their environment. The perception and signaling of ethylene in plants has intrigued many biologists since its discovery. The gaseous ethylene was established as a growth hormone in the early twentieth century, its biosynthesis pathway was revealed in the 1970s, the core ethylene signaling components were isolated in the 1990s, and the signaling mechanisms are further revealed in the last decade. Ethylene research has been at the very front of modern plant biology and has made a great impact on our understanding of plant biology at the molecular and genetic levels. This chapter describes how the signaling molecules were isolated and identified largely according to historical order (Fig. 2.1). Ethylene biosynthesis and biochemical characterization of signaling molecules are covered in other chapters in the book.

**Keywords** Genetic screen · Triple response · Ethylene signaling

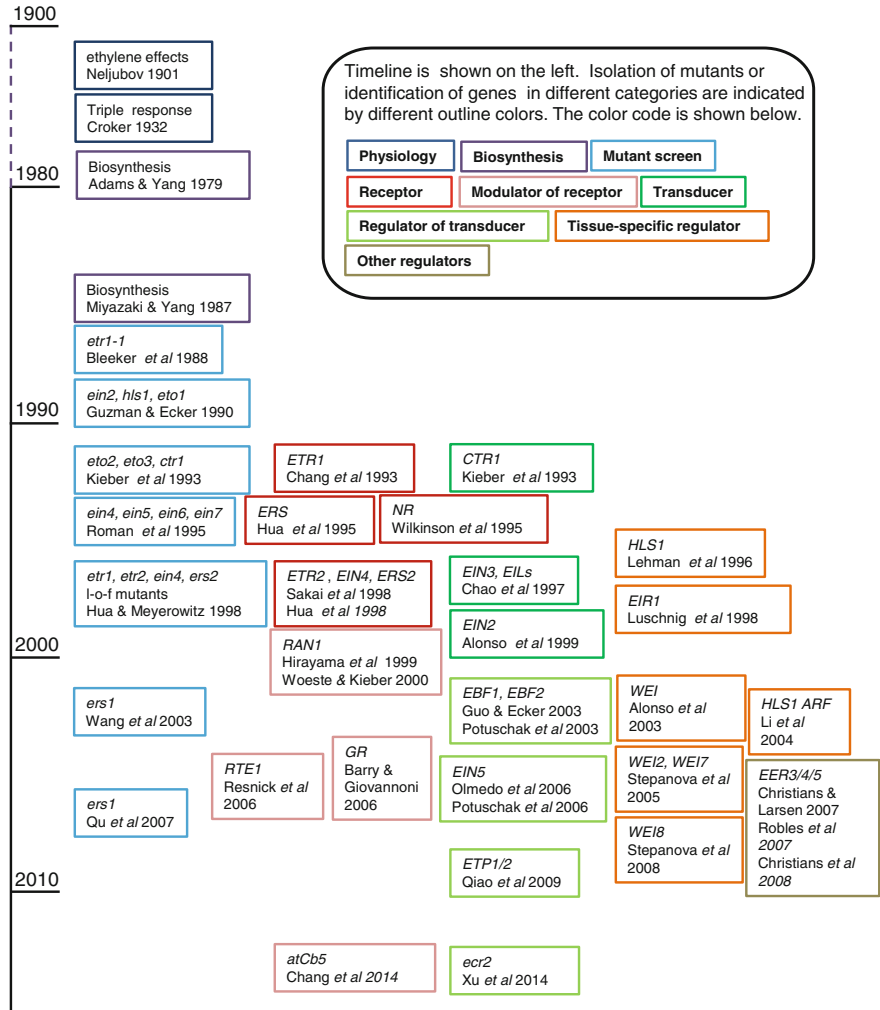
## 2.1 Overview

Ethylene was identified as a growth regulator in 1901 when Neljubov found that ethylene is the active component in illuminating gas that changed the growth orientation of pea (Abeles et al. 1992). Subsequent physiological studies revealed diverse effects of ethylene on plants, including senescence or ripening of plant organs, alteration of plant growth, biotic and abiotic stresses responses (Abeles et al. 1992). Among these responses, the “triple response” was used as a bioassay for ethylene until gas chromatography was introduced to determine the concentration of ethylene. This response refers to the horizontal growth of the apex (apical

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J. Hua (✉)

Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA  
e-mail: jh299@cornell.edu



**Fig. 2.1** Historical over view of identifying major ethylene signaling components

hook), short stem, and thick stem when pea seedlings grown in the dark are exposed to ethylene (Crocker 1932). It will become the widely used assay to uncover ethylene signaling mutants later.

Ethylene biosynthesis pathway was elucidated in the 1970s and 1980s. Ethylene is synthesized from methionine via S-adenosyl-L-methionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by ACC synthase and then to ethylene catalyzed by ACC oxidase (Adams and Yang 1979). ACC synthase produces, in addition to ACC, 5'-methylthioadenosine which is used to synthesize new methylene via a modified methionine cycle (Miyazaki and Yang 1987). Both ACC synthase and ACC oxidase were biochemically purified and later molecularly cloned (Kende 1993; Zarembinski and

Theologis 1994). These biosynthesis molecules are regulated by various environmental, developmental, and hormonal signals, indicating that ethylene is a signal for adaptive responses in plants.

How do plants detect and respond to ethylene? Plant cells must have receptor molecules that can detect varying amount of ethylene. Saturation of ethylene binding to tissues indicates the presence of ethylene binding molecules in plants. It was postulated that a transition metal might assist the binding of this molecule containing two hydrogenated carbons linked by a double bond. The effectiveness of ethylene at nanomolar concentrations indicates that some receptors have a high binding affinity for ethylene. However, biochemical purification of molecules binding to the gaseous ethylene was challenging. Same was true for purifying signaling molecules because molecular, physiological, and morphological responses to ethylene are complex.

The identification of ethylene signaling molecules would not have been possible if without the application of molecular genetics to plant research and the development of *Arabidopsis thaliana* as a model system in the 1980s. With its small genome, short life cycle, ease of genetics, it was adopted as a genetic system to study various plant processes such as flower development, flowering time, and embryogenesis among others (Meyerowitz and Somerville 1994). One of the processes studied was flower organ differentiation. Homeotic mutants that had one organ type (such as stamen) transformed into another organ type (for instance, petal) were studied, and mutant genes responsible for these transformations were cloned. Characterization of these genes at the genetic, molecular, and biochemical levels has elegantly revealed the mechanism of floral organ identity determination (Meyerowitz et al. 1991).

The power of molecular genetics in *Arabidopsis* demonstrated by flower development studies soon captivated plant researchers, and this approach was quickly utilized in many fields including plant hormone signaling. It is often a forward genetics screen, that is, to isolate mutants defective or abnormal in the process of interest without any prior assumption or knowledge on what they might be. A defective phenotype caused by a mutation indicates a disruption or alteration of the wild-type process by this particular mutation. By molecularly identifying the causal mutation, the gene that plays a critical role in that process would be found. The molecular identity of the gene might reveal how the process works or how it is regulated. Multiple mutants that affect the same process are likely to be isolated. Through analyzing the interaction of these mutants and their corresponding genes, a genetic pathway might be constructed which will set the framework for further molecular and biochemical studies of the process.

To isolate ethylene perception and signaling components, genetic screens were carried out to isolate mutants defective in ethylene responses. The screens used the highly ethylene-specific “triple response” as a report for any response defects. In this assay, *Arabidopsis* seedlings are grown on petri dishes and in the dark, and are exposed to ethylene by supplying ethylene directly or ethylene precursor ACC. Instead of being long and slender, a dark-grown seedling would have an exaggerated apical hood, a short thick hypocotyl, and a short root in ethylene. These

morphological features are likely adaptive as a germinating seedling underground produces ethylene to thicken its hypocotyl to push through soil and folds back its shoot tip to protect the delicate and essential shoot apex. These morphological features combined are unique to ethylene response as no other growth hormones or regulators would induce the exactly same response. Therefore, mutants that disrupt all these features are likely specifically defective in ethylene signaling rather than general growth process. This specific screen may have allowed the relatively rapid and efficient dissection of ethylene signaling pathway.

## 2.2 Mutants Isolated in Early Genetic Screens

The first ethylene mutant was reported in 1988 (Bleecker et al. 1988). From 75,000 M2 (progenies of mutagenized M1 plants) seedlings of EMS (ethyl methanesulfonate) mutagenized Col-0, three were found to grow tall in ethylene as if in air, and one of them was named *etr* (*ethylene response*) which was later renamed *etr1* (Bleecker et al. 1988). Two mutants with similar phenotypes to *etr1* were isolated in an independent screen and were named as *ethylene insensitive* (*ein*): *ein1* (later found to be allelic to *etr1*) and *ein2* (Guzman and Ecker 1990). Also identified in this second screen was a tissue-specific ethylene-insensitive mutant, named *hls1* (*hookless1*): only the apical hook, not hypocotyl or root, showed ethylene insensitivity. A different screen was carried out to identify mutants with phenotypes opposite to that of *ein* or *etr*. In this screen, the etiolated (dark-grown) seedlings were grown in air, and constitutive ethylene response mutants were isolated by their triple response morphology in air. The phenotype of the first such mutant can be reversed by inhibitors of ethylene biosynthesis, and it was therefore named *eto* (*ethylene overproducer*) or *eto1* (Guzman and Ecker 1990). More mutants with a phenotype similar to *eto1* were identified and they were named *eto2* and *eto3* (Kieber et al. 1993). Another mutant also exhibited a constitutive ethylene response phenotype, but this phenotype could not be blocked by inhibitors of ethylene biosynthesis. This mutant therefore was likely defective not in ethylene biosynthesis but in ethylene signaling and was named *ctr1* (*constitutive triple response1*) (Kieber et al. 1993). Additional mutants were identified from a number of mutagenesis pools induced by X-ray, diepoxybutane, or EMS (Roman et al. 1995). Five new loci were found: *ein4*, *ein5*, *ein6*, *ein7*, and *eir1*. All except *eir1* exhibited ethylene insensitivity or resistant similar to *etr1* and *ein2*. The *eir1* mutant had a resistant phenotype only in roots but not in hypocotyl or apex.

In sum, through genetic screens using the “triple response” phenotype, a large number of mutants were isolated by 1995. They are grouped into ethylene biosynthesis mutants (*eto1*, *eto2*, *eto3*), ethylene resistant or ethylene-insensitive mutants (*etr1*, *ein1*, *ein2*, *ein3*, *ein4*, *ein5*, *ein6*, *ein7*), constitutive ethylene response mutants (*ctr1*), or tissue-specific ethylene resistant mutants (*hls1*, *eir1*).

Although no mutagenesis was carried out for ethylene screens in agricultural plants until very recently (Ma et al. 2013), naturally occurring tomato mutants were

spotted and retained for their distinct features in fruit ripening. As ethylene is a key regulator of fruit ripening, some of these ripening mutants might be defective in ethylene biosynthesis or signaling. As will be discussed later, these mutants enable a comparative study of ethylene signaling and add to a more comprehensive view of ethylene biology in plants.

For mutants that affect almost all aspects of the ethylene response, they are likely defective in the early components of the core ethylene signaling process. Because the defect of these single mutants was often strong, it is likely that core ethylene signaling is through a linear pathway rather than parallel pathways. Double mutant analyses, epistasis analysis in particular, were carried out to order gene function in a pathway before they were molecularly cloned. Mutations conferring opposite phenotypes, such as *etr1* and *ctr1*, can be combined together; and the double mutant should show the phenotype of the downstream component in the linear regulatory pathway. This epistasis analysis on ethylene mutants indicates that *ETR1* and *EIN4* act upstream of *CTR1* which is upstream of *EIN2* (Roman et al. 1995). *EIN3*, *EIN5*, *EIN6*, and *EIN7* are downstream of *EIN2* but they could not be ordered because of their similar phenotypes.

The isolation and characterization of these mutants as well as the double mutant analysis therefore revealed a genetic pathway for core ethylene signaling, which forms the foundation for the molecular understanding of ethylene signaling.

## 2.3 Cloning of the Ethylene Receptor ETR1 Gene

*ETR1* was identified by map or position based cloning, and it was one of the first *Arabidopsis* genes identified by this approach. For map-based cloning, a mutant in one accession (ecotype or background) is crossed to a wild type but in a different accession. The F1 hybrid plant is selfed and the resulting F2 plants will segregate mutant and wild-type phenotypes. F2 plants will be genotyped by markers that are polymorphic between these two accessions to identify markers that are physically linked and thus cosegregate with the mutant phenotypes. When two close markers flanking the gene are identified, overlapping genomic fragments covering the region can be obtained. Genes in the region can be sequenced and compared between the wild type and the mutant to reveal potential causal mutations. Map-based cloning was a major task in the twentieth century because there was no available genome sequence information and very limited number of molecular markers even in *Arabidopsis*. “Chromosome walking” describes this process well because each step getting closer to the gene consists of using a known marker to isolate a genomic clone through library screening followed by identifying another marker in the clone isolated. The step will be repeated multiple times toward the direction of the gene till the gene is identified. It was not unusual for a chromosome walk to take 2–3 years to complete. The long walk led to the final molecular identification of *ETR1* (Chang et al. 1993). This was a milestone in plant biology not only for the

revelation of the first plant hormone receptor but also for the demonstration of utility of molecular genetics in plant physiology.

*ETR1* encodes a protein showing similarity to the two-component regulators that are utilized for signal perception and transduction in bacteria and yeasts (Chang et al. 1993). The first component has an amino (N)-terminal ligand-binding domain and a carboxyl (C)-terminal histidine kinase domain, while the second component has an N-terminal receiver domain and a C-terminal transcriptional activator domain. Autophosphorylation of histidine upon ligand binding and a subsequent phosphate transfer to the aspartate in the receiver domain activates transcription. The *ETR1* protein has a unique N-terminal domain followed by a histidine kinase-like domain and a receiver-like domain. This homology immediately suggests that *ETR1* is a signaling molecule and it could be an ethylene receptor using a signaling mechanism similar to the two-component system. Interestingly, all isolated ethylene-insensitive *etr1* mutant alleles have missense mutations in the unique N-terminal domain presumably affecting the binding of ethylene. This receptor model was supported by biochemical studies of *ETR1*. The wild-type *ETR1* protein but not the mutant form of *ETR1* (with *etr1-1* mutation), when expressed in yeasts, can bind ethylene; and the binding can be conferred with only the N-terminal domain of *ETR1* (Schaller and Bleecker 1995).

## 2.4 Cloning the *ETR1* Family Members

An intriguing feature of the *etr1* mutant alleles isolated thus far was their dominance, that is, an *etr1* heterozygous plant containing one mutant copy and one wild-type copy of the *ETR1* gene is ethylene insensitive similar to an *etr1* homozygous mutant. In addition, all four *etr1* mutants identified have missense mutations in the N-terminal domain, and no nonsense mutations were identified from the genetic screens. One explanation was that there are *ETR1*-like genes in *Arabidopsis* and a nonsense mutation conferring a loss of *ETR1* function will have no obvious ethylene response defect for it to be isolated from the mutant screens. This hypothesis was supported by the isolation of additional members of the *ETR1* gene family in *Arabidopsis*.

First was the *ETHYLENE RESPONSE SENSOR1* (*ERS1*) gene identified by its sequence homology to *ETR1* (Hua et al. 1995). A genomic DNA library was screened with the *ETR1* gene as a probe through low stringency hybridization, and the *ERS1* gene was isolated. *ERS1* encodes a protein with an N domain similar to that of *ETR1* and a histidine kinase domain but without a receiver domain. No existing ethylene mutants mapped to the region of *ERS1*, and therefore the function of *ERS1* was determined by transgenic approach. Targeted mutagenesis was used to introduce into the *ERS1* gene mutations mimicking the *etr1* dominant mutations, and the mutant forms of *ERS1* were transformed to the wild-type *Arabidopsis* plants. An ethylene-insensitive phenotype was observed in transgenic plants, and this insensitivity can be suppressed by *ctr1*. Therefore, *ERS1* potentially has a similar function as *ETR1*.

Subsequently, another *ETR1*-like gene *ETR2* was cloned (Sakai et al. 1998). The *ETR2* gene was first genetically isolated in a triple response screen as a mutant similar to *etr1* but with a distinct map position. Using chromosome walking, it was placed to a region where a homolog of *ETR1* was found. The protein encoded by this homolog, like *ETR1*, has an N-terminal domain, a histidine kinase domain, and a receiver domain. Sequencing this homolog in the *etr2* mutant identified a missense mutation in the N-terminal domain of the protein. Transgenic plants with the *etr2* mutant form of the homolog had ethylene insensitivity, indicating that *ETR2* is this homolog of *ETR1*.

Two other *ETR1* family members, *EIN4* and *ERS2*, were isolated by their sequence homology to *ETR2* (Hua et al. 1998). A genomic DNA library was screened at low stringency with *ETR2* as a probe, and clones positive at low but not high stringency belong to two genes. One of them was found to be *EIN4*, multiple alleles of which had previously been genetically identified as ethylene-insensitive mutants. All three *ein4* alleles had missense mutations in the N-terminal domain of *EIN4*. The second gene was named *ERS2* because it encodes a protein without a receiver-like domain similar to *ERS1*. No ethylene response mutants mapped to the *ERS2* genomic region. The *ERS2* gene was demonstrated to be involved in ethylene signaling using a transgenic approach similarly to that was employed for *ERS1*.

*ETR1*, *ERS1*, *ETR2*, *EIN4*, and *ERS2* are the five members of the *ETR1* gene family in *Arabidopsis*, and no additional genes were identified by low stringency hybridization with these genes as probes. Their encoded proteins are divided into subfamily I consisting of *ETR1* and *ERS1* and subfamily II consisting of *ETR2*, *EIN4*, and *ERS2*. All have the similar unique N-terminal domain and a histidine kinase-like domain. *ETR1*, *ETR2*, and *EIN4* have an additional receiver domain. All five members are involved in ethylene signaling and they all function upstream of *CTR1* as the ethylene resistance phenotypes of their dominant mutants or transgenic plants can be suppressed by the *ctr1* mutation.

*ETR1*-like genes are found in all higher plants examined, and they are extensively studied in tomato especially in the fruit ripening process. The *NR* (*Never Ripe*) gene was found to have a dominant missense mutant in an *ETR1*-like gene (Wilkinson et al. 1995), indicating a conserved function of *ETR1* gene families in *Arabidopsis* and tomato. Tomato has seven family members and only *NR* has no receiver domain (Klee and Giovannoni 2011). Interestingly, ethylene receptor proteins are rapidly degraded by ethylene treatment in tomato fruit, suggesting a level of regulation on receptors that was not observed in *Arabidopsis* (Kevany et al. 2007).

## 2.5 Identifying the Loss-of-Function (L-O-F) Mutants of the *ETR1* Family Members

Questions still remained with the finding of the *ETR1* family members. Are they all ethylene receptors? With their dominant mutant forms inhibiting ethylene responses, how do the wild-type forms regulate ethylene responses? From extensive

genetic screens carried out in different labs, only dominant mutants of *etr1*, *etr2*, and *ein4* containing missense mutations were identified, and the roles of the wild-type genes were not readily inferred from these mutant phenotypes. The dominance of a mutant form over the wild-type form may result from the following four scenarios: (1) The mutant form loses the wild-type activity and interferes the activity of the wild-type form. The mutant form then has a dominant interfering activity. (2) The mutant copy loses the wild-type activity and the activity from the wild-type copy in a heterozygous plant is not sufficient to support a normal process. This defect is called haploid insufficiency. (3) The mutant form has a higher activity than the wild-type form. The mutation results in a higher protein accumulation, a hyperactive protein, or a constitutively active protein. (4) The mutant form gains a new function that is not related to its wild-type function and this mutant is a neomorphic allele. In the first two scenarios, the l-o-f or null mutant of the gene should have a phenotype similar to the dominant mutant. In the third scenario, the l-o-f mutant would have an opposite phenotype to the dominant mutant. In the fourth scenario, the null mutant would have no mutant phenotype or a phenotype unrelated to that of the dominant mutant. As the biological function of a gene can be more easily inferred from its l-o-f mutants, it became critical to isolate such mutants of the *ETR1* family members to understand their function.

L-o-f mutants of a gene of interest were not readily available in the 1990s as they are now. Without any of the genetic resources such as indexed insertion mutant lines, suppressor screens were used to isolate l-o-f mutants of *etr1*, *etr2*, and *ein4* (Hua and Meyerowitz 1998). The absence of l-o-f mutants of these genes from a large scale of mutagenesis and screening could be due to lethality or no ethylene resistance phenotype in such mutants. A l-o-f mutant, if not lethal, should behave as a suppressor of the ethylene-insensitive mutant, that is, revert to an ethylene sensitive phenotype. With this hypothesis, suppressor screens were carried out for *etr1-1* and *etr1-2* mutants, respectively, to isolate mutants with a triple response morphology among the ethylene-insensitive tall seedlings. Among the isolated *etr1* suppressors, four were found to be intragenic (with mutations in the *ETR1* gene) through genetic means. Sequencing the *ETR1* gene revealed that these four mutants (named *etr1-5* to *etr1-8*) contained nonsense mutations or splicing mutations in the *ETR1* gene that would lead to truncated proteins. The *etr1-7* mutation in particular would result in a protein with a small portion of the N-terminal domain and no ETR1 protein could be detected by western blot in *etr1-7*, *etr1-5*, or *etr1-6*. Therefore, these four suppressors were l-o-f mutants or reduction of function mutants of *ETR1*.

Using a similar suppressor screen on dominant ethylene-insensitive mutants of *etr2* and *ein4*, four *etr2* l-o-f mutant alleles and eight *ein4* l-o-f mutant alleles were identified (Hua and Meyerowitz 1998). The *ERS2* l-o-f mutant was isolated from the then newly released 7,000 T-DNA insertion lines (Hua and Meyerowitz 1998). The T-DNA insertion sites in those lines were not known, but pools of genomic DNAs of these lines were available for PCR screening. Primers of *ERS2* combined with a T-DNA primer amplified a positive signal from the pool and further screening identified one line with a T-DNA inserted in the *ERS2* gene. The insertion would disrupt the function of the *ERS2* and create an *ers2* l-o-f mutant. No *ERS1*



T-DNA insertion mutant was found from any collections available then, but two such mutants were isolated later when more collections of T-DNA insertion lines were generated and released (Qu et al. 2007; Wang et al. 2003).

The l-o-f mutants of all five members of the *ETR1* family were largely ethylene sensitive, that is, they had a wild-type triple response to ethylene in contrast to the ethylene-insensitive dominant mutants (Hua and Meyerowitz 1998; Qu et al. 2007; Wang et al. 2003). The *etr1* l-o-f mutant exhibited an ethylene-independent phenotype, that is, it had a shorter hypocotyl than the wild type at all concentration of ethylene (Hua and Meyerowitz 1998). The wild-type ethylene response in these l-o-f mutants explains why only dominant mutants have been isolated from the ethylene response screen and indicates that the *ETR1* family members have either overlapping or no functions in ethylene signaling. To differentiate these two possibilities, mutant combinations were made among l-o-f mutants of different members. Interestingly and surprisingly, combination of these mutants led to constitutive ethylene responses: dark-grown seedlings exhibited triple response phenotype in air, and light-grown plants had reduced stature as if they were treated with ethylene (Hua and Meyerowitz 1998). These phenotypes were progressively more severe from the *etr1 ein4* double mutant to the *etr1 etr2 ein4* triple mutant and to the *etr1 etr2 ein4 ers2* quadruple mutant. In fact, the quadruple mutant would die at seedling stage or grow to reproductive stage without setting seeds (Hua and Meyerowitz 1998). Combination of l-o-f mutants of *ETR1* and *ERS1* (subfamily I) also induced a constitutive ethylene response phenotype to a degree even stronger than the *etr1 etr2 ein4 ers2* quadruple mutant (Qu et al. 2007; Wang et al. 2003).

These genetic analyses indicate that the *ETR1* family members are indeed ethylene signaling molecules. Combined molecular, genetic, and biochemical studies establish that these proteins are ethylene receptors. Furthermore, they show that ethylene receptors are negative regulators rather than positive regulators of ethylene responses (Hua and Meyerowitz 1998). The receptors are active in air to repress ethylene responses. They are inactivated by ethylene and as a consequence the downstream ethylene responses are activated. This mode of regulation was rather counter intuitive and surprising at the time it was discovered as receptors were often thought to be activated by ligands. Now we know many more examples of negative regulation (such as ligand triggered protein degradation) in plant signaling. Perhaps negative regulation and multiple receptors enable an effective tuning of responses over a large range of ethylene concentrations. Further study of the ethylene receptors in diverse plants including lower plants might shed light on the adaptation of this regulatory mode.

## 2.6 Isolation of Regulators of Ethylene Receptors

Several genes have been identified to be critical for the proper function of ethylene receptors. *RAN1* (*RESPONSIVE-TO-ANTAGONIST1*) was first genetically isolated as a mutant displaying a triple response in response to *trans*-cyclooctene, a

compound that competes with ethylene for binding to the receptor (Hirayama et al. 1999). The *ran1-1* mutant had a wild-type ethylene response and the *etr1-1* mutation suppressed the *ran1-1* mutant phenotype, indicating that *RAN1* acts very early in the ethylene signaling pathway. The *RAN1* gene was isolated by map-based cloning and it encodes a protein showing homology to a Menkes/Wilson disease-related copper transporter. The first two *ran1* alleles isolated had missense mutations and were not strong l-o-f alleles. *RAN1* cosuppression lines exhibited a constitutive ethylene response phenotype (Hirayama et al. 1999). Subsequently, a *ctr1*-like mutant (originally named *ctr2*) that died at 2 weeks stage was identified by a family screen where each M2 family or pool comes from a small number of M1 plants so that a M2 mutant plant that die before giving progenies can be recovered by its heterozygous siblings in the same M2 family pool. This gene was cloned by chromosome walking and it turned out to be *RAN1* (Woeste and Kieber 2000). This *ran1-3* allele had a conserved glycine mutated into an arginine and is a much stronger reduction of function allele of *RAN1* than *ran1-1* and *ran1-2*. Earlier physiological studies show that copper is needed for ethylene binding to the receptors. Therefore, *RAN1* is likely a copper transporter which is an essential element for ethylene perception. The unavailability of copper in the *ran1* reduction of function mutants may render the ethylene receptors inactive and induce constitutive ethylene responses similar to the l-o-f mutants of multiple ethylene receptors.

Another modifier of the ethylene receptor was identified from a suppressor screen of a weak dominant ethylene-insensitive *etr1-2* mutant. The mutation *rte1* (*reversion to ethylene sensitivity1*) suppressed the weak ethylene insensitivity of *etr1-2* (Resnick et al. 2006). Intriguingly, it did not suppress the *etr1-1* allele or ethylene resistant mutants of other ethylene receptor genes. The null mutants of *rte1* and *etr1* single and their double mutants had a similar phenotype, suggesting that *ETR1* and *RTE1* work together. The *RET1*-dependency of dominant ethylene resistant *etr1* mutants were tested in transgenic plants, and the *rte1* mutation suppressed mutant phenotypes of a subset of such mutant *ETR1* genes (Resnick et al. 2008). There was no clear correlation of *rte1* suppression with ethylene binding ability of the mutant *ETR1* protein, suggesting that *RTE1* is involved in the conformation changes of *ETR1* necessarily for its activation (Resnick et al. 2008).

This notion is further supported by the finding that *RTE1* is also required for signaling of the N-terminal domain of *ETR1* (Qiu et al. 2012). The *ETR1* N-terminus *etr1*<sup>1–349</sup> likely has a signaling output that requires *RTE1* without involving *CTR1*. *RTE1* might modulate conformation changes in *ETR1* and hence its activity. A regulation of *RTE*-like proteins on ethylene receptors might be present in other plant species as well. The *green-ripe* (*gr*) mutant in tomato had a dominant fruit ripening defect but had only a slight or no reduction in ethylene sensitivity (Barry and Giovannoni 2006). *GR* encodes a protein in a small family including additional *GRL1* and *GRL2*, and the *Arabidopsis* *RTE1* is the closest homolog of *GRL1* (Barry and Giovannoni 2006; Ma et al. 2012). Thus, the *gr* mutation might interfere with its related protein and affect ethylene signaling.

The role of *RTE1* in ethylene signaling was further investigated through its interacting proteins (Chang et al. 2014). Using the yeast split-ubiquitin system, a

cytochrome b5 (Cb5) isoform D was identified as a RTE1-interacting protein. All four ER-localized atCb5 (B, C, D, and E) proteins interact with RTE1 in plant cells. Single mutant of *atcb5* suppressed *etr1* dominant mutants that are *RTE1*-dependent but not *RTE1*-independent; and double mutant combinations of *atcb5* isoforms exhibited a weak ethylene insensitivity. Cytochrome b5 is known to perform electron transfer reactions, and how it works with RET1 to regulate ETR1 is not clear. An attractive model is that atCB5 may regulate oxidative folding of ETR1 through RTE1. Further biochemical and genetic studies of ethylene receptors, RTE1, and CB5s should reveal the details of effect and modulation of receptors by ethylene.

## 2.7 Cloning of Core Signaling Genes

Similar to the ethylene receptors and their modulators, core signaling molecules downstream of the ethylene receptors have been identified with molecular genetic approaches.

The *CTR1* gene was first genetically isolated as a mutant exhibiting a triple response phenotype in the absence of exogenous ethylene (Kieber et al. 1993). The *ctr1* mutant was the only signaling mutant with such a phenotype while others were ethylene overproducers. The *ctr1* mutation suppressed the ethylene-insensitive phenotypes of the dominant mutants of the *ETR1* family members (Hua et al. 1995, 1998; Kieber et al. 1993; Sakai et al. 1998). The *ctr1* mutant phenotype was suppressed by *ein2* and *ein3* mutations, indicating that *CTR1* acts very upstream in the ethylene signaling pathway (Roman et al. 1995). Using map-based cloning, *CTR1* was identified as a gene coding for a serine–threonine protein kinase closely related to the Raf protein kinase (Kieber et al. 1993). Therefore, it is a key signaling molecule in ethylene pathway possibly using a kinase activity to relay ethylene signals. A direct interaction of CTR1 and ETR1 was later identified, indicating that CTR1 is directly regulated by the receptors (Clark et al. 1998).

*EIN2* was genetically identified as a mutant with a strong ethylene-insensitive phenotype similar to that of *etr1-1*, and the suppression of *ctr1* by *ein2* indicates that EIN2 functions downstream of CTR1 (Roman et al. 1995). *EIN2* was molecularly identified by map-based cloning, and it encodes a large protein whose N-terminal domain shows similarity to the disease-related Nrap family of metal ion transporters (Alonso et al. 1999). The C-terminus did not show obvious homology to known proteins, but overexpressing this C-terminus but not the full-length of EIN2 induced a constitutive ethylene response phenotype in light-grown seedlings (Alonso et al. 1999). This suggests that C-terminus of EIN2 activates ethylene responses while the N domain might have a role in regulating its activity.

*EIN3* was genetically identified as ethylene-insensitive mutants from various mutagenesis by chemicals or T-DNA insertions (Chao et al. 1997; Roman et al. 1995). The three alleles, *ein3-1*, *ein3-2*, and *ein3-3*, all exhibited a weaker ethylene insensitivity compared to that of *ein2-1*. Because the T-DNA in *ein3-2* was tightly

linked to the *ein3* phenotype, the *EIN3* gene was cloned by plasmid rescue, that is, the T-DNA flanking genomic fragment (potentially containing the *EIN3* gene) was isolated together with the plasmid present in the T-DNA insertion (Chao et al. 1997). The predicted EIN3 protein is likely a transcriptional regulator because it has acid amino acid-rich motif, proline-rich region, and glutamine-rich region, all of which are possibly transcriptional activation domains. EIN3 protein is localized in the nucleus when expressed in protoplasts, further suggesting that EIN3 is a transcriptional regulator of ethylene responses.

Three *EIN3*-like genes were isolated when a cDNA library was screened with the *EIN3* genomic fragment, and they were named *EIL1*, *EIL2*, and *EIL3* (Chao et al. 1997). These genes are also involved in ethylene responses because *EIL1* and *EIL2* rescued the *ein3* mutant phenotype when overexpressed. Furthermore, a small fraction of the transgenic lines of overexpression either *EIN3* or *EIL1* exhibited constitutive ethylene response phenotypes in both the wild type and the *ein2* mutant background. Therefore, *EIN3* and *EIL1* function downstream of *EIN2* and are sufficient to induce ethylene responses when overexpressed.

## 2.8 Isolation of Regulators of Core Signaling Components

The overexpression studies suggest that the level of *EIN3* expression is important for ethylene responses (Chao et al. 1997). Because the EIN3 protein level is regulated by ethylene via a 26 proteasome-mediated pathway, F-box proteins that function to mediate protein degradation were investigated for potential roles in EIN3 regulation. Two F-box proteins EIN3-BINDING F-box1 (EBF1) and EBF2 were found to interact with EIN3, through either direct testing of EIN3 interaction with ethylene-induced F-box proteins (Guo and Ecker 2003) or obtaining EIN3 and EIL1 from yeast two-hybrid screens with EBF1 as a bait (Potuschak et al. 2003). L-o-f mutants of *EBF1* and *EBF2* were obtained through the available T-DNA insertion lines. Each of these *ebf1* and *ebf2* mutants exhibited hypersensitivity to ethylene due to an increase of EIN3 protein level. In addition, the *ebf1 ebf2* double mutant showed a constitutive ethylene response phenotype. These analyses establish that EBF1 and EBF2 are negative regulators of ethylene signaling through degrading the EIN3 and EIL1 proteins (Guo and Ecker 2003; Potuschak et al. 2003).

*EIN5* was initially genetically identified as a weak ethylene-insensitive mutant that is epistatic to *ctr1*, but its relative position to *EIN3* or *EIN2* in the signaling pathway was not clear (Olmedo et al. 2006; Roman et al. 1995). The *EIN5* gene was isolated by map-based cloning and it encodes a 5'–3' exoribonuclease *XRN4* (Olmedo et al. 2006). Independently, *XRN4* was tested and found to be the *EIN5* gene because the *xrn4* mutant accumulated a high level of *EBF1/2* transcripts similar to the *ein5* mutant (Potuschak et al. 2006). The l-o-f *ein5* mutation altered expression of many genes, and its target in ethylene response is the F-box coding *EBF1/2* as the l-o-f *ebf2-1* mutant partially suppressed the *ein5* phenotype (Olmedo et al. 2006; Potuschak et al. 2006). Therefore, the *EIN5* gene is a positive regulator

of *EIN3* through negative regulating *EBF1/2*. It may enhance the turnover of the *EBF1/2* transcript level (likely through an indirect mechanism) and subsequently promote the *EIN3* protein level to enhance ethylene responses.

Similar to *EIN3*, the stability of *EIN2* is regulated by 26S-proteasome and this regulation is conferred by two F-box proteins (Qiao et al. 2009). Yeast two-hybrid screen with the *EIN2* C-terminal end identified an F-box protein *EIN2-TARGETING PROTEIN* (*ETP1*), and its homolog *ETP2* was found to interact with *EIN2* as well. Knocking out the expression of *ETP1/2* by artificial miRNA conferred a constitutive ethylene response phenotype, while overexpression of *ETP1* or *ETP2* resulted in ethylene insensitivity. Furthermore, ethylene downregulates the level of *ETP1/2* transcripts and knocking out *ETP1/2* elevated the protein level of *EIN2*. Together, these results indicate that *EIN2* is regulated at the protein level by *ETP1/2* as part of the ethylene response.

A potential regulator of *CTR1* is recently genetically identified (Xu et al. 2014). An enhancer screen of a weak allele of *ctr1-10* was carried out to isolate component that might be directly involved in receptor regulation of *CTR1*. The *ecr2* (*enhancing ctr1-10 ethylene response2*) mutant, when combined with *ctr1-10*, had a similar phenotype to the strong allele *ctr1-1*. Genetic studies indicate that *ECR2* acts downstream of the ethylene receptors but upstream of *EIN3*. Molecular identification of this gene will reveal if and how it may facilitate the activation of *CTR1* by the receptors.

## 2.9 Investigation of Tissue-Specific or Subtle Ethylene Mutants

Studies of mutants affecting ethylene responses in all tissues have identified core ethylene signaling components and their regulators. Interestingly, studies of mutants with tissue-specific ethylene defects have revealed interplay of ethylene with other plant hormones.

The *ethylene-insensitive root1* (*eir1*) mutant had a root-specific ethylene insensitivity which was reminiscent of auxin mutants such as *axr1* and *aux1* (Roman et al. 1995). *EIR1* was cloned by transposon tagging, and it encodes a protein similar to bacterial membrane transporters (Luschnig et al. 1998). *EIR1* (also known as *PIN2*) belongs to a large family of *PIN* proteins that function as auxin efflux carriers to transport auxin in plants (Paponov et al. 2005). The involvement of auxin efflux in tissue-specific response indicates that ethylene influences root elongation via auxin.

The *hls1* mutant has a hook-specific ethylene insensitivity (Guzman and Ecker 1990). Map-based cloning showed that *HLS1* encodes an acetyltransferase (Lehman et al. 1996), but its direct biological target is not known. Suppressor screen of *hls1* revealed a role of *HLS1* in regulating auxin response (Li et al. 2004). The *hls2* phenotype was suppressed by a mutation in the *ARF2* (*AUXIN RESPONSE FACTOR2*) gene that is known to regulate auxin response. In addition, ethylene-induced

*ARF2* accumulation in an *HLS1*-dependent manner, indicating that *HLS1* is an important factor mediating ethylene effect on auxin.

Additional mutants were identified from a sensitive genetic screen using ethylene (provided as the ethylene precursor ACC) at a lower concentration than conventionally used. Seedlings with an ethylene-insensitive phenotype weaker than that of *ein5-1* were categorized as *wei* (*weak ethylene insensitivity*) (Alonso et al. 2003). Several loci were identified, and most of them have been identified largely through map-based cloning. Two of them turned out to be new alleles of known ethylene signaling genes: *wei4* was a dominant allele of *ERS1* and *wei5* was a semidominant mutant of *EIL1*. These mutants may provide genetic materials for probing the structure and function of the core signaling molecules.

Four other *wei* mutants, *wei1*, *wei2*, *wei7*, and *wei8*, had root-specific ethylene insensitivity (Alonso et al. 2003; Stepanova et al. 2005, 2008). The *wei1* mutant is found to be a recessive allele of *TIR1* which encodes a SCF protein ubiquitin ligase mediating auxin perception (Alonso et al. 2003). This further indicates an involvement of auxin in ethylene response. The *WEI2* gene was cloned by a map-based approach in conjunction with testing T-DNA mutants of the candidate genes (Stepanova et al. 2005). It encodes ASA1 (ANTHRANILATE SYNTHASE  $\alpha 1$ ),  $\alpha$ -subunit of AS1 that catalyzes the conversion of chorismate to anthranilate in Trp biosynthesis. *WEI7* was cloned through a rough mapping followed by testing putative AS  $\beta$  subunit (*ASB*) genes residing in the region (Stepanova et al. 2005). The most divergent one among the five *ASB* genes were found to be defective in all *wei7* alleles, indicating that this *ASB1* is the *WEI7* gene. *WEI2* and *WEI7* are regulators of auxin production through Trp, as their mutant phenotypes could be rescued by anthranilate, Trp, or auxin, and they were defective in ethylene-induced auxin response. These analyses further establish the role of auxin in root-specific ethylene responses.

The molecular identification of *WEI8* has further shed light on the auxin biosynthesis pathway (Stepanova et al. 2008). Map-based cloning reveals that *WEI8* encodes a tryptophan aminotransferase (*TAA1*) that catalyzes the conversion of Trp to indole-3-pyruvic acid in the essential branch of auxin biosynthetic pathway. *TAA1* and its homolog *TAR2* were expressed in specific cell types in roots and apical hook and were induced by ethylene. These results thus revealed the molecular link of auxin biosynthesis with tissue-specific ethylene response as well as a previously unidentified pathway for auxin biosynthesis.

## 2.10 Other Ethylene Response Mutants

Further utilization of the triple response phenotype has yielded additional ethylene related mutants. The *eer* (*enhanced ethylene response*) mutants were identified as having extremely short hypocotyls in the presence of ethylene (Christians and Larsen 2007; Christians et al. 2008; Robles et al. 2007). The *eer3* mutant was an ethylene overproducer but was hypersensitive to ethylene even in the presence of

ethylene biosynthesis inhibitors. The *EER3* gene was identified as a prohibitin coding gene *PHB3* by map-based cloning (Christians and Larsen 2007). A strong allele of the T-DNA insertion line subsequently identified had a constitutive ethylene response, and this phenotype appears to be independent of *ctr1* or *ein3*. Strikingly, the *eer3* mutations, although conferring a strong ethylene response growth phenotype, had little effects on ethylene regulated gene expression. A mutant of *PHB3* was later isolated from a genetic screen for mutants not accumulating NO in response to H<sub>2</sub>O<sub>2</sub> (Wang et al. 2010). The *phb3/eer3* mutant had multiple defects in NO response and stress responses. These results suggest that PHB3 may be involved in forming transcriptional complexes and regulate expression of sets of genes. In respective to ethylene responses, PHB3 may negatively regulate expression of some downstream components of ethylene signaling which perhaps directly confer morphological changes in response to ethylene.

Similar to *eer3*, the *eer4* mutant had drastically reduced hypocotyl length in the presence of ethylene and was partially ethylene insensitive at the level of gene expression. Unlike *eer3*, this phenotype could be suppressed by *ein2* and *ein3*. The *EER4* gene encodes a transcription factor with a putative TFIID-interacting domain (Robles et al. 2007). It may be involved in regulating a previously unknown process to modulate the core signaling pathway.

The *eer5* mutant also had reduced hypocotyl length in ethylene compared to the wild type, and the mutant has a slight alteration in ethylene response at gene expression level (Christians et al. 2008). Map-based cloning revealed that *EER5* encodes a protein with a domain found in COP9 signalosome (CNS), and the *EER5* protein could interact with EIN2 and CSN in a protein pulled down assay (Christians et al. 2008). The target of the *EER5* function in ethylene response is unclear, and the combination of the *eer5* l-o-f mutation with *ctr1* or *ein3* appeared to have additive effects. Recent study indicates that *EER5* is a component of a TREX-2 complex that is associated with nuclear pore complex (Lu et al. 2010). Therefore, it may directly or indirectly affect mRNA export and/or protein degradation of an ethylene signaling or response gene.

## 2.11 Concluding Remarks

The field of ethylene signaling has advanced significantly in the last 30 years, and we now have a good molecular picture of the core signaling pathway and the regulation of the major components. This has been greatly facilitated by forward genetics which has led to revealing and sometimes surprising discoveries. With biology moving to the postgenomics era, combination of classical approaches with new “omics” approaches in diverse plants will continue to unveil the mechanisms of sensitivity, efficiency, fine tuning, and diversity in ethylene signaling.

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