Chi-Kuang Wen *Editor*

Ethylene in Plants

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Preface

Ethylene, the first identified gaseous hormone, has commercial importance in agriculture and profound effects on various aspects of plant processes throughout the life cycle. Extensive studies have been performed to unravel mechanisms of ethylene actions, with application to agricultural practices. Historical breakthroughs in ethylene study are (1) the identification of ethylene as a gaseous hormone, (2) biochemical elucidation of the coupling of methionine recycling (the Yang cycle) and ethylene biosynthesis, and (3) isolation of ethylene-forming enzymes and the corresponding genes to validate the biochemically deduced pathway.

In the past two decades, rapid and significant advances have led to the understanding of ethylene signal transduction and regulation of its biosynthesis, with isolation of the involved components and studies of the underlying mechanisms. Moreover, dissecting hormone signaling crosstalk and interactions at the molecular level has furthered our knowledge about the networking of ethylene with other plant growth substances in response to external and internal cues.

This book represents the vast expertise of researchers devoted to research into this important molecule. It describes the historical breakthroughs in the role of ethylene to provide background knowledge. In addition, it highlights significant advances in ethylene signaling, biosynthesis and its crosstalk as well as interactions with other stimuli to emphasize significant breakthroughs in the field. Evolutionary perspectives of ethylene as a plant hormone are addressed. Finally, the ethylene research tools outlined may facilitate ethylene studies inside and outside of the field.

This book is conceptually divided into four parts: Chap. 1 for ethylene biosynthesis and its regulation, Chaps. 2–6 for ethylene signaling, Chaps. 8–11 for the networking of ethylene with other signals, and Chaps. 12–14 for ethylene research tools. Chapter 7, not in the four categories, involves ethylene biosynthesis and signaling from an evolutionary perspective.

The chapter authors have been very active in related areas, with pioneering contributions that have made significant advances in the field. As the Editor of the book, I extend my gratitude to all the authors, whose efforts and invaluable contributions have made the book possible and regret that we could not include contributions from experts in related fields.

Chi-Kuang Wen

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Chapter 1
Ethylene Biosynthesis and Regulation in Plants

Juan Xu and Shuqun Zhang

Abstract Ethylene, a gaseous plant hormone, influences plant growth, development, and response to various stresses and pathogen infection. Ethylene is synthesized from *S*-adenosylmethionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC). In plants, ACC synthase (ACS) and ACC oxidase (ACO), two key enzymes in the ethylene biosynthetic pathway, are tightly regulated both transcriptionally and posttranscriptionally to modulate ethylene biosynthesis. This chapter summarizes the ethylene biosynthetic pathway and its regulation in higher plants, with a particular focus on the regulation of ACS, generally the rate-limiting enzyme of ethylene biosynthesis. Increasing evidence demonstrates that stability and turnover of the ACS protein is tightly regulated by phosphorylation, dephosphorylation, and ubiquitination-mediated proteasomal degradation. Together with the spatiotemporal-specific expression of the *ACS* gene family, multilevel regulation of cellular ACS activity can fine-tune the kinetics and magnitude of ethylene biosynthesis in response to diverse endogenous and environmental cues, which is critical to ethylene physiology.

Keywords Ethylene biosynthesis · ACC synthase (ACS) · ACC oxidase (ACO) · Transcriptional regulation · Ubiquitin–proteasome system (proteasomal degradation) · Protein phosphorylation and dephosphorylation · Multilevel regulation

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1.1 Introduction

The gaseous plant hormone ethylene is an important regulator of plant growth, development, and responses to abiotic/biotic stresses. Ethylene-regulated processes are closely related to endogenous ethylene content and are initiated by elevated ethylene production. While all plants produce ethylene, overall level of ethylene is usually low. During various developmental stages and stress events, ethylene production can be dramatically induced, such as in senescing plants, ripening fruits, stressed or infected plants, which can in turn affect local or neighboring cells (Yang and Hoffman 1984; Kende 1993; Wang et al. 2002). Thus, crucial to the functions of ethylene is the tight regulation of its biosynthesis. Furthermore, unlike auxin or other plant hormones, ethylene does not need to be actively transported or degraded in plant cells, making ethylene biosynthesis the only key regulatory point for plants to control ethylene levels (Burstenbinder and Sauter 2012). In lower plants (algae, mosses, ferns), ethylene is synthesized through unidentified pathway(s) that are different from that in higher plants. In this chapter, therefore, only the ethylene biosynthesis and regulation in higher plants are described and discussed.

Ethylene is synthesized from *S*-adenosylmethionine (SAM), an activated form of methionine (Met), via 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman 1984). In contrast to the simple chemical nature and biosynthetic pathway of ethylene, the regulation of ethylene biosynthesis is rather complex and involves complicated integration of internal and external signals. The two key ethylene biosynthetic enzymes, ACC synthase (ACS) and ACC oxidase (ACO), are both encoded by multigene families. With distinct spatial and temporal expression patterns, they are the primary regulation points in ethylene biosynthesis. In addition to transcription regulation of *ACS* and *ACO* genes, ACS protein turnover mediated by ubiquitination and phosphorylation/dephosphorylation also plays an important role in controlling cellular ACS activity. This chapter summarizes our current knowledge of ethylene biosynthesis and its regulation in *Arabidopsis*, tomato, and other plants, with a specific focus on the regulation of ethylene biosynthesis in development and stress responses.

1.2 Ethylene Biosynthesis Pathway

The ethylene biosynthetic pathway has been intensively studied from the mid-1960s to the 1980s. The identification of methionine, SAM, and ACC as pathway precursor/intermediates were major breakthroughs in defining the ethylene biosynthetic pathway in higher plants (Lieberman et al. 1966; Adams and Yang 1977, 1979). Ethylene is synthesized from SAM, an activated form of methionine and a common precursor to many biosynthetic pathways. SAM is converted to ACC by ACS, and ACC is then oxidized by ACO to form ethylene (Yang and Hoffman 1984; Kende 1993).

1.2.1 Enzymes and Precursor/Intermediates in the Ethylene Biosynthesis Pathway

The identification of the precursors/intermediates to ethylene was a key step in elucidating the biosynthesis of this important hormone. Because of the simple two-carbon chemical structure of ethylene, a number of compounds, including linolenic acid, β-alanine, methionine, and others, were originally proposed as precursors of ethylene (Yang 1974). The discovery of methionine as a precursor of ethylene opened a new chapter in the understanding of ethylene biosynthesis (Lieberman and Kunishi 1965; Lieberman et al. 1966; Yang et al. 1966). Ethylene is derived from C-3,4 of Met in vivo, as indicated by the efficient conversion of ^{14}C-labeled Met in apple fruit tissue (Lieberman et al. 1966). When ^{35}S-Met is converted to ethylene, the release of ^{35}S-labeled 5′-methylthioadenosine (MTA) and its hydrolysis product, 5-methylthioribose (MTR), first indicated SAM to be an intermediate in the biosynthesis of ethylene (Fig. 1.1) (Adams and Yang 1977). Shortly after, ^{14}C-labeled Met is found to be converted to an unknown compound under anoxic conditions, which was subsequently identified as the nonprotein amino acid ACC, the immediate precursor of ethylene. ACC is then converted to ethylene in an oxygen-dependent manner (Fig. 1.1) (Yu et al. 1979b). Together, these studies revealed the complete ethylene biosynthetic pathway in higher plants, i.e., ethylene is formed from methionine via SAM and ACC (Yang and Hoffman 1984). These individual steps of ethylene synthesis are catalyzed by SAM synthetase (ATP: L-methionine *S*-adenosyltransferase), ACS (*S*-adenosyl-L-methionine methylthioadenosine-lyase), and ACO, respectively (Kende 1993).

1.2.2 The Methionine or Yang Cycle

Besides functioning as a precursor for ethylene, methionine also participates in other important physiological processes, including sulfation, protein synthesis, and methylation of protein and nucleic acids. Because of the relatively low and stable abundance of methionine in plant cells, it was reasoned that there is a recycling mechanism to maintain the methionine pool (Baur and Yang 1972). Characterization of radioactive metabolites from isotope-labeled methionine and other intermediates allowed Yang and colleagues to discover the methionine cycle in higher plants (Yang and Hoffman 1984; Miyazaki and Yang 1987), which has been called the Yang cycle in honor of Shang Fa Yang. In the Yang cycle, MTA, released as a byproduct when SAM is converted to ACC by ACS, is subsequently recycled to methionine (Fig. 1.1) (Miyazaki and Yang 1987). In each cycle from methionine to ethylene, one molecule of ATP is consumed to generate SAM, and an aminobutyrate group is added to regenerate methionine, while the methyl group of the original methionine is preserved through each round of the cycle. Therefore, ethylene can be produced continuously without depleting the methionine pool. This

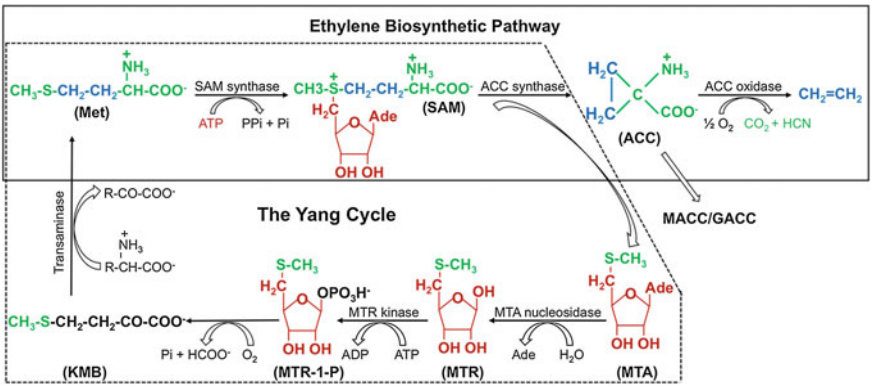

Fig. 1.1 Ethylene biosynthetic pathway and the Yang cycle. Ethylene is synthesized from Met via SAM and ACC. MTA, a byproduct when SAM is converted to ACC by ACS, is subsequently recycled to Met via a pathway known as the methionine cycle or the Yang cycle. ACC undergoes oxidative cleavage to form ethylene, a process catalyzed by ACO. In addition, ACC can be conjugated with malonic acid or glutathione to form MACC or GACC. Met: methionine; SAM: *S*-adenosyl-L-methionine; ACC: 1-aminocyclopropane-1-carboxylate; MTA: 5′-methylthioadenosine; MTR: 5′-methylthioribose; MTR-1-P: 5′-methylthioribose-1-phosphate; KMB: 2-keto-4-methylthiobutyric acid; MACC: malonyl-ACC; GACC: 1-(γ-L-glutamylamino) ACC

methionine salvage pathway not only plays an important role in sustained ethylene production, but is also involved in polyamine and nicotianamine biosynthesis (Miyazaki and Yang 1987; Shojima et al. 1989; Ravanel et al. 1998).

Stored apples can produce ethylene sustainably for months without any sulfur source for de novo synthesis of methionine, indicating that ethylene production is mainly dependent on methionine recycling via the Yang cycle (Baur and Yang 1972). The significance of the Yang cycle and its contribution to ethylene production is also supported by genetic studies of *Arabidopsis eto3* (*ethylene-overproducer3*) in the *5-methylthioribose kinase* (*mtk*) mutant background (Bürstenbinder et al. 2007). Mutation of the single *Arabidopsis MTK* gene disrupts phosphorylation of MTR, resulting in impaired methionine recycling. In the *eto3* mutants, ethylene production level is high due to a point mutation in the ACS9 protein that leads to its stabilization (Chae et al. 2003). Ethylene production is significantly reduced in *mtk eto3* double mutants, compared to *eto3* single mutants. However, this reduction can be eliminated by methionine feeding, indicating that the Yang cycle is required for continuously high rate of ethylene biosynthesis. Nonetheless, ethylene production in *mtk eto3* seedlings is still higher than production in wide-type seedlings, indicating that de novo synthesized Met can contribute when ethylene is synthesized at high rates (Bürstenbinder et al. 2007). Consistent with this, increased de novo Met synthesis was found in parallel with elevated ethylene production in tomato ripening fruit, which also supports de novo Met synthesis being involved in high rates of ethylene production (Katz et al. 2006).

1.2.3 Formation of ACC Derivatives

Ethylene cannot be degraded or actively transported in plants. However, the localized concentration of its immediate precursor, ACC, can be controlled strictly by localized cellular ACS activity and the formation of ACC derivatives. ACC can be diverted from its route to ethylene by conjugating with malonic acid to form malonyl-ACC (MACC) by the enzyme ACC malonyltransferase (Hoffman et al. 1982). It was reported that regulation of the activity of ACC malonyltransferase may play a role in controlling ethylene production (Liu et al. 1985; Gallardo et al. 1991). MACC is a major ACC conjugate in higher plants (Peiser and Yang 1998). A second ACC conjugation, 1-(γ-L-glutamylamino) ACC (GACC), has also been identified in tomato fruits (Martin et al. 1995). Its formation is catalyzed by a γ-glutamyltransferase. ACC conjugation could be an important mechanism to decrease the local ACC concentration. At present, whether MACC can be converted to ethylene in plants remains unclear. It was generally believed that ACC conjugation is essentially irreversible, thus creating a sink for ACC (Hoffman et al. 1983). However, there is also evidence that high levels of MACC can be hydrolyzed to some extent to free ACC by inducible MACC-hydrolase activity (Jiao et al. 1986; Hanley et al. 1989).

Besides conjugation, ACC can be transported in plants, leading to ethylene synthesis in the receiving tissue, such as stressed or senescent organs (Yoon and Kieber 2013). Conjugation and translocation are common mechanisms in regulating the levels of plant hormones. In the case of ethylene, these regulations may occur at the level of its immediate precursor, ACC, therefore providing a similar regulatory mechanism for ethylene biosynthesis as other plant hormones.

1.3 Two Key Enzymes in the Ethylene Biosynthetic Pathway: ACS and ACO

Two key reactions that are specific to ethylene biosynthesis pathway are the conversion of SAM to ACC and then ACC to ethylene, catalyzed by ACS and ACO, respectively (Kende 1993). ACS activity is labile and presents at very low levels in tissues that do not produce a large amount of ethylene, while its activity is highly elevated under conditions that promote ethylene formation. In contrast, ACO is constitutively present in most vegetative tissues. As a result, ACS is thought mostly to be the committing and generally rate-limiting enzyme of ethylene biosynthesis (Yang and Hoffman 1984; Sato and Theologis 1989; Zarembinski and Theologis 1994; Wang et al. 2002). However, emerging evidence indicates that ACO can also be the limiting factor in ethylene production under certain physiological conditions (Dorling and McManus 2012).

1.3.1 A Brief Historical Overview of the Identification of ACS and ACO

Soon after the identification of ACC as the immediate precursor of ethylene, ACS activity was identified in tomato pericarp homogenates (Boller et al. 1979; Yu et al. 1979b). A soluble enzyme in tomato fruit extract was found to be capable to convert SAM to ACC with a *Km* of 13 μM, of which the activity can be competitively inhibited by aminoethoxyvinylglycine (AVG) and be activated by pyridoxal phosphate (Boller et al. 1979; Yu et al. 1979b). The ACS activity was shown to be enhanced by factors that promote ethylene production and to be a limit factor in ethylene production in many cases (Yang and Hoffman 1984). Purification and characterization of ACS protein became a major research focus after its importance in ethylene biosynthesis was recognized. However, the low abundance and instability of ACS protein made its purification a challenging task.

Wounded tomato pericarps have relatively high ACS activity, and were the material of choice for purification of ACS protein. Based on known kinetic parameters and molecular mass of ACS, ACS was partially purified using a combination of conventional and high-performance liquid chromatography approach. Mouse monoclonal antibodies were then prepared using partially purified ACS preparation as an antigen (Acaster and Kende 1983; Bleecker et al. 1986). The monoclonal antibody that effectively removed 90–98 % of the ACS activity from crude or partially purified enzyme preparations immunopurified an ACS protein of 50 kD (Bleecker et al. 1986). Based on these pioneering works, different ACS isoforms were subsequently isolated in various plant species (Kende 1989). Amino acid sequencing of the purified ACS led to the cloning of *ACS* gene and structure analysis of ACS protein (Sato and Theologis 1989; Van der Straeten et al. 1990). The structure of the ACS enzyme resembles the subgroup I family of pyridoxal 5′-phosphate (PLP)-dependent aminotransferases (Alexander et al. 1994; Capitani et al. 1999; Huai et al. 2001). As a result, the activity of ACS enzymes can be strongly inhibited by rhizobitoxine and AVG, compounds that react with PLP (Yang and Hoffman 1984).

ACO catalyzes the final step of ethylene synthesis, the conversion of ACC to ethylene, releasing CO_2 and cyanide. An initially wrong assumption that ACO was an integral membrane protein hindered the identification of ACO (Yang and Hoffman 1984). Unlike ACS, which was purified by conventional biochemical approach, ACO was successfully isolated by expression of a functional *ACO* cDNA in yeast. In 1990 Hamilton et al. identified *pTOM13,* a gene induced in ripening tomato fruit and encoding an ethylene-forming enzyme (EFE), which was later named *ACO1*. Expression of *pTOM13* antisense RNA reduces ethylene synthesis in a gene dosage-dependent manner during fruit ripening or wounding responses (Hamilton et al. 1990). Furthermore, when *pRC13,* a corrected version of *pTOM13*, was expressed in yeast, it was able to catalyze the conversion of ACC to ethylene. Amino acid sequence analysis and structure prediction of pRC13 indicated that this protein was likely soluble and might require cofactors, providing clues vital to the

first successful purification of the ACO enzyme (Hamilton et al. 1991). Indeed, with supplementary Fe^{2+} and ascorbate, ACO purified from melon retained full enzymatic activity (Ververidis and John 1991). Subsequently, ACO was purified to near homogeneity from apple fruit and shown to function as a monomer. In this report, the stoichiometry of the ACO-catalyzed reaction was determined as follows: ACC + Ascorbate + O_2 → C_2H_2 + HCN + CO_2 + dehydroascorbate (Dong et al. 1992). In this reaction, cyanide is unable to react destructively with the proximal iron center at the active site of ACO. It was discovered recently that cyanoformate, $[NCCO2]^-$, which forms and then decomposes to carbon dioxide and cyanide, shuttles the potentially toxic cyanide away from the low dielectric active site of ACO before it breaks down in the higher dielectric medium of the cell (Murphy et al. 2014).

1.3.2 ACS, the Rate-Limiting Enzyme in Ethylene Biosynthesis

ACS is encoded by a multigene family in plants. In *Arabidopsis*, there are nine ACS isoforms (ACS1-2, ACS4-9, ACS11), of which ACS1 is enzymatically inactive as a homodimer but can form functional heterodimers with other ACS isoforms (Yamagami et al. 2003; Tsuchisaka and Theologis 2004b). Similarly, at least nine ACS isoforms have been identified in tomato (LeACS1A, LeACS1B, and LeACS2-8) (Jiang and Fu 2000; Alexander and Grierson 2002). ACS isoforms show high sequence similarity in their N-terminal catalytic domains, but are more divergent in their short noncatalytic C-termini. Based on the presence or absence of phosphorylation sites in their C-terminal sequences, ACS proteins can be classified into three groups (Fig. 1.2a). Type I ACS isoforms, which include *Arabidopsis* ACS1, ACS2, and ACS6, have an extended C-terminal domain containing one calcium-dependent protein kinase (CPK) and three mitogen-activated protein kinase (MAPK) phosphorylation sites. Type II ACS isoforms, which include *Arabidopsis* ACS4, ACS5, ACS8, ACS9, and ACS11, have only a single potential CPK phosphorylation site embedded within a specific domain called TOE (Target of ETO1), which is required for its interaction with ETO1 (ETHYLENE OVERPRODUCER1, an E3 ligase component that directly interacts with the target ACS proteins for their degradation, see Sect. 1.4.1) during ACS degradation. In contrast, Type III ACS isoforms, including *Arabidopsis* ACS7, have the shortest C-terminal extensions and lack both known phosphorylation sites and a TOE domain (Chae and Kieber 2005; Yoshida et al. 2005).

ACS functions as a dimeric enzyme. Recombinant apple ACS in *Escherichia coli* was found to be homodimer (White et al. 1994). The activity of catalytically inactive forms of ACS can be partially restored when they are coexpressed with wild-type ACS protein, indicating that ACS functions as a dimer (Tarun and Theologis 1998). Biochemical characterization of *Arabidopsis* ACS isoforms

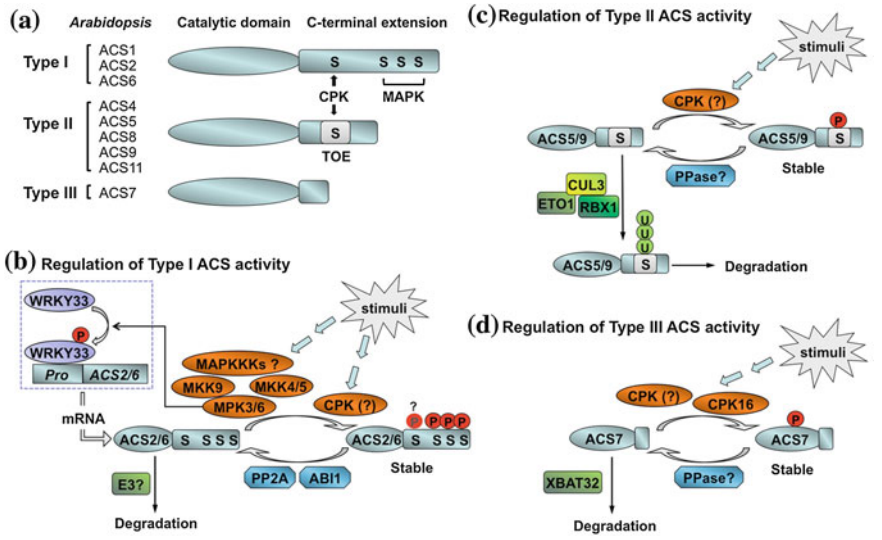

Fig. 1.2 Regulation of cellular ACS activity, a rate-limiting step in ethylene biosynthesis. **a** Classification of ACS members into three subgroups based on the presence and absence of MAPK and/or CPK phosphorylation sites in the C-termini of ACS. Only *Arabidopsis* ACS isoforms are shown. **b** Regulation of *Arabidopsis* Type I ACS by stress/pathogen-responsive MPK3/MPK6 cascade at the transcriptional and posttranslational levels. Phosphorylation of ACS2/ACS6 by MPK3/MPK6 leads to the stabilization of ACS protein. In addition, the expression of *ACS2* and *ACS6* genes is also upregulated by MPK3/MPK6 activation via another MPK3/MPK6 substrate, WRKY33. Dual-level regulation of Type I ACS by MAPKs and possibly CPK(s) greatly enhances the cellular ACS activity and ethylene biosynthesis. Phosphatases involved in the dephosphorylation of ACS2/ACS6 have also been identified. **c** Stability regulation of Type II ACSs such as *Arabidopsis* ACS5 by ETO1-containing E3 ligase that recognizes the TOE domain in their C-termini. It is postulated that CPK phosphorylation is involved in regulating this ubiquitination process, therefore, the stability of Type II ACS protein. **d** Phosphorylation and stability regulation of Type III ACS isoforms. *Arabidopsis* ACS7, a Type III ACS, can be phosphorylated by a CPK in vitro in its catalytic domain, which appears to play a role in ethylene induction during root gravitropism. ACS7 can be degraded via the ubiquitin-26S proteasome pathway that requires the XBAT32 E3 ligase

revealed that each *Arabidopsis* ACS could form a homodimer with distinct biochemical properties, including different optimal pH values, substrate affinities, and K_{cat} values, thus providing another layer of regulation of ethylene biosynthesis (Yamagami et al. 2003). While further studies demonstrated that all ACS isoforms could form heterodimers. However, only the heterodimers formed between members of the same subgroup are functional. The only exception is that ACS7, a Type III ACS, can form functional heterodimers with members of both Type I and Type II branches (Tsuchisaka and Theologis 2004b). Homo- and heterodimerization between ACS isoforms have recently been confirmed in vivo, using bimolecular fluorescent complementation (BiFC), in transgenic *Arabidopsis*. Functional heterodimerization of ACS may act as a regulatory mechanism to enhance isozyme

diversity and provide physiological versatility in various cells/tissues during plant growth and development (Tsuchisaka et al. 2009).

ACS isoforms have very low activity and abundance in vivo and are transcriptionally and posttranscriptionally regulated in response to both endogenous developmental and exogenous environmental stimuli. The role of ACS in ethylene biosynthesis was intensively investigated before it was determined to be the rate-limiting enzyme. Changes of endogenous ACC content in ripening fruit closely correlates with ethylene production rates (Hoffman and Yang 1980). Auxin-induced ethylene production, which involves the conversion of SAM to ACC, can be inhibited by cycloheximide, a translational inhibitor, indicating that de novo synthesis of ACS is required for enhanced ethylene production (Yoshii and Imaseki 1982). In response to stresses such as pathogen infection and wounding, ethylene production and ACC levels increase dramatically, which can be countered by AVG or cycloheximide treatment (Boller and Kende 1980; Yu and Yang 1980; Kende and Boller 1981; Riov and Yang 1982). This strong correlation between ethylene production and endogenous ACC levels suggests that conversion of SAM to ACC by ACS is generally the rate-limiting step of ethylene biosynthesis. Furthermore, exogenous ACC, but not SAM, can greatly increase ethylene production (Yu et al. 1979a; Apelbaum and Yang 1981; Hogsett et al. 1981), suggesting that ACO activity is constitutive, while ACS activity is limiting and represents a regulatory point of ethylene production. Many studies in recent years help us understand more about the regulation of ethylene production at the ACS level, which will be described in detail in Sects. 1.4–1.6.

1.3.3 ACO, the Ethylene-Forming Enzyme

ACO is encoded by small gene families in plants, generally comprised of 3–5 members that show differential regulation in response to various developmental and environmental cues. Because of the originally suggested "rate-limiting" role of ACS in ethylene biosynthesis, regulation of the ACO activity has been much less studied. However, the expression of *ACO* can be induced rapidly and dramatically in a number of physiological processes, including ripening, senescence, and wound-healing responses, indicating that the regulation of ethylene production also occurs at ACO level (Barry et al. 1996; Blume and Grierson 1997).

ACO may become a limiting factor when high levels of ethylene are produced under certain developmental and stress conditions. During cotton (*Gossypium hirsutum*) fiber elongation, ethylene production induced by various treatments is closely correlated with the accumulated transcripts of *ACO*, but not *ACS*, genes (Qin et al. 2007). In poplar [*Populus tremula* (L.) × *P. tremuloides* (Michx)], the expression of an *ACO* gene, *PttACO1*, is specifically upregulated on the upper side (but not on the lower side) to induce ethylene synthesis during gravitational stimulation of tension wood, and ACO activity increased in parallel to *PttACO1* expression. The asymmetric induction of *PttACO1* genes and ACO activity

contributes to differential ethylene production within the poplar stem, which causes profound effects on the pattern and rate of wood development (Andersson-Gunneràs et al. 2003). Further studies of cell/tissue-specific expression patterns of *PttACO* and *PttACS* revealed potential reasons for the important role of ACO, but not ACS, in this particular physiological process: *PttACO1* is strongly expressed in developing xylem, while the expression of the *PttACS* genes is generally more prominent in phloem/cambia tissues (Love et al. 2009). Differential functions of ACS and ACO enzymes in ethylene production, in response to different internal and external cues, might be a result of spatiotemporal-specific regulation of their genes (Dorling and McManus 2012).

1.4 Posttranscriptional Regulation of Cellular ACS Activity

The rapid induction of ethylene biosynthesis suggests the involvement of post-translational regulation. Studies during the past 10 years demonstrate that regulation of ACS protein stability and turnover, which involves kinases, phosphatases, and the ubiquitin-proteasome system, plays a pivotal role in controlling ethylene production during development and stress-related responses (Chae and Kieber 2005; Argueso et al. 2007; McClellan and Chang 2008; Lyzenga and Stone 2012; Xu and Zhang 2014). Phosphorylation and dephosphorylation, coupled with targeted protein degradation by the proteasome pathway, can rapidly regulate ethylene levels in plants, thus allowing a quick response after the perception of internal and external stimuli (Fig. 1.2).

1.4.1 Ubiquitin–Proteasome Degradation System in ACS Protein Turnover

Early studies found that ACS protein stability varies during different developmental stages. For instance, the half-life of ACS in pericarp tissue of ripening tomato fruits is much longer than that of green fruits (2 h vs. 30–40 min) (Kende and Boller 1981). In suspension cultured cells of parsley and tomato, elicitor-induced ACS activity is insensitive to transcriptional inhibitors, supporting a posttranscriptional mechanism of ACS activity regulation (Chappell et al. 1984; Felix et al. 1991). Further studies of *Arabidopsis ethylene-overproducer* (*eto*) mutants provided direct evidence that the ACS protein is indeed posttranscriptionally regulated by the ubiquitin-proteasome pathway (Chae and Kieber 2005).

Etiolated seedlings of *eto1, eto2,* and *eto3* mutants constitutively display a triple response phenotype due to the overproduction of 10 to 100-fold more ethylene compared to wild-type plants (Guzman and Ecker 1990; Kieber et al. 1993; Woeste et al. 1999). *eto2* and *eto3* mutants were subsequently found to have dominant

mutations in the C-termini of ACS5 and ACS9, respectively. Specifically, *eto2* is a result of a single base-pair insertion in *ACS5* that causes a frameshift and replacement of its 12 C-terminal amino acids (Vogel et al. 1998); whereas *eto3* has a missense mutation in the C-terminal domain of ACS9 that changes V457 to a D (Chae et al. 2003). In the *eto2* mutant, elevated ethylene production is the result of a significantly prolonged half-life of ACS5, rather than an increased enzymatic activity. Likewise, the *eto3* mutation enhances the stability of ACS9 protein as well (Chae et al. 2003).

The characterization of the *eto1* mutant revealed a molecular mechanism underlying regulation of ACS5 stability (Wang et al. 2004). Unlike *eto2* and *eto3*, *eto1* is a recessive mutant (Woeste et al. 1999). Cloning of *ETO1* revealed that it encodes a component of E3 ligase that possesses a BTB (broad-complex/tramtrack/bric-a-brac) domain (Wang et al. 2004). BTB domain-containing proteins have been shown to link CUL3-based ubiquitin ligases to substrate proteins, directing the target protein for ubiquitin-dependent degradation by the 26S proteasome (Pintard et al. 2004). Using in vitro pull-down assays, ETO1 was demonstrated to interact directly with both ACS5 and CUL3, indicating that ETO1 indeed serves as a substrate-specific adaptor protein that directs ACS5 for degradation (Wang et al. 2004). Furthermore, studies of the *clu3*^{hyp} mutant (double homozygous *cul3a-3 cul3b-1* mutant) indicated that CLU3 participates in ACS5 protein degradation and modulates ethylene production. The elevated ethylene production in the *clu3*^{hyp} mutant is remarkably enhanced by the *eto1* mutation (Thomann et al. 2009). Together, these results suggest that the ETO1-CUL3 ubiquitin ligase plays a critical role in regulating the stability of the ACS5 protein.

In *Arabidopsis*, two close paralogs of EOT1, ETO1-LIKE1 (EOL1) and EOL2, function together with ETO1 to downregulate ethylene production (Christians et al. 2009). These three BTB proteins specifically target type II ACS proteins, but not type I or type III ACSs (Yoshida et al. 2005; Christians et al. 2009). Type II ACS proteins carry a unique C-terminal *cis*-acting sequence called a TOE domain, which is the recognition site for ETO1/EOL1/EOL2 proteins. Fusion of the TOE domain to other proteins could result in rapid degradation of the chimeric proteins in a ETO1-dependent manner (Yoshida et al. 2006). Together with the findings from *eto2* and *eto3* mutants, it can be concluded that the C-terminal sequence of Type II ACS proteins is critical for their stability. Recently, it was shown that light destabilizes ETO1 and EOLs and therefore, stabilizes the ACS5 protein, presenting a novel control point that regulates ethylene biosynthesis in response to environmental cues (Yoon and Kieber 2013).

Additional E3 ligase components have also been identified to regulate the stability of ACS. Recently, a monomeric ring-type E3 ligase, XBAT32, was shown to mediate proteasomal degradation of ACS4 and ACS7, Type II and Type III ACS, respectively (Prasad et al. 2010; Lyzenga et al. 2012). Similar to *eto1*, *xbat32-1* seedlings display a number of ethylene-overproduction phenotypes (Prasad et al. 2010). The degradation rate of transgenic HA-ACS7 in an *xbat32-1* background is greatly decreased compared to that in a wild-type background (Lyzenga et al. 2012). It is interesting that despite the lack of any known regulatory sequences

within the shortened C-terminal tail of ACS7, turnover of this enzyme can still be mediated by the ubiquitin proteasome system, suggesting the presence of additional unidentified *cis*-regulatory sequences in the ACS protein.

Mutations of other E3 ligase components could also result in phenotypes related to ethylene overproduction. For instance, RNAi suppression of RUB1/2 (Related to Ubiquitin ½), which is required for the function of SCF-type E3 ligase complexes through their covalent attachment to CULLINs, leads to an increase in ethylene biosynthesis and triple responses (Bostick et al. 2004). In addition, mutation of RCE1 (RUB1-CONJUGATING ENZYME 1) results in increased ethylene production, which is associated with enhanced ACO activity (Larsen and Cancel 2004). However, at this stage, whether ACS or ACO is directly regulated by the RUB-dependent pathway is unknown.

At present, no E3 ligase has been identified that targets Type I ACS proteins. Nonetheless, MG132, a specific inhibitor of the 26S proteasome, can greatly enhance stability of the ACS6 protein, suggesting involvement of the ubiquitin-proteasome pathway in regulating the stability of Type I ACS (Joo et al. 2008). Thus, the ubiquitin-proteasome degradation system is involved in the turnover of all three types of ACSs to modulate ethylene biosynthesis (Fig. 1.2). Further studies demonstrate that ACS phosphorylation by MAPKs and CPKs is a key mechanism to antagonize ubiquitination and stabilize ACS proteins.

1.4.2 Phosphorylation Regulation of ACS Isoforms by MAPKs and CPKs

It have been long recognized that protein phosphorylation and dephosphorylation play important roles in the regulation of ACS activities, based on pharmacological studies. General Ser/Thr protein kinase inhibitors K252a and staurosporine could block elicitor-induced ACS activity in cultured tomato cells (Grosskopf et al. 1990; Felix et al. 1991; Spanu et al. 1994). In contrast, treatment of the cultured tomato cells with the protein phosphatase inhibitor calyculin A rapidly increased ACS activity in the absence of elicitors (Felix et al. 1994; Spanu et al. 1994). Since ACS activity was not affected by treatment with these inhibitors or protein phosphatase in vitro, it was speculated that protein phosphorylation and dephosphorylation possibly regulate ACS activity by controlling the rate of enzyme turnover, rather than affecting its catalytic activity directly (Spanu et al. 1994).

In tobacco, activation of SIPK (SALICYLIC ACID-INDUCED PROTEIN KINASE) and WIPK (WOUNDING-INDUCED PROTEIN KINASE), two stress/pathogen-responsive MAPKs, induces high levels of ethylene production, accompanied by a dramatic increase in ACS activity (Kim et al. 2003). The identification of the first pair of plant MAPK substrates revealed that *Arabidopsis* ACS2 and ACS6 can be directly phosphorylated by MPK3 and MPK6, orthologs of tobacco WIPK and SIPK, respectively (Liu and Zhang 2004; Han et al. 2010). MPK3/MPK6

phosphorylate ACS2 and ACS6 on three Ser residues in their C-termini. These three Ser residues are highly conserved in specific ACS isoforms, and have become the criterion to define Type I ACS isoforms (Liu and Zhang 2004; Yoshida et al. 2005). Detailed biochemical and genetic analyses demonstrated that phosphorylation of ACS2/ACS6 by MPK3 and MPK6 dramatically improves the stability of ACS proteins in vivo, resulting in higher cellular ACS activity and elevated ethylene production. Analogously, $ACS6^{DDD}$, a gain-of-function ACS6 mutant that mimics the phosphorylated form of ACS6, is much more stable than the wild-type ACS6 protein (Liu and Zhang 2004; Han et al. 2010). Further study revealed that phosphorylation of ACS2/ACS6 by MPK3/MPK6 prevents ACS proteins from being degraded by the ubiquitin-proteasome machinery, therefore increasing the stability of ACS/ACS6 proteins (Joo et al. 2008). Together, these findings demonstrate that phosphorylation of Type I ACS isoforms by MAPKs can enhance their stability (Fig. 1.2b), representing an important regulatory mechanism of ethylene production in plant stress/immunity responses.

An increasing body of evidence also implicates the involvement of CPK(s) in regulating ACS turnover. Tomato LeACS2 was found to be phosphorylated by LeCPK2 at Ser-460 of its C-terminus (Tatsuki and Mori 2001; Kamiyoshihara et al. 2010). This conserved CPK-phosphorylation site exists in both type I and type II ACS proteins, and it was shown that phosphorylation at both the CPK and MAPK target sites is required for ACS stability in wounded pericarp (Kamiyoshihara et al. 2010). Moreover, in cotton, GhACS2 was found to be phosphorylated by GhCPK1 in vitro at Ser-460, significantly increasing its enzymatic activity (Wang et al. 2011). In *Arabidopsis*, CPK4 and CPK11, two ABA-activated CPKs, were recently shown to phosphorylate ACS6 at its C-terminus, resulting in ACS6 protein stabilization and increased ethylene production during root growth (Luo et al. 2014). All these evidences reveal an important role of phosphorylation by CPKs in regulating Type I, and possibly Type II, ACS protein stability (Fig. 1.2).

Although the conserved CPK phosphorylation site is embedded in the TOE domain of Type II ACS proteins, which is the ETO1/EOL1/EOL2 recognition sequence, thus far, evidence is lacking to directly support a role for CPK phosphorylation in the stability regulation of Type II ACS proteins. On the other hand, despite the lack of any known regulatory sequences within the short C-termini of Type III ACS isoforms, it is interesting that *Arabidopsis* ACS7 can be phosphorylated by a CPK in vitro in its catalytic domain, which appears to play a role in ethylene induction during root gravitropism (Huang et al. 2013). As mentioned earlier, ACS7 can be degraded via the ubiquitin-26S proteasome pathway that requires the XBAT32 E3 ligase (Lyzenga et al. 2012) (Fig. 1.2d). However, whether phosphorylation of ACS7 by CPK(s) is antagonistic to regulation of its proteasomal degradation remains unknown.

1.4.3 Dephosphorylation Regulation of ACS Isoforms by Protein Phosphatases

As mentioned above, calyculin A, a protein phosphatase inhibitor, greatly stimulated ACS activity, suggesting the involvement of phosphatase(s) in downregulating ethylene biosynthesis (Felix et al. 1994; Spanu et al. 1994). The identities of the phosphatases had remained elusive until recently. Overexpression of *AP2C1*, which encodes a Ser/Thr protein type 2C phosphatase that can negatively regulate MPK6, suppresses wounding-induced ethylene production (Schweighofer et al. 2007). In a more recent report, protein phosphatase 2A was shown to fine-tune ethylene production by negatively regulating the activity of Type I ACS isoforms, while positively influencing the abundance of Type II ACS isozymes (Skottke et al. 2011). The immunoprecipitated PP2A complexes can specifically dephosphorylate a phosphopeptide, corresponding to the C-terminus of ACS6, in vitro (Skottke et al. 2011). ABI1, another protein phosphatase 2C that negatively regulates ABA signaling, was found to directly dephosphorylate both ACS6 and MPK6, and therefore, negatively regulates ethylene biosynthesis during oxidative stress (Agnieszka et al. 2014). Together, all these studies indicate that Type I ACSs and MPK6, and possibly MPK3, are key targets of phosphatases to antagonize phosphorylation-mediated stabilization of ACS proteins.

1.5 Transcriptional Regulation of ACS Genes

ACS genes are differentially regulated at the transcriptional level by signaling pathways that are responsive to either endogenous or exogenous stimuli, or both. This is another key mechanism in regulating cellular ACS activity, in addition to the posttranslational regulation discussed in the previous section. The combination of transcriptional and posttranscriptional regulation allows for "fine-tuning" of ethylene production in different cells/tissues, at different growth/developmental stages, and also in response to biotic and abiotic stresses.

1.5.1 Developmental Regulation of ACS Genes

Ethylene production is tightly regulated during distinct stages of plant growth and development, including germination, senescence, floral organ abscission, and fruit ripening (Yang and Hoffman 1984; Kende 1993; Zarembinski and Theologis 1994; Wang et al. 2002; Argueso et al. 2007). Studies of *ACS* expression showed that *ACS* genes exhibit cell- and tissue-specific expression patterns and are differentially regulated in various developmental stages (Tsuchisaka and Theologis 2004a; Tsuchisaka et al. 2009). In *Arabidopsis*, *ACS6* is the most common transcript

among the *ACS* gene family and is highly expressed in roots, mature leaves, and inflorescence stems. *ACS2, 4, 7,* and *8* are moderately expressed in roots and cotyledons, while *ACS1, 5, 9,* and *11* are expressed in their respective specific tissues, and have a relatively low expression level in the whole plant (Tsuchisaka and Theologis 2004a; Dugardeyn et al. 2008). GUS reporter-aided analysis of the promoter activities of *Arabidopsis ACS* genes revealed that multiple members are expressed at any specific growth and developmental stage (Wang et al. 2005). These unique and overlapping expression patterns indicate that *ACSs* may have specific but redundant functions during development, as revealed by phenotypic characterization of single, double and high-order mutants in more recent studies (Tsuchisaka et al. 2009).

Tomato is an ideal model system for understanding the role of ethylene in fruit development and ripening. Two ethylene regulatory systems have been proposed, based on levels of ethylene production and the different feedback mechanisms of ethylene biosynthesis in tomato and other climacteric plants (McMurchie et al. 1972; Alexander and Grierson 2002). Regulatory System-1 functions during vegetative growth, in which the basal level of ethylene is produced and ethylene is negatively feedback regulated. System-2 operates during fruit ripening to produce a high level of ethylene, which then positively regulates its own biosynthesis. This positive feedback of System-2 is an important mechanism to ensure a quick fruit ripening process that usually starts from one specific region of a fruit (Alexander and Grierson 2002). *LeACS1A* and *LeACS6*, which are negatively regulated by ethylene, have been shown to be the main *ACS* genes responsible for the basal production of ethylene in the preclimacteric period (Barry et al. 2000). During the transition from preclimacteric period to climacteric fruit ripening, *LeACS2* and *LeACS4* are induced by ethylene through a positive feedback regulation to initiate and maintain System-2 activity (Barry et al. 2000; Alba et al. 2005). Recently, several studies revealed that RIN (RIPENING INHIBITOR), a key MADS transcription factor that controls tomato fruit ripening, upregulates *LeACS2* and *LeACS4* via direct binding to CArG *cis*-elements in their promoters (Ito et al. 2008; Martel et al. 2011; Fujisawa et al. 2013). Promoter analysis of *LeACS6* further revealed that the *cis*-elements responsible for negative regulation (System-1) located to −347 and −266 bp regions upstream of its transcription start site (Lin et al. 2007). At present, the trans-acting transcription factor(s) that bind to this *cis*-element region remain unidentified. Furthermore, how these transcription factor(s) are regulated by ethylene or developmental cues is unknown. More research is needed for us to fully understand how *ACS* genes are regulated, either negatively or positively.

In addition to developmental regulation and negative and positive regulation by ethylene, *ACS* genes are also responsive to other hormonal signals. Auxin induces the expression of three ACS genes, *ACS 6, 8,* and *11* (Paponov et al. 2008). Brassinosterioids (BR) and cytokinins also induce ethylene biosynthesis, mainly, however, via increased stability of a subset of ACS proteins (Chae and Kieber 2005; Hansen et al. 2009).

1.5.2 *Induction of* ACS *Gene Expression in Response to Stress/Pathogen Invasion*

In addition to its roles in plant development, ethylene also regulates plant responses to many environmental stresses and thus is known as the "stress hormone". A wide range of abiotic and biotic stresses including wounding, flooding, drought, ozone, hypoxia, herbivore and pathogen attack induce ethylene production in plants. Because of the central role of ACS enzymes in ethylene production, the impact of different stressors on *ACS* gene regulation has been thoroughly investigated by both traditional and whole genome analyses (Tsuchisaka and Theologis 2004a; Peng et al. 2005; Wang et al. 2005; Broekaert et al. 2006).

One of the best studied abiotic stresses involved in ethylene induction is wounding. Wounding of pericarp tissue of tomato fruit rapidly induces high levels of ethylene (Boller and Kende 1980; Yu and Yang 1980). Further studies revealed that *LeACS2* and *LeACS4*, two genes responsible for ethylene production during tomato fruit ripening, are super-induced in pericarp tissue by wounding during various stages of ripening. Wound response *cis*-elements were found to exist in the promoters of both *LeACS2* and *LeACS4* (Lincoln et al. 1993). Thus, increased ethylene may function as a signal mediating wound response in tomato plants (O'Donnell et al. 1996). In *Arabidopsis* hypocotyl, wounding induces the expression of *ACS2, 4, 6, 7, 8* and *11*, but suppresses *ACS1* and *ACS5* (Tsuchisaka and Theologis 2004a). In response to hypoxia, mRNA transcripts of *ACS2, 6, 7,* and *9* accumulate in *Arabidopsis* (Peng et al. 2005). Tsuchisaka and Theologis (2004a, b) investigated the spatial-temporal expression patterns of *ACS* genes in *Arabidopsis* under different stresses conditions, revealing specific and overlapping patterns of *ACS* gene induction in different tissues.

It has been long recognized that plants invaded by pathogens produce high levels of ethylene (Boller 1991). The ethylene production induced by pathogen invasion is generally much higher than production induced by abiotic stresses. For example, the induction of ethylene production by herbivore attack exceeds that produced by a similar physical wounding (Von Dahl and Baldwin 2007). Both *PsACS2* and *PsACS3* are strongly upregulated during weevil attack in *Picea sitchensis* (Ralph et al. 2007). In *Nicotiana attenuata* that is invaded by *Manduca sexta*, increased transcript accumulation of *NaACS3a* is required for oral secretion-induced ethylene burst (von Dahl et al. 2007).

Data mining using Genevestigator (Zimmermann et al. 2004) allowed an overview of *ACS* expression regulation following various biotic stresses (Broekaert et al. 2006). *ACS2* is strongly upregulated upon attack by *Pseudomonas syringae*, *Botrytis cinerea*, and *Alternaria brassicicola*, while *ACS5* and *ACS11* expression tends to be downregulated in response to *P. syringae* infection (Broekaert et al. 2006). Challenge by necrotrophic fungi, such as *B. cinerea,* triggers very high levels of ethylene production in infected plant tissues (Elad 1990). In *Arabidopsis*, both transcriptional and posttranscriptional regulation of *ACS2* and *ACS6* are required for full ethylene induction in response to *B. cinerea* infection (Han et al.

2010; Li et al. 2012). The residual ethylene induction in the *acs2 acs6* double mutant suggests the involvement of additional ACS isoforms (Han et al. 2010). Further studies revealed that *ACS7*, *ACS8*, and *ACS11* also contribute to *B. cinerea*-induced ethylene production (Li et al. 2012). These unique and overlapping expression patterns of *ACS* genes could fine-tune ethylene production levels and contribute to the appropriate response of plants faced with particular threats. Plants in their natural environment are often simultaneously or sequentially attacked by various parasites. A recent report showed that the pathogen *P. syringae*-triggered ethylene production is required for systemically induced susceptibility to herbivory, thus indicating an important role for ethylene production in plant defense against multiple enemies (Groen et al. 2013).

1.6 Regulation of ACS at Multiple Levels: Integration of Signaling Pathways

In addition to the involvement of multiple ACS isoforms, ACS regulation by multiple signaling pathways and/or a single pathway at multiple levels appears to be key to heightened induction of ethylene biosynthesis. Plants produce high levels of ethylene when challenged by necrotrophic fungal pathogens. In *Arabidopsis* infected with *B. cinerea*, ACS genes encoding all three isoenzyme types contribute to induction of ethylene biosynthesis (Li et al. 2012). ACS2 and ACS6, two Type I isoforms, are regulated by MPK3 and MPK6 via two different mechanisms (Fig. 1.2): (1) by direct phosphorylation and stabilization of ACS2 and ACS6 proteins (Liu and Zhang 2004; Joo et al. 2008; Han et al. 2010) and (2) by activation of *ACS2* and *ACS6* gene expression (Li et al. 2012). Phosphorylation of ACS2 and ACS6 by MPK3/MPK6 results in their stabilization and further, enhanced gene expression could increase de novo synthesis of ACS2/ACS6 proteins (Liu and Zhang 2004; Han et al. 2010; Li et al. 2012). A key transcription factor, WRKY33, another substrate of MPK3/MPK6, can directly bind to the promoters of *ACS2* and *ACS6* and activate their gene expression (Mao et al. 2011; Li et al. 2012). Moreover, upregulation of *ACS2* and *ACS6* gene expression by WRKY33 is required for MPK3/MPK6-induced ethylene production. De novo synthesis of ACS proteins resulting from gene activation, coupled with their phosphorylation by MPK3/MPK6 and stabilization, provides a vital supply of ACS enzymes to maintain a high rate of ethylene production (Li et al. 2012) (Fig. 1.2b). In this situation, long-lasting activation by MPK3/MPK6 is critical in driving both processes.

When there is only a transient activation of MPK3/MPK6, such as in wounded *Arabidopsis*, there is only temporary low-level ethylene induction (Arteca and Arteca 1999) and activation of *ACS* gene expression (Tatsuki and Mori 1999). However, due to the transient nature of MAPK activation, which returns to a basal level within about 0.5–1 h (Zhang and Klessig 1998), de novo synthesized ACS

may not have the chance to be phosphorylated, and will be degraded quickly. As a result, regulation of ACS activity at dual levels by the MPK3/MPK6 cascade is an important mechanism underlying the kinetics and levels of ethylene production, in response to rapidly changing environments.

Regulation of ACS activity may also occur through the interplay of multiple upstream signaling pathways. Both CPK and MAPK signaling pathways are activated in response to environmental stimuli, and they may also work synergistically, antagonistically, or independently in promoting ethylene biosynthesis at multiple levels. Ludwig et al. showed that a balanced interplay between the MAPK and CPK signaling pathways controls stress-induced ethylene production (Ludwig et al. 2005). It was shown that stress-induced activation of SIPK and WIPK is compromised in CPK-VK plants, in which an activated form of tobacco CPK2 lacking its autoinhibitory and the calmodulin-like domains is transient expressed. This inhibition requires ethylene synthesis and perception based on analyses using inhibitors of either ethylene synthesis (AVG) or ethylene perception (silver thiosulphate) (Ludwig et al. 2005). It was proposed that simultaneous activation of these two signaling pathways, in response to one stimulus, offers a back-up system to guarantee multiple activation events. However, they might subsequently exert regulatory effects on each other, allowing for fine tuning of partially overlapping defense responses. Once high levels of ethylene are produced, an ethylene-mediated feedback crosstalk occurs to reset these two signaling systems to respond to stimuli in the most appropriate way (Ludwig et al. 2005).

Type I ACS isoforms can be phosphorylated by both CPK and MAPK (Liu and Zhang 2004; Kamiyoshihara et al. 2010). It was shown that inhibition of either CPK or MAPK decreased LeACS2 accumulation in wounded tomato fruit, indicating that the two signaling pathways may act together to regulate LeACS2 stability (Kamiyoshihara et al. 2010). These findings highlight the complexity of phosphorylation signaling pathways in regulating ethylene biosynthesis.

1.7 Summary and Future Directions

ACS, a frequently rate-limiting enzyme that catalyzes the committing step of ethylene biosynthesis, is regulated at multiple levels in response to various endogenous and environmental cues. First, the stability of ACS proteins is tightly regulated at the posttranscriptional level by the ubiquitin–proteasome system, which is dependent on the phosphorylation status of the ACS protein, allowing rapid changes in total cellular ACS activity and ethylene production rates. Second, different *ACS* genes are differentially regulated at the transcription level, allowing specificity of spatiotemporal *ACS* expression and ethylene production. Third, multiple ACS isoforms, often members from different isoform groups, are involved in ethylene induction in response to a single stimulus. A potential fourth level of regulation of ACS enzymes is the homo- and heterodimerization of ACS isoforms to produce

distinct enzymatic properties, which may represent another layer of complexity to fine-tune ethylene biosynthesis (Tsuchisaka and Theologis 2004b).

At this stage, our understanding of ethylene biosynthesis regulation is still fragmented. Recent advances in how plants sense invading pathogens allow us to picture a pathway from the sensing of exterior stimuli, to the signaling transduction pathways (e.g., MAPK), to the regulation of ACS activity, at both transcriptional and posttranslational levels (Fig. 1.2b). At this stage, it is still not possible for us to directly measure ethylene induction in specific tissues/cells because of instrument limitations. However, the localization of ethylene biosynthesis should coincide with where the limiting enzyme is induced, which can be determined by elucidating (1) the expression and induction patterns of specific ACS isoforms, or ACO when it is limiting; and (2) the requirement of specific ACS or ACO isoforms for ethylene induction, based on genetic analysis. Studies by Theologis's group advanced our knowledge on both fronts (Tsuchisaka and Theologis 2004a; Tsuchisaka et al. 2009). The generation of a variety of high-order *acs* mutants led to the identification of specific isoforms involved in *B. cinerea*-induced ethylene production (Li et al. 2012). More research using these valuable tools should allow us to identify specific combinations of ACS isoforms required for ethylene induction in response to specific internal/external stimuli. We also need information about the spatiotemporal expression patterns of these ACS isoforms. To that end, a tool set of *ACS* promoter-driven GUS reporters is available (Tsuchisaka and Theologis 2004a). By superimposing the spatiotemporal activation of specific signaling pathways, we should be able to infer the distinct locations (i.e., cell/tissue-specificity) of ethylene production.

Different stimuli induce different levels of ethylene, which can vary by hundreds of folds. Both the magnitude and kinetics of signaling processes are critical to levels of ethylene biosynthesis. At this stage, we still do not know how much ethylene is required to trigger a specific ethylene-regulated response. It is possible that ethylene production could be limited to a specific set of cells/tissues, resulting in a very high local concentration of ethylene, while overall ethylene levels (normalized to the whole plant/organ) remain low. Thus, it is critical for us to determine when and where ethylene is produced. Together with tissue/cell-specific expression of ethylene receptors and downstream signaling components, we can begin to understand the ethylene signaling processes in plants, starting from the sensing of external/internal cues, to the induction of ethylene, and the ethylene sensing/signaling pathways.

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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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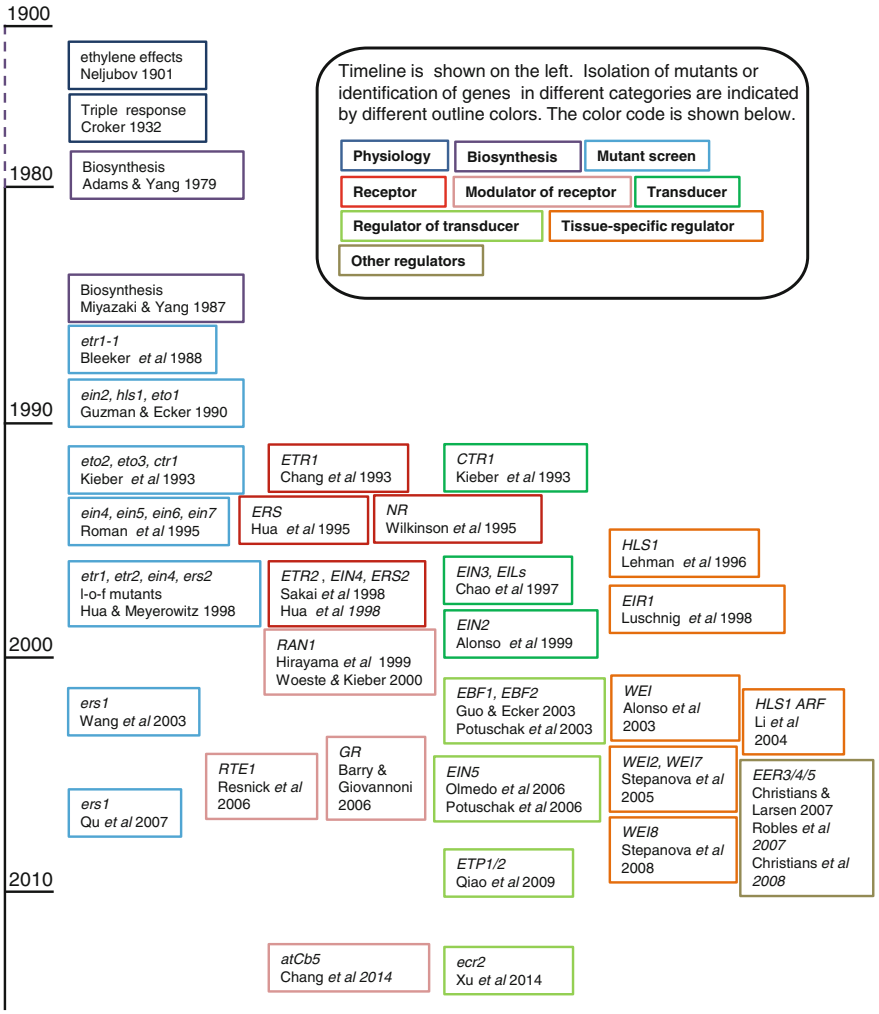

Fig. 2.1 Historical over view of identifying major ethylene signaling components

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 *ein*4, 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 $etr1^{1-349}$ 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–theorine 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 Nramp 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 proteosome-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-proteosome 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 *wie8*, 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 α1), α-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 β 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_2O_2 (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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Chapter 3
Ethylene Receptors—Biochemical Events

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Abstract The first step in ethylene perception occurs when the ethylene molecule binds to a receptor. A large number of studies have increased our understanding about how ethylene binds to the receptors and a signal is transduced. These studies have shown that a copper ion is a required cofactor that is delivered by the RAN1 (RESPONSIVE TO ANTAGONIST1) copper transporter. Additionally, biochemical studies have determined that the receptors are functional protein kinases. However, receptor protein kinase activity is not required for responses to ethylene. Rather, this activity seems to modulate responses to ethylene. Even though the exact nature of receptor output is unknown, it is clear that the receptors affect the activity of the CTR1 (CONSITUTIVE TRIPLE RESPONSE1) protein kinase. A model for how ethylene affects receptor signaling is presented.

Keywords Ethylene receptors · Histidine kinase · Protein–protein interactions · Copper · Ethylene binding

3.1 Overview

The first step in ethylene signal transduction is the binding of ethylene to the ethylene receptors. These receptors have been studied for many decades but the mechanism by which these receptors transduce the ethylene-binding signal to downstream elements is unclear. This chapter summarizes what is known about the

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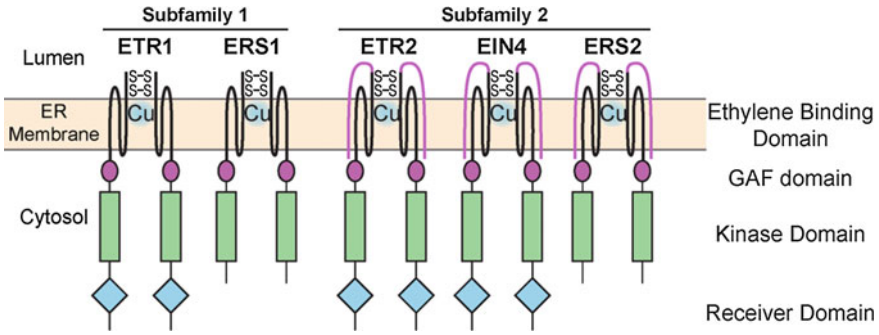

Fig. 3.1 The domain structures of the ethylene receptors from *Arabidopsis thaliana* are shown. Each receptor contains an *ethylene binding*, *GAF*, and *kinase domain* as shown. Three of the five also contain a receiver domain. *ETR1* and *ERS1* are in *subfamily 1* and *subfamily 2* includes *ETR2*, *EIN4*, and *ERS2*. *Subfamily 2* receptors are characterized by additional amino acids at the N-terminus that may form a fourth transmembrane helix or act as a signal peptide

biochemistry of the ethylene receptors and provides a model for how signal output may be regulated.

Individual plant species contain multiple ethylene receptor isoforms that, along with many downstream signaling components, are localized predominantly in the membranes of the endoplasmic reticulum (Ju and Chang 2012). All of the isoforms have similar domain structures that are similar to bacterial two-component receptors (Fig. 3.1) (Chang et al. 1993). All are predicted to contain three transmembrane α-helices that comprise the ethylene-binding domain. Following this input domain is a GAF (cGMP-specific phosphodiseterases, adenyl cyclases FhlA) and kinase domain. A subset of the receptor isoforms also contain a receiver domain at their C-termini. These three domains are thought to mediate signal output from the receptors. The receptors form dimers that are stabilized at their N-terminus by two disulfide bonds (Schaller et al. 1995).

Based upon sequence comparisons of the ethylene-binding domains, the receptors fall into two subfamilies (Wang et al. 2006). Bacterial two-component receptors are well studied and are known to function by histidine autophosphorylation followed by a histidine to aspartate phosphorelay (West and Stock 2001). However, the biochemical events that underlie ethylene signaling from the receptors are not clear. The best-studied receptors are from *Arabidopsis thaliana*. In *Arabidopsis*, there are five receptor isoforms called ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE INSENSITIVE4 (EIN4), ETHYLENE RESPONSE SENSOR1 (ERS1), and ERS2. ETR1 and ERS1 are in subfamily 1 and the remaining isoforms belong to subfamily 2 (Fig. 3.1). Most of what we know about the ethylene receptors comes from research on the receptors from *Arabidopsis* and this will form the focus of this chapter.

Even though not the focus of this chapter, it worth noting that most research indicates that the major downstream target for the ethylene receptors is the protein kinase, CTR1 (Kieber et al. 1993). CTR1 is a negative regulator of ethylene

signaling, thus, in air it represses ethylene responses. When ethylene binds to the receptors, it is thought to lead to inhibition of CTR1 and thus responses to ethylene. More about these interactions can be found in Chap. 4.

3.2 Ethylene-Binding Domain

The ethylene-binding domain of ethylene receptors is composed of three transmembrane α-helices. In order for the ethylene receptor to function, the ethylene-binding domain must be able to provide a stable binding pocket and effectively respond to ethylene. Here we will highlight what is currently known about how ethylene binds to the receptor and how this signal is transmitted through the binding domain.

3.2.1 Requisite Copper Cofactor

It has long been known that ethylene binds to receptors with high affinity. However, the question of how ethylene binds remained elusive for many years. Several lines of evidence suggested that a metal cofactor is required for this high affinity binding. Initially, it was known that olefins such as ethylene are able to form complexes with metals due to the presence of electron rich π-orbitals. Early work showed that in addition to ethylene, exposure to carbon monoxide caused ethylene responses in plants at higher concentrations. Because carbon monoxide is only biologically active when bound to metalloproteins, this led to speculation that ethylene too requires a protein–metal complex to elicit a physiological response (Burg and Burg 1967). Further work established that silver is able to block ethylene responses, which suggested that copper functions as the natural cofactor since, like silver, it is a Group 11 metal (Beyer 1976). This idea was supported by a study confirming that copper and ethylene form a stable coordination complex that is capable of functioning within a protein (Thompson et al. 1983).

A direct test of this model was made possible with the development of an exogenous yeast expression system for functional ethylene receptors (Schaller and Bleecker 1995). Using this system, Rodriguez et al. (1999) showed that of the many transition metals tested, copper ions were one of the few that acted as a cofactor for ethylene binding to ETR1. They also showed that copper co-purified with ETR1 with a stoichiometry of 1 copper ion per receptor dimer suggesting the dimer is the functional unit of these receptors. An ethylene insensitive mutant, *etr1-1*, was identified and shown to contain a C65Y mutation (Bleecker et al. 1988; Chang et al. 1993). When this mutation was introduced into ETR1, it was incapable of binding ethylene and copper did not co-purify with the etr1-1 protein, indicating that C65 is involved in coordination of the copper ion (Rodriguez et al. 1999).

Interestingly, of the many transition metals besides copper tested, only silver and gold ions were capable of supporting ethylene-binding activity in exogenously

expressed ETR1 (Rodriguez et al. 1999; Binder et al. 2007). Silver has long been known to inhibit ethylene responses in plants (Beyer 1976), whereas gold ions do not block ethylene responses (Binder et al. 2007). This has led to a model where silver ions may occupy the ethylene-binding pocket and prevent downstream signaling upon ethylene binding. However, this model for the action of silver ions may be too simplistic since it has recently been shown that only the subfamily 1 receptors, ETR1 and ERS1, are able to bind ethylene in the presence of silver yet all the receptors except for ETR2 can mediate the effects of silver ions on plants (McDaniel and Binder 2012). This suggests that there may be a second metal-binding site on the receptors outside of the ethylene-binding domain. Another interesting observation is that silver ions only support approximately 30 % of the ethylene-binding activity seen with copper ions (Rodriguez et al. 1999; Binder et al. 2007; McDaniel and Binder 2012). This is not due to differences in the K_d for ethylene or half-time of ethylene release (McDaniel and Binder 2012), suggesting that fewer active ethylene-binding sites are generated with silver ions compared to copper ions. One interpretation of this is that there may be multiple copper ions per receptor dimer with each copper capable of binding ethylene (McDaniel and Binder 2012). Even though this seems to be in conflict with the earlier study by Rodriguez et al. (1999), in this earlier study it was noted that not all the receptors were active and capable of binding ethylene leaving open the possibility that active receptors contain more than one copper ion per dimer.

Further support that copper ions are required for ethylene binding to the receptors comes from studies on mutations in RAN1 (RESPONSIVE TO ANTAGONIST1), a protein in *Arabidopsis* with homology to the yeast Ccc2 copper transporter. Multiple mutants of RAN1 have been identified, including partial loss-of-function mutants (*ran1-1*, *ran1-2*) and null mutants (*ran1-3*, *ran1-4*). (Hirayama et al. 1999; Himelblau and Amasino 2000; Woeste and Kieber 2000) The *ran1-1* and *ran1-2* plants respond normally to ethylene. However, paradoxically, application of *trans*-cyclootene mimics the effects of ethylene in these mutants. This is interesting because *trans*-cyclootene inhibits ethylene responses in wild-type plants (Sisler et al. 1990). Addition of copper to the growth media for *ran1-1* and *ran1-2* plants leads to rescue of the wild-type *trans*-cyclootene response. In other words, *trans*-cyclootene blocks ethylene responses in these mutants when grown with excess copper (Hirayama et al. 1999). The *ran1-3* and *ran1-4* plants mimic receptor loss-of-function plants showing a phenotype similar to constitutive ethylene signaling, indicating that efficient delivery of copper is also needed for the production of functional ethylene receptors (Himelblau and Amasino 2000; Woeste and Kieber 2000). This idea was further supported by the observation that the growth of *ran1-1* and *ran1-2* plants is more sensitive to reductions in copper levels (Binder et al. 2010). Additionally, it was shown that ETR1 expressed in ccc2-deficient yeast was incapable of binding ethylene. Ethylene binding was restored when the mutant yeast were complemented with RAN1 (Binder et al. 2010). Together, these results highlight the importance of effective copper delivery to the receptors in both an exogenous expression system and in vivo.

3.2.2 The Ethylene-Binding Pocket

As mentioned previously, the ethylene-binding pocket lies within the three membrane spanning N-terminus of the ethylene receptors (Schaller and Bleecker 1995; Rodriguez et al. 1999). Because this ethylene-binding domain is membranous, a crystal structure has remained elusive. Thus, to assess the structure and function of the ethylene-binding pocket, both genetic and chemical studies have been applied. Collectively, these studies have led to a clearer description of both the ethylene-binding pocket and signaling within the receptor.

To evaluate the role of specific amino acids in the ethylene-binding domain, point mutations have been made on residues located in the ethylene-binding domain of ETR1. Residues to study have been chosen based on random mutagenesis screens leading to defects in ethylene signaling in plants and based on conservation of residues with putative ethylene-binding domains across many organisms (Bleecker et al. 1988; Chang et al. 1993; Rodriguez et al. 1999; Wang et al. 2006). Interestingly, seven residues have been shown to be required for ethylene binding, all of which are predicted to lie along the same face of transmembrane helices one and two of the ethylene-binding domain (Fig. 3.2) (Schaller and Bleecker 1995; Wang et al. 2006). The previously mentioned C65 residue in ETR1 that is thought to be involved in copper coordination is among these seven amino acids. This has led to speculation that these residues coordinate a copper ion and make up the ethylene-binding pocket. Additionally, it has been shown that mutations in residues surrounding the seven residues required for ethylene binding lead to reduced ethylene binding, indicating a possible role for these residues in structurally supporting the ethylene-binding pocket within this domain (Wang et al. 2006).

Further insight into the nature of the ethylene-binding pocket has been gained by examining binding of *trans*-cyclooctene to ETR1. *trans*-cyclooctene, a chiral, strained alkene, functions as a competitive inhibitor of ethylene binding to the receptor (Sisler et al. 1990). The R-enantiomer of *trans*-cyclooctene is five times more effective at blocking ethylene binding to ETR1 compared to the S-enantiomer, indicating asymmetry in the binding pocket (Pirrung et al. 2008). This result is surprising due to the symmetry and simplicity of the ethylene molecule, however, it provides unique insight into the ethylene-binding pocket.

3.2.3 Signal Transduction

In the presence of air, the ethylene receptor signals function to suppress the ethylene response. Upon ethylene binding, this signaling is turned off and ethylene responses occur. By mutating the seven previously mentioned residues required for ethylene binding, the receptor remains locked in a signaling state, as if in air. While mutation of other residues in the three helices of the ethylene-binding domain has no effect on ethylene binding to the receptor, many of these residues have been shown to

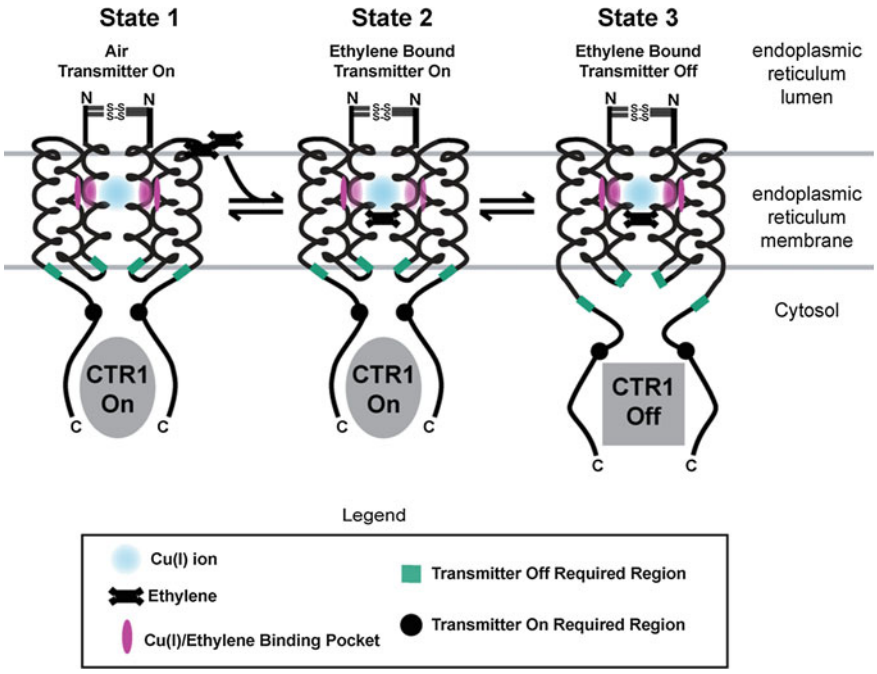

Fig. 3.2 A three-state model for receptor signaling. The ethylene-binding domain of ETR1 is depicted. In this model, the receptor forms a homodimer that is stabilized at its N-terminus with two disulfide bonds. Each monomer is contains three transmembrane helices. Mutational studies have defines regions in these helices that affect ethylene binding and receptor function. One region is important for the binding of copper ions and ethylene. Another area is required for turning off the receptor, and a third area maintains the receptor in a signaling state. In his model, in air *State 1* is favored. Upon binding of ethylene, the receptor enters an intermediate conformation where ethylene is bound, yet the receptor is still signaling (*State 2*). With ethylene bound, the receptor is in equilibrium with *State 3* where receptor signaling is turned off. This leads to less *CTR1* activity and causes a release of inhibition of downstream signaling. Adapted from Wang et al. (2006)

effect signaling none the less (Fig. 3.2). In addition to the seven residues required for ethylene binding, 13 other residues have been identified that, when mutated, lead to a receptor that is capable of binding ethylene but incapable of responding to it. These residues are thought to play a role in propagating conformational changes that lead to alterations in signaling when ethylene is bound to the receptor. Interestingly, two other residues have been identified that are required for maintaining receptor function. When mutated, ethylene binding is unaffected, but the receptors are locked in the "off" state leading to constitutive ethylene responses in the absence of ethylene (Fig. 3.2) (Wang et al. 2006).

These mutational studies on ETR1 have led to a three-state model for receptor function (Fig. 3.2). In this model, in air the receptor is predominantly in the first state and transmitting to CTR1. CTR1 represses ethylene responses. Upon ethylene binding, the receptor enters state two in which ethylene is bound but the receptor is

still keeping CTR1 active. The seven residues that facilitate ethylene binding are required for this receptor signaling state. With ethylene bound, the receptor is in equilibrium with the third signaling state where the receptor is no longer transmitting signal to CTR1. In this signaling state, a conformational change in the receptor is thought to occur that diminishes CTR1 activity and releases downstream signaling from the inhibitory effects of CTR1. Transition of the receptor from state two to state three is mediated by the 13 previously mentioned residues that are required for turning off the receptor upon ethylene binding. The existence of this transition state of the receptor in the presence ethylene is supported experimentally by physiological studies examining the response of plants to saturating ethylene concentrations compared to receptor null plants (Hua and Meyerowitz 1998; Hall and Bleecker 2003; Wang et al. 2003). Receptor null plants show a stronger ethylene response than plants saturated with ethylene. Because signaling states two and three are theoretically in equilibrium, when plants are saturated with ethylene, a portion of the receptors would remain in a signaling on state with ethylene present. Whereas, in receptor null plants, downstream ethylene signaling components would be fully activated, as no receptors would exist to suppress them.

3.3 Output Domains

While the N-terminal domain of the ethylene receptors is primarily involved in signal input, the C-terminal domain is predominately responsible for signal output. As mentioned above, the receptors contain a GAF domain, a histidine kinase-like domain and, in some of the receptors, a receiver domain. In this section, we will discuss the biochemical function and role of each of these domains in signal output.

3.3.1 GAF Domain

All of the ethylene receptors contain a GAF domain. GAF domains were first described as non-catalytic cGMP-binding sites found in the phosphodiesterase of vertebrate rod photoreceptors (Charbonneau et al. 1990) and have since been found to bind a diversity of small molecules (Kanacher et al. 2002; Sardiwal et al. 2005; Tucker et al. 2006; Cann 2007; Levdikov et al. 2009; Ulijasz et al. 2009). However, in most cases the ligand remains unknown, and in some cases there is no evidence of a ligand binding. In the latter case, the GAF domain is thought to serve a structural role in the protein (Levdikov et al. 2009). The GAF domain in the *Synechocystis* ethylene-binding protein, SynETR1, has been shown in vitro to bind the chromophore phycocyanobilin and to be capable of blue green photoconversion (Ulijasz et al. 2009). However, the GAF domain of ETR1 from *Arabidopsis* is unlikely to bind a ligand and is missing the cysteine found in the GAF domain of the *Arabidopsis phytochromes* that is required for chromophore binding (Aravind

and Ponting 1997). Instead, the GAF domain in plant ethylene receptors has been suggested to mediate higher order heteromeric interactions between the receptor homodimers (Gao et al. 2008; Grefen et al. 2008). These higher order heteromeric interactions are thought to amplify receptor signaling, thus allowing *Arabidopsis* to respond to ethylene concentrations 300 times less than the K_d of ethylene binding to the receptors (Binder et al. 2004a; Chen et al. 2010). In addition, in *Arabidopsis* ETR1, the GAF domain appears to be capable of signaling independent of the kinase and receiver domains (Xie et al. 2006; Qiu et al. 2012). This so called "N-terminal signaling" is independent of the protein kinase CTR1, but is promoted by RTE1 (REVERSION TO SENSITIVITY1) (Qiu et al. 2012). RTE1 is a protein that interacts with ETR1 and will be discussed more in Chap. 4.

3.3.2 Kinase Domain

In addition to a GAF domain, the ethylene receptors all contain a histidine protein kinase-like domain. In bacteria, histidine protein kinase domains are known to have autokinase activity, phosphotransfer activity, and in some cases, additional phosphatase activity (Stewart 2010). This domain can be split into two subdomains: a dimerization and histidine phosphotransfer domain (DHp) that is characterized by an H box, and a catalytic and ATP-binding domain (CA) that is characterized by a N, G1 (D), F, and G2 (G) box (Wolanin et al. 2002; Stewart 2010). As the name suggests, the DHp subdomain is required for histidine protein kinase homodimer formation. Homodimerization is necessary for autophosphorylation which is thought to occur in *trans* to the H box histidine (Parkinson and Kofoid 1992; Wolanin et al. 2002). This phospho-accepting histidine serves as an intermediate for transfer of the phosphoryl group to an aspartate in the response regulator's receiver domain (Parkinson and Kofoid 1992). Mutation of the phospho-accepting histidine has been shown to eliminate both autophosphorylation and phosphatase activity, while mutation of other residues in the H box can individually affect either autophosphorylation or phosphatase activity (Parkinson and Kofoid 1992). Within the CA domain, the N, G1, F, and G2 boxes are involved in ATP binding (Wolanin et al. 2002). Residues in the N box coordinate the divalent metal cofactor required for ATP binding, while the F box makes up part of the ATP lid that is flanked by the G1 and G2 boxes with the G1 box forming a flexible hinge at the end of the ATP lid (Parkinson and Kofoid 1992; Wolanin et al. 2002). Mutation of the N, G1, or G2 boxes has been shown to eliminate autokinase activity; however, it is not uncommon for a histidine protein kinase CA domain to lack one of these boxes (Parkinson and Kofoid 1992; Wolanin et al. 2002).

In plants, the ethylene receptors have been subdivided into two families based on phylogenetic analysis and structural features (Wang et al. 2006). Subfamily 1 members contain all of the conserved motifs of the histidine protein kinase domain required for histidine kinase activity, while subfamily 2 members lack one or more of these features (Moussatche and Klee 2004; Wang et al. 2006). Consistent with

their domain features, in vitro analysis of the *Arabidopsis* subfamily 1 ethylene receptors, ERS1 and ETR1, suggests they have histidine kinase activity while analysis of subfamily 2 members, ETR2, ERS2, and EIN4, suggests they lost their histidine kinase activity and together with ERS1 acquired serine/threonine kinase activity (Gamble et al. 1998; Moussatche and Klee 2004). In *Arabidopsis*, histidine kinase activity appears to be restricted to subfamily 1 members. However, this is not the case in tobacco (*Nicotiana tabacum*). Of the four ethylene receptors identified in tobacco, NtETR1, a subfamily 1 member, and NTHK1 and NTHK2, both subfamily 2 receptors, were examined for kinase activity. As with *Arabidopsis*, NtETR1 was shown to have histidine kinase activity and both of the subfamily 2 proteins were shown to have serine/threonine kinase activity in vitro in the presence of Mn^{2+} (Xie et al. 2003; Zhang et al. 2004; Chen et al. 2009). However, the subfamily 2 member NTHK2 was shown to have both serine/threonine and histidine kinase activity (Zhang et al. 2004). Whereas ETR1, ERS1, and NtETR1 have histidine kinase activity in the presence of Mn^{2+}, NTHK2 has histidine kinase activity in the presence of Ca^{2+}. It is not known whether any of the *Arabidopsis* subfamily 2 members have histidine kinase activity when Ca^{2+} is supplied as the metal cofactor, however, this seems unlikely given their divergent histidine protein kinase domain (Gamble et al. 1998; Moussatche and Klee 2004; Zhang et al. 2004; Chen et al. 2009). Although all of the ethylene receptors examined to date show serine/threonine and/or histidine kinase activity in vitro, direct biochemical evidence for kinase activity in vivo has not been shown for any of the ethylene receptors. In fact, whether or not ETR1 and ERS1 have histidine kinase activity in vivo has been called into question based on the finding that under physiologically relevant cellular ratios of Mg^{2+} to Mn^{2+}, where Mg^{2+} concentrations are 50- to 100-fold higher than that of Mn^{2+}, ERS1 only shows autophosphorylation on serine residues and ETR1 shows no autophosphorylation (Moussatche and Klee 2004).

In bacterial two-component histidine kinases, binding of ligand to the N-terminal domain modulates activity of the kinase domain. Binding of ethylene to the ethylene receptors also appears to modulate activity of their kinase domain. As discussed above, ETR1 is capable of autophosphorylation in vitro in the presence of Mn^{2+} (Gamble et al. 1998; Moussatche and Klee 2004). Ethylene and the structurally similar compound cyanide (interestingly a byproduct of ethylene biosynthesis) have both been shown to reduce autophosphorylation of ETR1 in vitro (Voet-van-Vormizeele and Groth 2008). Reduction of ETR1 autophosphorylation by cyanide requires both the ethylene-binding domain and the metal cofactor copper (Voet-van-Vormizeele and Groth 2008; Bisson and Groth 2012). Interestingly, this reduction in autophosphorylation is blocked in the presence of silver ions (Bisson and Groth 2012) correlating with the inhibitory effects of silver ions on ethylene perception. Additionally, the ethylene antagonist 1-methylcyclopropene blocks the cyanide-induced reduction in ETR1 autophosphorylation (Voet-van-Vormizeele and Groth 2008). Interestingly, an in vivo study on two tomato ethylene receptors, LeETR4 and NEVER-RIPE (NR), shows that both receptors are highly and multiply phosphorylated in the absence of ethylene and have reduced phosphorylation in the presence of ethylene (Kamiyoshihara et al. 2012). However, it

remains to be determined whether this represents autophosphorylation or phosphorylation by another protein kinase.

Even though the in vitro studies mentioned above suggest that the ethylene receptors have kinase activity that is modulated by ligand binding and genetic complementation studies with truncated ETR1 show the importance of the kinase domain in ethylene signal output, complementation studies with kinase deficient versions of ETR1 suggest that kinase activity is not required for signaling (Gamble et al. 2002; Wang et al. 2003; Binder et al. 2004b; Qu and Schaller 2004; Xie et al. 2006; Hall et al. 2012). Instead ETR1 kinase activity appears to modulate its responsiveness and sensitivity to ethylene as well as recovery from ethylene after its removal (Binder et al. 2004b; Qu and Schaller 2004; Hall et al. 2012). The targets for ethylene receptor histidine kinase activity remain to be determined. Likely targets include histidine phosphotransfer and response regulatory proteins that have been shown to physically interact with the ethylene receptors in *Arabidopsis*, and perhaps, modulate ethylene responses (Urao et al. 2000; Hass et al. 2004; Scharein et al. 2008; Scharein and Groth 2011). Similarly, it is possible that the receptors phosphorylate CTR1 or EIN2 since they are co-localized to the ER membrane with the receptors and physically interact with the receptors (Ju and Chang 2012). This will be discussed more in Chap. 4.

Whether or not receptor serine/threonine activity is required for signaling or modulates the plant's responsiveness and/or sensitivity to ethylene has not been extensively tested. However, when overexpressed in *Arabidopsis*, the subfamily 2 ethylene receptor NTHK1 caused increased sensitivity of etiolated seedlings to the ethylene precursor 1-aminocyclopropate-1-carboxylic acid, while the kinase deficient version of NTHK1 maintained wild-type sensitivity to 1-aminocyclopropate-1-carboxylic acid (Chen et al. 2009). This suggests that serine/threonine kinase activity may be required for wild-type sensitivity to ethylene.

3.3.3 Receiver Domain

Some of the ethylene receptors are hybrid kinases and contain a C-terminal receiver domain. In dicots, receiver domains have been found in both subfamily 1 and subfamily 2 members, but in all monocots studied to date, receiver domains have only been identified in subfamily 2 members (Binder et al. 2012).

Receiver domains contain six conserved residues: three aspartic acid residues (two of which can also be a glutamic acid residue), a lysine, a serine/threonine, and a phenylalanine/tyrosine residue (Bourret 2010). The three aspartic acid residues form an acidic pocket and, along with the lysine, coordinate a metal cofactor in the active site. One of these aspartic acid residues also serves as the site of phosphorylation (Bourret 2010). The serine/threonine and phenylalanine/tyrosine residues are involved in conformational changes of the receiver domain and signal output (Bourret 2010). The *Arabidopsis* ETR1 receiver domain was crystalized and despite showing low sequence similarity to the well-studied *Escherichia coli* CheY

receiver domain, they showed high structural conservation (Müller-Dieckmann et al. 1999). The most interesting difference between the ETR1 and CheY receiver domains is the orientation of their γ loops. The γ loop is thought to be involved in molecular recognition (Müller-Dieckmann et al. 1999). In CheY, the backbone carbonyl of an asparagine residue in the γ loop participates in cation ligation. However, the backbone carbonyl of the corresponding cysteine in ETR1 is facing away from the acidic pocket and would not be able to participate in cation ligation unless the γ loop underwent a major conformational change (Müller-Dieckmann et al. 1999). The orientation of the γ loop in the other ethylene receptors and the biological implications of their orientation are not currently known.

In prokaryotes, receiver domains are usually attached to an effector domain where they act as a phospho-mediated on/off switch for controlling the output of their effector domain (Bourret 2010). Most of these effector domains regulate transcription and in these cases, phosphorylation of the receiver domain is thought to result in its dimerization which promotes DNA binding and transcriptional activation of the effector domain (Bourret 2010; Gao and Stock 2010). The *Arabidopsis* ETR1 receiver domain is not attached to an effector domain but is found as a dimer both in solution and in the crystal form (Müller-Dieckmann et al. 1999). Based on comparison to bacterial CheY and CheB receiver domains, phosphorylation of the ETR1 receiver domain is predicted to result in monomerization of the domain (Müller-Dieckmann et al. 1999).

In prokaryotes, some receiver domains are part of the histidine kinase protein. These are known as hybrid kinases that often participate in multistep phosphorelays where the phospho group is passed from the phospho-accepting histidine in the kinase domain to the aspartic acid of the receiver domain. This is followed by transfer of the phospho group to the phospho-accepting histidine of a histidine phosphotransfer protein and then to the aspartic acid of another receiver domain containing protein (Bourret 2010). Even though some of the ethylene receptors are hybrid kinases containing both a sensor histidine kinase and a receiver domain, phosphotransfer through the receiver domain is not required for most responses to ethylene. For instance, *etr1 etr2 ein4* triple loss-of-function mutants are lacking the three receptor isoforms with a receiver domain, yet they still respond to ethylene (Hua and Meyerowitz 1998). Complementation studies with a truncated ETR1 lacking the receiver domain support this and indicate that the receiver domain, like histidine kinase activity, is involved in the control of sensitivity and responsiveness to ethylene as well as recovery from ethylene after its removal (Binder et al. 2004b; Qu and Schaller 2004). These subtle roles of the receiver domain appear to be via a phosphotransfer mechanism. However, the receiver domain also appears to have histidine kinase-independent roles. For instance, ethylene stimulates nutational bending that requires the ETR1 receiver domain, but not histidine kinase activity (Binder et al. 2006). Domain-swapping experiments show that the receiver domain from EIN4 cannot substitute for the ETR1 receiver domain (Kim et al. 2011). Thus, the receiver domain seems to have multiple functions.

3.4 Concluding Remarks

The binding of ethylene to ethylene receptors is known to occur in the transmembrane domains of the receptors. This binding event requires a copper cofactor. However, the mechanism for signal output through the ethylene receptors is still not understood and appears to have diverged from the simple histidine to aspartate phosphorelay found in bacterial systems. Even though ethylene modulates histidine kinase activity of ETR1, numerous studies indicate that histidine kinase activity and the receiver domain are not required for signal transduction. Rather, kinase activity and the receiver domain affect sensitivity and responsiveness to ethylene as well as recovery after removal of ethylene. Models are developing that posit that ethylene is modulating receptor–protein interactions leading to ethylene responses. The interaction of the ethylene receptors with downstream signaling components and the implications thereof will be discussed in Chap. 4.

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Chapter 4
The Role of Protein–Protein Interactions in Signaling by the Ethylene Receptors

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Abstract Protein–protein interactions of the ethylene receptors are involved in propagation and modulation of the ethylene signal. Interactions of the receptors with critical pathway components such as CTR1 and EIN2 are likely to involve multiple members of the receptor family. Additional interactions, such as that involving the receptor ETR1 and regulatory protein RTE1, may allow for isoform-specific signal output. Ethylene receptors also form higher order complexes with each other, suggesting a cooperative mechanism for amplification of the ethylene signal. A model incorporating the role of physical interactions in signal transmission by the receptors is described.

Keywords Ethylene receptors · Histidine kinase · Cooperativity · Endoplasmic reticulum · ETR1 · CTR1

4.1 Introduction

Ethylene is perceived in plants by receptor families. In *Arabidopsis thaliana*, where the receptors have been studied in most detail, there are five members to the receptor family: ETR1 (ETHYLENE RESPONSE1), ETR2, ERS1 (ETHYLENE RESPONSE SENSOR1), ERS2, and EIN4 (ETHYLENE INSENSITIVE4) (Fig. 4.1). The ethylene receptors of dicots and monocots fall into two subfamilies based upon structure and amino acid sequence, all containing an ethylene-binding domain, a GAF (cGMP-specific phosphodiesterases, adenyl cyclases, FhlA)

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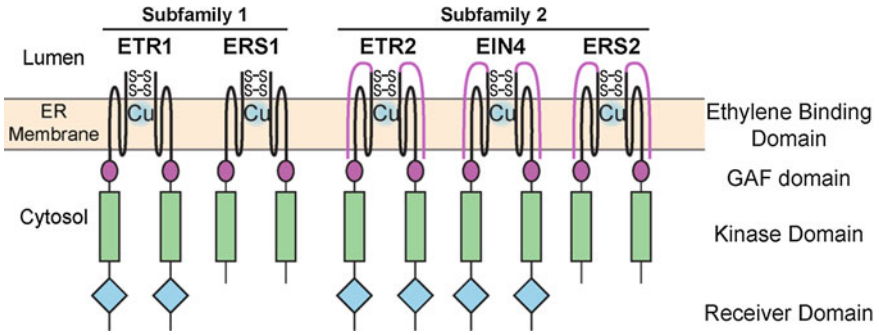

Fig. 4.1 Domain structures of the ethylene receptors from *Arabidopsis thaliana*. The receptors are disulfide-linked homodimers localized to the endoplasmic reticulum. Each receptor contains an ethylene-binding, a GAF, and a histidine kinase-like domain. Three of the five receptors also contain a receiver domain. The ethylene-binding domain includes a copper cofactor (Cu). The receptors are present as two subfamilies based on phylogenetic analysis and structural features. The subfamily-2 receptors contain additional amino acids at the N-terminus that may function as a fourth transmembrane helix or a cleavable signal peptide (*highlighted in purple*)

domain, and a histidine kinase-like domain. Three of the five receptors in *Arabidopsis*, also contain a receiver domain (Fig. 4.1). Biochemical characteristics of the receptors are detailed in Chap. 3. In this chapter, we focus on the roles that protein–protein interactions play in signaling by the receptors. To this end, we discuss (1) the interactions between the receptors and the primary downstream signaling components, CTR1 (CONSTITUTIVE TRIPLE RESPONSE1) and EIN2; (2) the specific interaction between ETR1 and RTE1 (REVERSION-TO-ETHYLENE-SENSITIVITY1); (3) the role that cooperative receptor–receptor interactions may play in amplifying the ethylene signal; and (4) additional interactions, the significance of which is only beginning to be understood.

4.2 Interactions with Downstream Components of the Primary Signal Transduction Pathway

The ethylene receptors are predominantly localized to membranes of the endoplasmic reticulum (ER), their topology being such that the ethylene-binding domain is present within the ER membrane itself and the signal output domain is cytosolic. Not surprisingly, other proteins involved in the primary response to ethylene such as CTR1 and EIN2 are also localized to the ER (Fig. 4.2) (Chen et al. 2002; Gao et al. 2003; Grefen et al. 2008; Bisson et al. 2009; Bisson and Groth 2010).

CTR1 is a Ser/Thr protein kinase that acts just downstream of the receptors to inhibit downstream signaling (Kieber et al. 1993). Several lines of evidence indicate that CTR1 interacts with ethylene receptors. First, although CTR1 contains no transmembrane domains itself, it is found associated with membranes of the ER.

Fig. 4.2 Primary ethylene signal transduction pathway as defined by genetic interactions. Initial signaling elements in the pathway are shown. Those gene products associated with the ER as either integral or peripheral membrane proteins are highlighted in *gray*

The ER association of CTR1 is dependent on the ethylene receptors, mutations of both subfamily-1 and subfamily-2 receptors reducing the levels of membrane-associated CTR1 (Gao et al. 2003). Second, a physical association of CTR1 with the ethylene receptor ETR1 is supported by two-hybrid analysis, in vitro-binding experiments, and co-purification analysis from *Arabidopsis* extracts (Clark et al. 1998; Gao et al. 2003; Huang et al. 2003). Studies on the interaction of CTR1 with other members of the receptor family are more limited than with ETR1, although interactions are observed with ERS1 and ETR2 based on yeast two-hybrid analysis (Clark et al. 1998; Cancel and Larsen 2002). The two-hybrid studies demonstrate that both the kinase and receiver domains of the receptors can interact with CTR1 (Clark et al. 1998). Interaction of CTR1 with the kinase domain could potentially allow CTR1 to interact with all five members of the receptor family; the functional significance of interaction with the receiver domain is unknown.

Studies suggest that differences exist among the receptors and their ability to interact with and regulate CTR1. In yeast two-hybrid studies, the strongest CTR1 interaction is observed with ETR1, the interactions with ERS1 and ETR2 being progressively weaker (Clark et al. 1998; Cancel and Larsen 2002). A greater affinity of CTR1 for subfamily-1 receptors compared to subfamily-2 receptors may explain the predominant role of ETR1 and ERS1 in the regulation of ethylene signal transduction in *Arabidopsis* (Wang et al. 2003; Qu et al. 2007). Studies on the receptor-dependent association of CTR1 with membranes also indicate that the amount of CTR1 associated with the ER does not always correlate with signaling from the receptor/CTR1 complex (Gao et al. 2003). For instance, loss of ETR1 results in an increase in membrane-associated CTR1, which is opposite to predictions based upon the receptor dependence for association of CTR1 with the ER (Gao et al. 2003; Qu et al. 2007). If the level of membrane-associated CTR1 is directly proportional to the signaling output from the receptors, then this increase in CTR1 levels is predicted to result in stronger suppression of the ethylene response. However, *etr1* null plants actually exhibit an increased sensitivity to ethylene (Cancel and Larsen 2002; Qu et al. 2007). A similar conflict with this model is found in the observation that kinase-inactive ETR1 recruits less CTR1 to the ER than wild-type ETR1 (Hall et al. 2012). Here, plants with lower levels of CTR1 suppress ethylene responses to a greater extent than plants with higher levels (Hall et al. 2012). Taken together, these results indicate that CTR1 associates with receptors in a non-stoichiometric fashion and that, furthermore, there exist isoform-specific differences in the ability of the receptors to regulate CTR1.

EIN2 acts downstream of CTR1 in the ethylene signal transduction pathway and, like the ethylene receptors and CTR1, is localized to the ER membrane (Bisson et al. 2009). EIN2 is an integral membrane protein, its N-terminal portion containing the transmembrane segments and being related to the Nramp family of metal transporters. The C-terminal portion of EIN2 contains a large soluble domain that is cleaved in response to ethylene, after which it translocates to the nucleus to control the transcriptional response to ethylene (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012). The five *Arabidopsis* receptors can interact directly with the soluble domain of EIN2 (Bisson et al. 2009; Bisson and Groth 2010). The interaction with EIN2 occurs through the kinase domains of the receptors, and studies with ETR1 indicate that autophosphorylation modulates this interaction, a non-phosphorylatable form of ETR1 exhibiting higher affinity for EIN2 (Bisson et al. 2009). As noted in Chap. 3, ETR1 kinase activity modulates but is not required for the well-characterized ethylene responses, making the significance of this receptor–EIN2 interaction unclear. However, it is possible that this represents a further mechanism to modulate ethylene responses. Taken together, the interactions of the ethylene receptors with CTR1 and EIN2 point the existence of a signaling complex at the ER membrane. These interactions, involving the first three elements in the ethylene signaling pathway, could facilitate the regulatory mechanism by which ethylene perception controls proteolytic cleavage of EIN2 in a CTR1-dependent manner.

4.3 Interactions Between ETR1 and RTE1

RTE1 encodes a transmembrane protein that physically associates with ETR1 (Dong et al. 2008, 2010). *RTE1* was first identified as a gene required for the ethylene insensitivity conferred by the dominant *etr1-2* mutation (Resnick et al. 2006). Genes similar to *RTE1* are found in other plants. For instance, the *GREEN-RIPE* (*GR*) gene of tomato appears to have a similar function to *RTE1* (Barry and Giovannoni 2006). Additional *RTE1*/*GR*-like genes are found in tomato and *Arabidopsis* and there may be subfunctionalization within this gene family (Ma et al. 2012).

RTE1 appears to predominantly regulate ETR1 function, having little if any effect on other members of the *Arabidopsis* receptor family. For example, overexpression of *RTE1* results in a reduction in ethylene sensitivity that is largely dependent on ETR1 (Resnick et al. 2006; Zhou et al. 2007). Additionally, *rte1* null mutants phenocopy *etr1* null mutants, resulting in enhanced ethylene sensitivity (Resnick et al. 2006; Zhou et al. 2007). Interestingly, missense mutations like *etr1-2* that confer RTE1-dependent ethylene insensitivity are specific to ETR1. When introduced into the other receptor isoforms, the plants are not ethylene insensitive (Resnick et al. 2006; Rivarola et al. 2009). By contrast, missense mutations in ETR1, such as *etr1-1*, that confer ethylene insensitivity independently of RTE1 are effective at causing ethylene insensitivity when introduced into other receptor isoforms (Rivarola et al. 2009).

Genetic analysis indicated a role of RTE1 in regulating ETR1 activity, the presence of RTE1 facilitating the ability of ETR1 to suppress ethylene responses (i.e., facilitating the role that ETR1 plays in the absence on ethylene). RTE1 physically associates with ETR1 based on in vivo and in vitro assays, truncation analysis indicating that RTE1 interacts with the N-terminal half of ETR1 that contains the transmembrane and GAF domains (Dong et al. 2010). A missense mutation of RTE1 that results in a loss-of-function phenotype decreased the affinity of RTE1 for ETR1, indicating that the RTE1–ETR1 interaction is necessary for function. The interaction of RTE1 with ETR1 may serve to stabilize ETR1 in a state-1 or state-2 conformation (see Chap. 4), whereby the receptor activates CTR1 to suppress the downstream ethylene response. Interestingly, expression of *RTE1* is induced by ethylene, suggesting that RTE1 may be a negative feedback regulator for ETR1, serving to desensitize ETR1 to ethylene. A specific regulator of ETR1 function may have arisen in part due to the predominant role that ETR1 plays in the *Arabidopsis* ethylene response.

Recent research suggests that ETR1 and RTE1 may mediate ethylene signaling in part via a CTR1-independent pathway (Qiu et al. 2012). These researchers found that expression of the N-terminal half of ETR1 containing the ethylene-binding and GAF domains could partially reverse the constitutive triple-response phenotype of *ctr1*. This reversal was RTE1 dependent. This supports the existence of additional ethylene signaling pathways that function independently of the canonical CTR1-dependent pathway, as has been suggested by several independent studies (Kieber et al. 1993; Roman et al. 1995; Larsen and Chang 2001; Hall and Bleecker 2003; Binder et al. 2006). This result also indicates that signaling can occur from the N-terminal half of ETR1 to this pathway. It is unclear whether or not ETR1 is signaling via RTE1 or is simply dependent on RTE1 to maintain the proper conformation for this signaling. It is possible that RTE1 is regulating events occurring within the ER, which raises the possibility that ETR1 has signal outputs to both the ER lumen and cytosol. Support for this hypothesis is a recent study where RTE1 was found to associate with the ER-localized cytochrome b5 to modulate ETR1 function (Chang et al. 2014). This suggests that the ethylene receptors may function in, or be affected by, redox reactions. For instance, ETR1 may mediate H_2O_2 signaling independent of its role as an ethylene receptor (Desikan et al. 2005, 2006). Thus, ETR1 may function in perception and transduction of two signals, ethylene and reactive oxygen species.

4.4 Higher Order Receptor Complexes and Cooperative Signaling

Most models for ethylene signal transduction suggest a fairly simple linear pathway. However, these models do not explain the ability of plants to respond to ethylene across a concentration range that spans approximately six orders of magnitude (Chen and Bleecker 1995; Binder et al. 2004a). It is likely that multiple mechanisms facilitate this wide range of ethylene responsiveness. One possibility,

as described above, is the presence of negative feedback regulators such as RTE1. Another possibility, inspired by our understanding of how similar systems function in bacteria, is cooperative signaling mediated by receptor–receptor interactions.

The ethylene receptors form homodimers that are stabilized by two disulfide bonds (Schaller and Bleecker 1995; Hall et al. 2000; Gao et al. 2008; Chen et al. 2010). These dimers represent the simplest functional unit of the receptors, in which one ethylene molecule binds per receptor dimer (Rodriguez et al. 1999). However, the receptor dimers can also form higher order complexes with each other via non-covalent interactions that are possibly mediated by the GAF domain (Gao et al. 2008; Grefen et al. 2008). It has been suggested that CTR1 may also facilitate or participate in this clustering (Mayerhofer et al. 2012). If the ethylene receptors exist as clusters, then it is likely that cooperative signaling occurs. Such models of cooperative signaling have been invoked for the evolutionarily related histidine kinase-linked chemoreceptors of bacteria to explain the high sensitivity and wide-dynamic range of the receptors (Bray et al. 1998).

In this model for cooperative signaling, clustering allows for conformational changes that occur in one receptor that binds ethylene to be transmitted to other receptors in the cluster that lack ethylene, thereby amplifying the ethylene signal (Maddock and Shapiro 1993; Gestwicki and Kiessling 2002; Francis et al. 2004; Wolanin and Stock 2004). Cooperative signaling between the ethylene receptors may explain the observation that *Arabidopsis* plants respond to ethylene at levels approximately 300-fold below the K_d of the receptors for ethylene (Schaller and Bleecker 1995; Binder et al. 2004a; McDaniel and Binder 2012). Cooperative signaling may also help explain the dominant ethylene insensitivity conferred by mutant receptors such as *etr1-1* (Gao and Schaller 2009). Ethylene-insensitive mutations in the binding sites of the receptors such as *etr1-1* display stronger dominance than predicted for a lesion solely within one receptor isoform (Gamble et al. 2002). Additionally, a truncated etr1-1 protein lacking the kinase and receiver output domains still confers dominant ethylene insensitivity (Gamble et al. 2002; Xie et al. 2006; Gao et al. 2008; Qiu et al. 2012). One explanation for this is that the truncated receptor influences the signaling state of the surrounding, full-length receptors. Finally, the ethylene insensitivity of *etr2-1* is partially dependent on ETR1 (Cancel and Larsen 2002).

Physical clustering of the receptors may also allow for trans-phosphorylation between the receptor isoforms. For instance, the histidine kinase of ETR1 could phosphorylate the receiver domains of ETR1 and EIN4. Support for this hypothesis comes from studies on seedling growth recovery following treatment then removal of ethylene, this growth recovery response being dependent upon ETR1 histidine kinase activity. Interestingly, the growth recovery response is substantially slower in the *etr1 etr2 ein4* triple loss-of-function mutant, which lacks the three receptor isoforms with receiver domains, than in the *etr1* single mutant (Binder et al. 2004b). Furthermore, the slow growth recovery of the triple mutant can be rescued by any of the three receptor isoforms containing a receiver domain as well as by a chimeric ETR1 receptor containing the EIN4 receiver domain, but is not rescued by ERS1, ERS2, or a truncated ETR1 lacking the receiver domain (Binder et al. 2004b; Kim et al. 2011).

4.5 Additional Interactions of the Ethylene Receptors

Current data support the existence of ethylene–receptor signaling complexes in which the receptors interact with integral and peripheral proteins of the endoplasmic reticulum (Ju et al. 2012). Gel filtration analysis of ethylene receptors solubilized from *Arabidopsis* supports the existence of such complexes and also suggests that the complexes may contain isoform-specific components (Chen et al. 2010). All five receptors from *Arabidopsis* were identified as components in large protein complexes but the size of these complexes varied depending on which receptor was examined. Interestingly, the complex size associated with ERS1 was affected by ethylene binding, while the complex size associated with ETR1 was not, suggesting that ethylene may regulate composition of the complexes in an isoform-specific manner (Chen et al. 2010).

Analysis of the receptor complexes suggested that additional components besides CTR1, EIN2, RTE1, or additional members of the receptor family participate in these signaling complexes (Chen et al. 2010). Additional proteins have been identified that form physical interactions with the ethylene receptors, however, little is known about the functional implications of these interactions. In *Arabidopsis*, the ethylene receptors interact with phosphotransfer proteins and the affinity of ETR1 for at least one of these is phosphorylation dependent (Urao et al. 2000; Scharein et al. 2008; Scharein and Groth 2011). One possibility is that these phosphotransfer proteins represent downstream targets for the receptor histidine kinase that functions to modulate ethylene responses. This possibility is supported by the observation that the *Arabidopsis* Response Regulatory Protein2 (ARR2) modulates ethylene responses (Hass et al. 2004; Mason and Schaller 2005). A phosphorylation-dependent two-component signaling pathway, along with the potential RTE1-dependent pathway described earlier, implies that ETR1 may signal to several pathways outside of the canonical CTR1-dependent signaling pathway. It also suggests that different domains of ETR1 may signal to different downstream components.

There is also evidence that the receptors of *Arabidopsis* and tomato interact with tetratricopeptide repeat (TPR) proteins (Lin et al. 2008, 2009). These proteins are poorly characterized in plants but are related to proteins in humans that interact with heterotrimeric G-proteins and the small GTPase Ras. It remains to be determined if this interaction could represent another signaling pathway and/or modulate the receptor–CTR1 interaction.

4.6 A Model for Signal Output from the Receptors

In Fig. 4.3, we present a model that emphasizes the significance of physical interactions in mediating signaling from the ethylene receptors. In this model, ethylene is perceived by a family of receptors (ETR1, ETR2, ERS1, ERS2, and

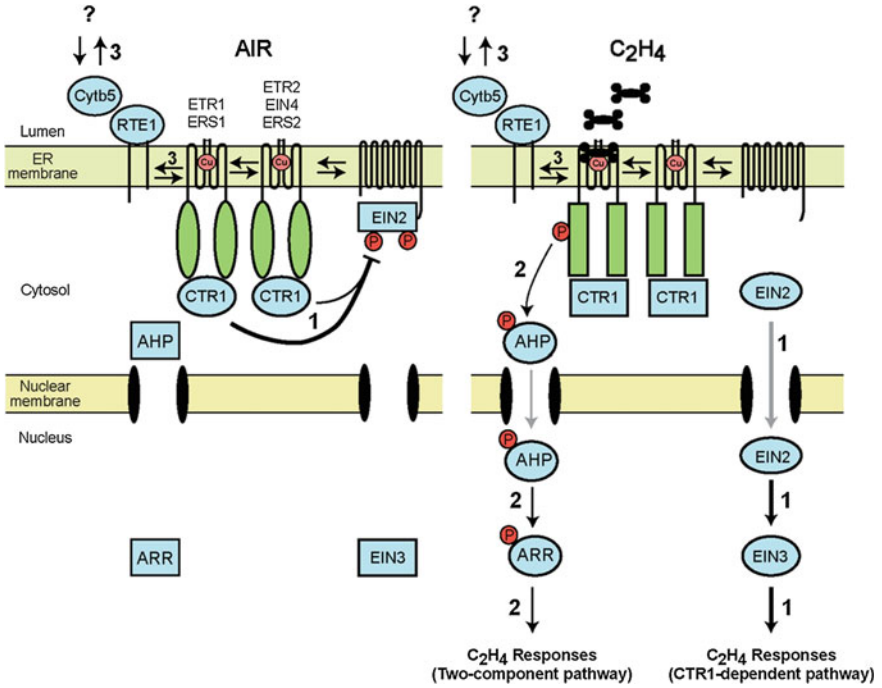

Fig. 4.3 A model for ethylene signal transduction in *Arabidopsis.* Interactions between the receptors and signaling elements of the pathway are indicated. Potential signaling pathways from the receptors are indicated by the numerals 1, 2, and 3. The canonical pathway (*pathway 1*) involves interaction of the receptors with CTR1. In air, the ethylene receptors activate CTR1, which in turn, phosphorylates and inhibits EIN2. In the presence of ethylene, the receptors are inactivated and CTR1 activity is reduced, potentially via conformational changes in the receptors. As a result, EIN2 phosphorylation decreases and is proteolytically cleaved, its C-terminus translocating to the nucleus to initiate the ethylene response. The receptors may also physically associate with EIN2. Several CTR1-independent signaling pathways may also exist. A two-component pathway (*pathway 2*) initiated from the subfamily-1 receptors involves phosphotransfer (AHP) and response regulator (ARR) proteins. RTE1 stabilizes ETR1 through physical interactions and may also mediate signaling to cytochrome b5 in the ER lumen (*pathway 3*). The RTE1-cytochrome b5 pathway could also mediate responses to other signals such as reactive oxygen species. The *thickness of arrows* indicates the relative contributions of these pathways to the ethylene response. Cooperativity in signaling by the receptors may occur due to their physical interaction, such that binding of ethylene to one receptor affects the signaling state of neighboring receptors that do not have ethylene bound. *Ovals* and *squares* indicate different conformations of the proteins, with ovals generally indicating the active form of the protein where known. *Thicker arrows* indicate a greater relative contribution to ethylene signaling. *Gray arrows* indicate translocation to the nucleus of signaling elements

EIN4 in *Arabidopsis*) predominantly localized to the ER membrane. The primary output from the receptors is to CTR1. Here, the receptors function in a largely overlapping manner to physically associate with CTR1 and to regulate CTR1 activity. When ethylene binds, a conformational change in the receptors is

transmitted to CTR1 and CTR1 activity is reduced. Even though all five receptor isoforms are likely involved in regulating CTR1, evidence suggests that the subfamily-1 receptors of *Arabidopsis* play a larger role than the subfamily-2 receptors in this regulation. The reduction in CTR1 activity results in activation of EIN2 via proteolytic cleavage, and stimulation of the ethylene response. The model depicted in Fig. 4.3 also includes several other potential outputs from the receptors. One potential output is based on participation in a two-component signaling pathway dependent on His-Asp phosphorylation, and physical interaction with phosphotransfer proteins. A second potential output is an RTE1-dependent signaling pathway that involves the N-terminal portion of ETR1. The downstream target for this is unknown but could involve a signaling element within the ER lumen such as cytochrome b5. Alternatively, RTE1 may mediating responses from other ligands. Because these additional pathways likely represent outputs that are secondary to the CTR1-mediated pathway, as well as being receptor isoform-specific, thinner arrows are used to indicate their signaling role. This model also depicts cooperative signaling where the binding of ethylene to one receptor may affect the signaling state of neighboring receptors to amplify the signal from a single binding event. Cooperative signaling could regulate signaling to the CTR1-dependent pathway as well as the additional CTR1-independent pathways.

4.7 Concluding Remarks

Ethylene binds to its cognate receptors in plants to mediate the variety of responses associated with this phytohormone. Physical interactions between the receptors and downstream signaling components are vital to signal transduction. The majority of the responses we associate with ethylene are dependent on signaling from the receptors to CTR1 and are likely transmitted via conformational changes in the receptors, transduction to CTR1 facilitated by physical interaction of the receptors and CTR1. The receptors also interact with other proteins. These interactions may serve to modulate signaling through the CTR1-dependent pathway and/or allow for signaling through alternative CTR1-independent pathways. There is increasing evidence for isoform-specific interactions of the receptors with downstream signaling elements. The existence of such interactions supports subfunctionalization of the receptors, explaining in part why ethylene receptors exist as multi-member families in plants. Future studies will undoubtedly reveal additional components of the ethylene–receptor signaling complexes.

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Chapter 5
Regulatory Components of Ethylene Signal Transduction

Chi-Kuang Wen, Wenyang Li and Hongwei Guo

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

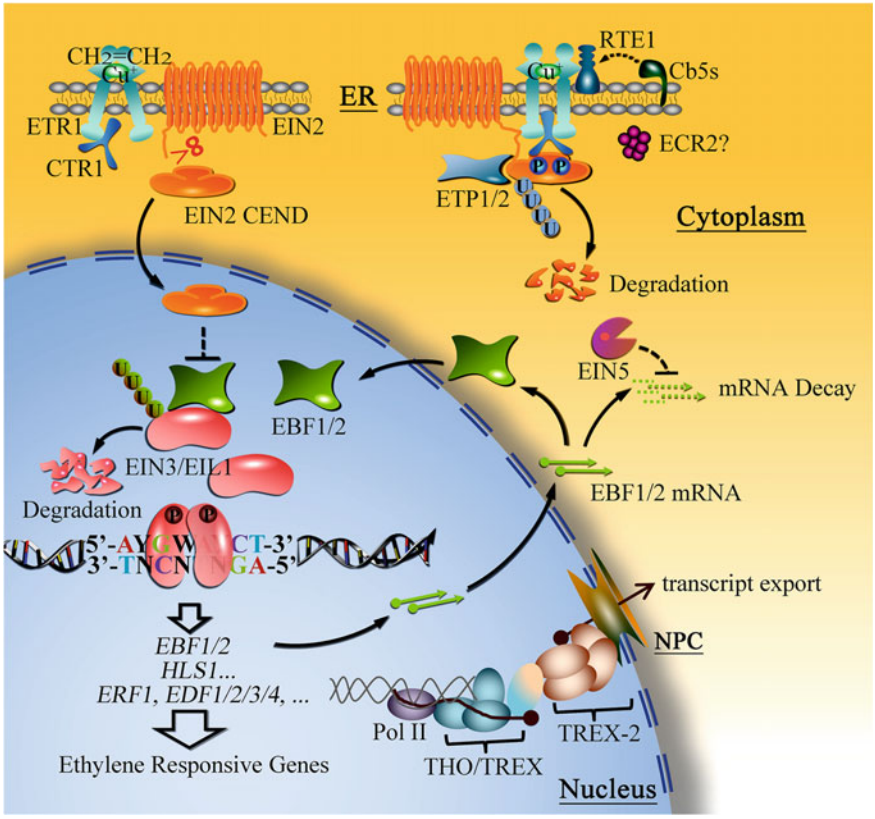

Fig. 5.1 Multilevel regulations of ethylene signaling. Involvement of RTE1 in ETR1 ethylene receptor signaling and ECR2 in CTR1 functions; ECR2 may act downstream of the ethylene receptors and upstream of EIN3/EIL1. ETP1/ETP2 are F-box proteins for EIN2 degradation, and EBF1/EBF2 for EIN3 degradation. 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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Chapter 6
Ethylene Signaling from the Endoplasmic Reticulum Membrane to the Nucleus

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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

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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

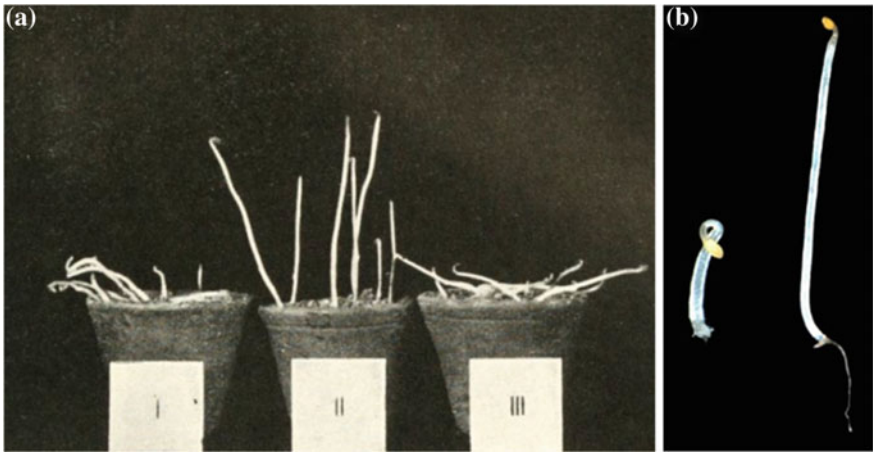

Fig. 6.1 The ethylene-signaling pathway was genetically dissected by exploiting the "triple response" in dark-grown seedlings treated with ethylene. **a** Discovery of the triple response phenotype of dark-grown pea seedlings by the Russian scientist Neljubov (1901). The pea seedlings showed *horizontal 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.

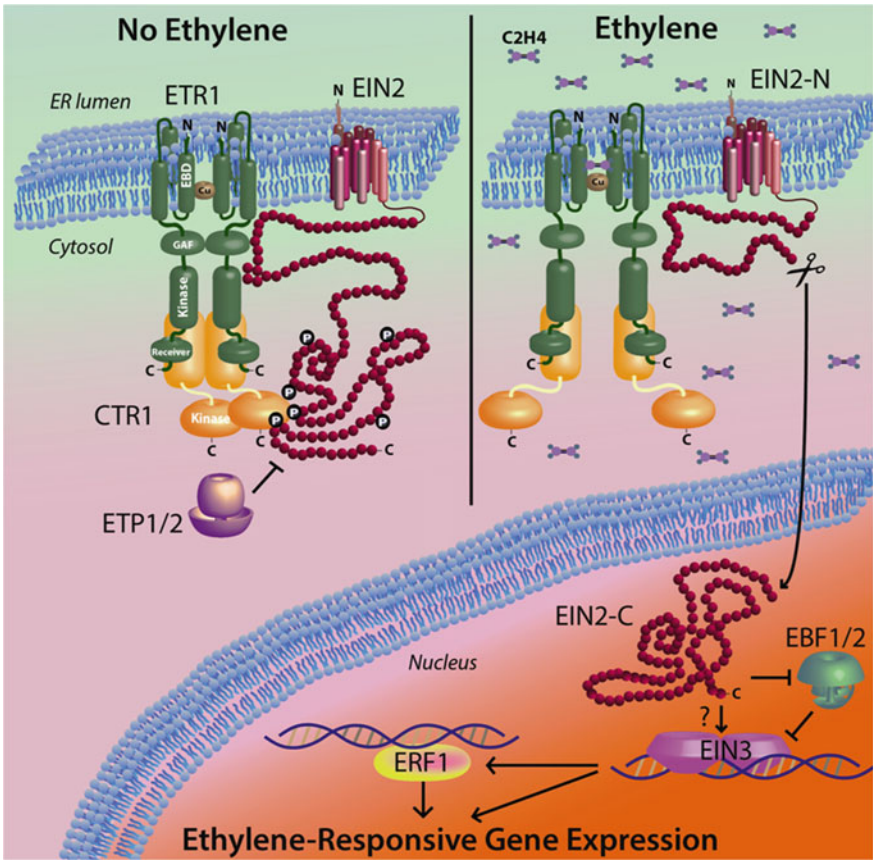

Fig. 6.2 Current model of the ethylene-signaling pathway in *Arabidopsis*. Without ethylene (*left side*), the ethylene receptor dimer (represented by ETR1) at the ER membrane activates the protein kinase CTR1, which is physically associated with the receptor's kinase and receiver domains. The CTR1 dimer 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

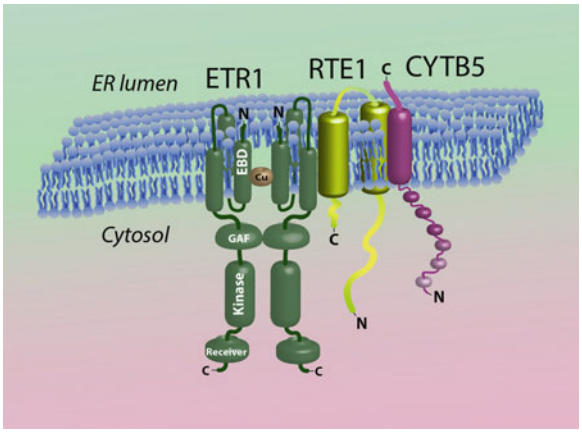

Fig. 6.3 Additional interactions of the ethylene-signaling complex at the ER membrane. Reversion-To-Ethylene Sensitivity1 (RTE1) interacts specifically with the *Arabidopsis* ETR1 ethylene receptor (Dong et al. 2008, 2010), promoting the active signaling form by an unknown mechanism (Resnick et al. 2008; 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

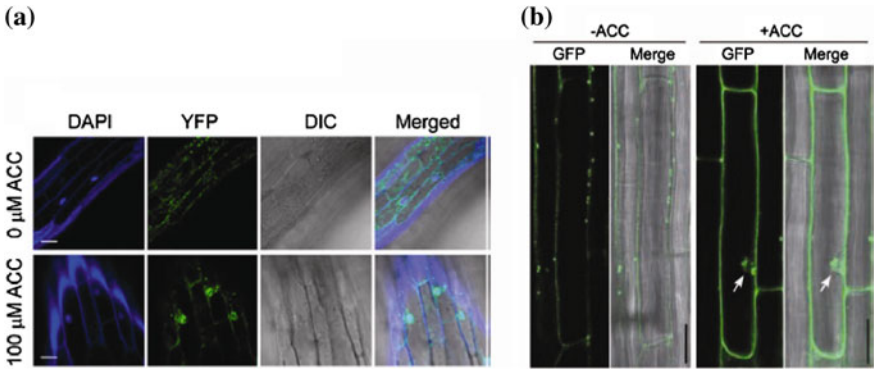

Fig. 6.4 Nuclear localization of the EIN2 C-end upon ethylene treatment **a** Confocal microscopy showing ethylene-responsive nuclear localization of EIN2 WT-YFP in *Arabidopsis* hypocotyl cells of four-day-old dark-grown wild-type seedlings transformed with *35S-EIN2 WT-YFP*. Seedlings were treated 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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Chapter 7
An Evolutionary Perspective on the Plant Hormone Ethylene

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Abstract The plant hormone ethylene plays diverse roles in growth, development, and stress responses, and has been well studied in *Arabidopsis* and other flowering plants, with somewhat sparser information among other land plants. There has been increasing interest in the evolution of this hormone, as studies of mosses, lycopods, ferns, and gymnosperms have made it clear that ethylene functions as a plant hormone across the land plants (embryophytes). Hormones present a particularly interesting problem in evolutionary biology because they require cooperating components for biosynthesis, perception, signaling, and response, and the sequence by which these different parts of the hormone system become operational is not always immediately obvious. In the case of ethylene, biosynthesis appears to have an ancient origin in the Archaeplastida, whereas ethylene signaling was assembled from a combination of prokaryotic, eukaryotic, and plant-specific elements. The gene for ethylene perception appears to have originated in cyanobacteria and entered the plant lineage with the endosymbiotic acquisition of the chloroplast, possibly originally functioning for environmental sensing. Most likely, ethylene as a plant hormone arose during the evolution of the charophyte algae, which ultimately gave rise to land plants. Ethylene's roles as a hormone have undergone considerable modification during the course of plant evolution, and yet elements of the pathways involved are surprisingly well conserved. Study of the genomes and biology of diverse plants and plant relatives is helping to reveal this history. Knowledge of the process by which it has evolved helps clarify the relationships among different aspects of ethylene signaling, suggests mechanisms that could be of

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theoretical and practical importance, and reveals ways in which natural systems have solved problems that may be of agricultural interest.

Keywords Ethylene · Evolution · Biosynthesis · Perception · Response · Algae · Charophytes · Liverworts · Mosses · Ferns · ACC · ACO

7.1 Introduction

Ethylene is a tremendously important plant hormone. It has profound effects on many aspects of plant growth and development, including seed germination, fruit ripening, organ abscission and senescence (Chaps. 8–10 this book; McManus 2012). Ethylene also mediates responses to biotic and abiotic stresses (Chap. 11 this book; McManus 2012), including adaptive responses in aquatic and semi-aquatic environments (Osborne et al. 1996; Yasumura et al. 2012; Voesenek and Sasidharan 2013). An understanding of the evolutionary history of the plant ethylene system has the potential to yield novel insights into how the system works and has worked in the past, what it can be used for, and what its essential components are.

In order to retrace the evolutionary history of the plant ethylene system, two kinds of information are required: (1) knowledge of the evolutionary history of plants and their closest relatives, and their place in the tree of life, and (2) comparative information on the presence and characteristics of ethylene biosynthesis and/or responses across the tree of life. Bringing together this phylogenetic and comparative information allows us to infer when and how each component of the plant ethylene system was acquired and how these components were assembled. Furthermore, we can gain insights into how, subsequent to its acquisition, the plant ethylene system gained an increasingly diverse set of roles in signaling stress responses and developmental processes in plants. Knowledge of the phylogeny of plants and their relatives has improved dramatically in recent decades, while comparative information on physiology and biochemistry of these organisms has lagged somewhat. This chapter summarizes the phylogenetic context, synthesizes comparative information, discusses the evolutionary implications, and highlights gaps in our knowledge of how the plant ethylene system evolved.

7.2 Phylogenetic Context

Plants are eukaryotes, which are one of the three great domains of life, and are more closely related to Archaea than to bacteria (Fig. 7.1; He et al. 2014). Eukaryotes contain an endosymbiotic organelle, the mitochondrion, the ancestor of which is thought to have been an α-proteobacterium (Andersson et al. 1998; Thrash et al. 2011). During the establishment of the mitochondrion, a large number of genes from the proteobacterial ancestor were transferred to the nuclear genome (Koonin

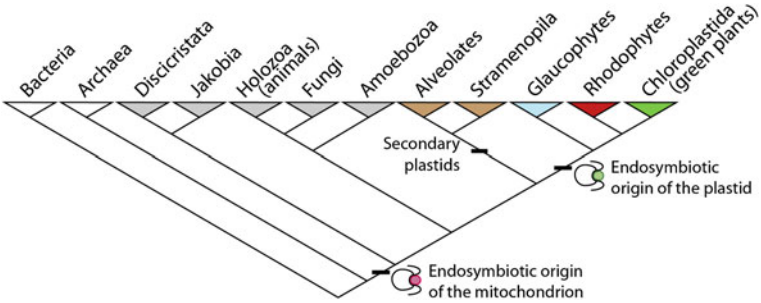

Fig. 7.1 Overview of the tree of life. *Filled triangles* represent major clades of eukaryotes. *Colored triangles* indicate plastid-containing lineages. A more detailed phylogeny of green plants is shown in Fig. 7.2. Phylogenetic relationships among eukaryotes follow He et al. (2014)

2010). Thus, eukaryotic nuclear genomes represent a mosaic of genes of diverse prokaryotic origin (Timmis et al. 2004).

Important in the evolutionary history of plants was the establishment of a second endosymbiotic organelle, the plastid, which descended from a formerly free-living cyanobacterial cell (Delwiche 1999; Cavalier-Smith 2000; Keeling 2010). Three groups of organisms evolved from this endosymbiotic event, the Glaucophyta, the red algae (Rhodophyceae), and the green lineage (Chloroplastida) (Fig. 7.1). The latter of which includes both green algae and land plants (embryophytes). Together these three plant lineages are known as the Archaeplastida (Adl et al. 2012). In much the same way as establishment of the mitochondrion led to the incorporation of large numbers of α-proteobacterial genes into the nuclear genome, so too did establishment of the plastid involve transfer of large numbers of cyanobacterial genes into the nuclear genome (Martin et al. 2002; Timmis et al. 2004). Thus, the gene content of plant genomes traces its ancestry to, minimally, the genomes of three highly divergent groups of prokaryotes; the Archaea (ancestral genome), the α-proteobacteria (mitochondrion), and the cyanobacteria (plastid). Some eukaryotes (e.g., brown algae and diatoms, dinoflagellates, and euglenoids) acquired their plastids indirectly by ingesting a red or green alga and retaining its plastids (Delwiche 1999; Cavalier-Smith 2000; Keeling 2010). Interestingly, endosymbiont-derived genes are not only important in organellar biology. A number of key plant biochemistries are originally derived from organelles, including the ability to sense and respond to ethylene.

Land plants (embryophytes) are a monophyletic group of organisms that evolved from within the green algae (as has been recognized at least since Bower 1908). Specifically, land plants share a most recent common ancestor with some members of the charophyte green algae and are more distantly related to the chlorophyte green algae, which include the model unicellular green alga *Chlamydomonas reinhardtii* (Karol et al. 2001; Timme and Delwiche 2010). However, the precise phylogenetic relationships between plants and their closest relatives remain controversial (Cooper 2014). Figure 7.2 shows the consensus view of the green algal and land plant phylogeny and highlights the phylogenetic position of the important experimental or genomic model organisms. It is clear that any search for the origins of land plant

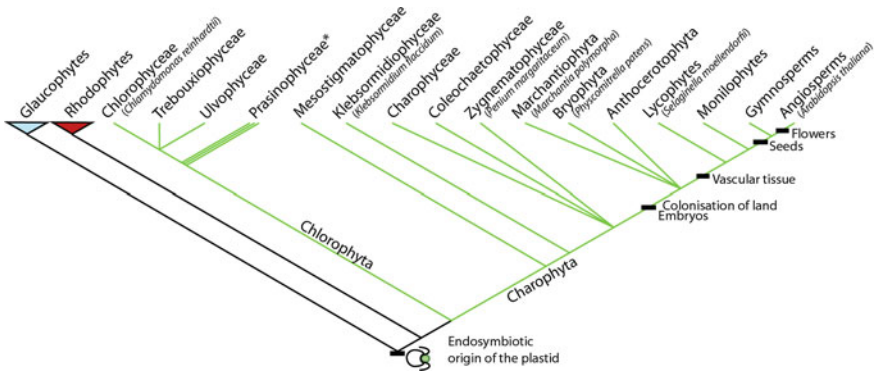

Fig. 7.2 Phylogeny of the plants (Archaeplastida): Glaucophytes, Rhodophytes, and Chlorophytes. The *green* lineage (Chloroplastida) can be divided into the Chlorophyta, often referred to as chlorophytes, and the Charophyta. The Charophyta includes both green algae and land plants (embryophytes); note that "Charophyceae" are the stoneworts, a clade within the Charophyta that have been important in physiological research because of their giant cells. *The Prasinophyceae are not a monophyletic group and are shown here as an arbitrary number of lineages

traits will lead to the charophyte green algae, making genome sequencing (e.g., Hori et al. 2014) and model system development (e.g., Sørensen et al. 2014) of representative charophytes fundamentally important for plant evolutionary biology.

7.3 Evolution of a Plant Hormone

Plant hormone systems can be conceptualized as a linear series of components (Fig. 7.3) (Zhang and Ho 2010). First, a hormone is synthesized in response to an exogenous trigger or a developmental cue. The hormone can subsequently be perceived locally or can be transported to a site remote from the site of biosynthesis, thereby communicating the signal to that part of the plant where the phenotypic response is required. In order to elicit a response, the hormone must be perceived, typically by a dedicated receptor, and the signal processed by a signal transduction pathway. For many plant hormones, the molecular mechanisms of the entire process have been elucidated in the model angiosperm *Arabidopsis thaliana*. The plant ethylene system is among the best known plant hormone systems, and is unique in that long distance transport per se is bypassed, since ethylene gas is freely diffusible across cell membranes, evoking mainly local responses.

In order for plants to have a complete and functional hormone system, all of these different elements of the system need to operate together regardless of their origin. For ethylene, it is possible that a perception/signaling system was first acquired by cyanobacteria to respond to environmental ethylene gas, independent of the endogenous ethylene biosynthesis pathway. Ethylene is a relatively reactive gas and is present at low concentration in most natural environments. Although it is

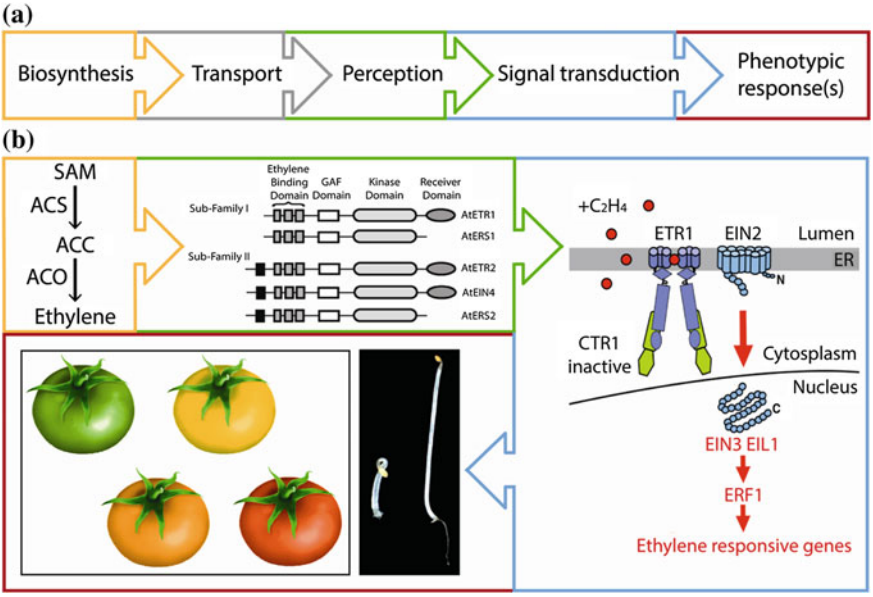

Fig. 7.3 **a** Conceptual outline of a hormone system. **b** Summary of the plant ethylene system starting with hormone biosynthesis, eliminating the transport section as ethylene diffuses freely throughout cells, signal transduction, and physiological responses. Perception is depicted by representing the five different *Arabidopsis* ethylene receptors according to Binder (2008) (Reprinted with permission from Plant Science); see Chap. 3 for more information. Signal transduction is depicted according to Ju et al. (2012). See Chap. 6 for more details. Phenotypic responses are illustrated by two examples: the *Arabidopsis* triple response and climacteric fruit ripening of tomato

possible that atmospheric ethylene gas played more of a role in earth's early history (e.g., volcanic activity, UV radiation) than it does today, there are no available records on early atmospheric ethylene concentrations.

The components of the plant ethylene system are discussed in detail in Chaps. 2–6. Here, we synthesize reports of a biological role for ethylene in organisms across the tree of life and discuss their evolutionary implications. First, we will consider ethylene biosynthesis, we then explore ethylene perception and phenotypic responses, before turning our attention specifically to the origin of the components of the ethylene biosynthesis and signaling pathways.

7.4 Ethylene Biosynthesis

Ethylene biosynthesis has been reported in a diversity of organisms that are widely distributed across the tree of life. These include bacteria (Lynch 1972; Primrose 1976), fungi (Ilag and Curtis 1968; Lynch 1972), slime molds (Amagai and Maeda 1992),

brown algae (Broadgate et al. 2004), red algae (Garcia-Jimenez et al. 2013), chlorophyte green algae (e.g., Vanden Driessche et al. 1988; Maillard et al. 1993; Plettner et al. 2005), and most groups of land plants (e.g., Thomas and Harrison 1983; Rohwer and Bopp 1985; Law et al. 1985; Chernys and Kende 1996; Osborne et al. 1996). This wide distribution of ethylene production across the tree of life suggests that ethylene plays an important role in the life cycles of diverse organisms living in different habitats and separated from each other by large evolutionary distances, although there may be a few cases where ethylene production might not be part of an ethylene hormone system. The role of ethylene outside of plant systems is poorly characterized, but small, hydrophobic molecules can be produced as metabolic by-products.

Three ethylene biosynthesis pathways are known (Fig. 7.4): (1) the enzymatic conversion of the substrate 2-oxoglutarate (=α-ketoglutarate; AKG) into ethylene by an ethylene-forming enzyme (EFE; 2-oxoglutarate-dependent ethylene/succinate-forming enzyme). This pathway was discovered in the bacterium, *Pseudomonas syringae*, but is now also known for additional bacterial species and several fungi (Jacobsen and Wang 1968; Nagahama et al. 1991; Weingart and Volksch 1997);

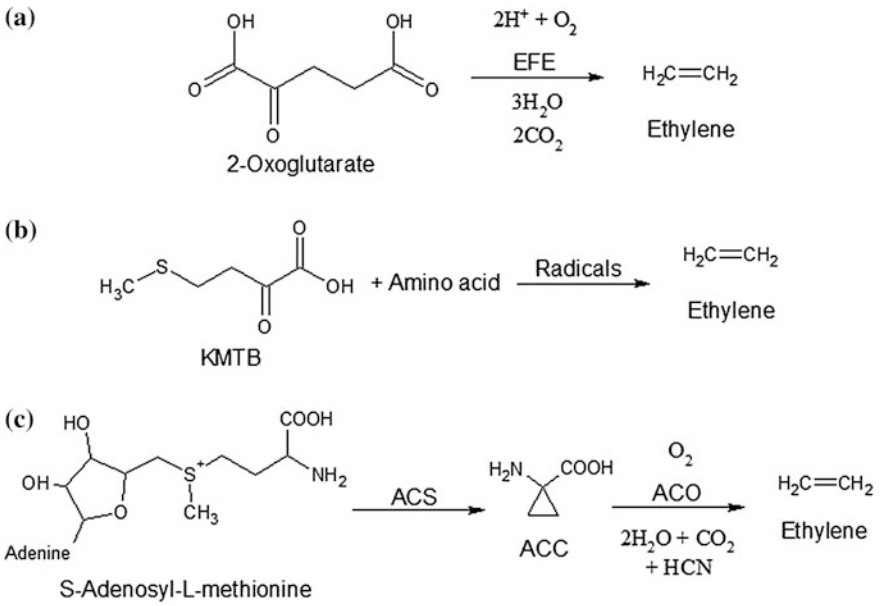

Fig. 7.4 The three known ethylene biosynthesis pathways. **a** The bacterial and fungal pathway using 2-oxyglutarate as substrate and the ethylene-forming enzyme (EFE). **b** The nonenzymatic bacterial pathway using 2-keto-4-methylthiobutyric acid (KMBA). **c** The plant-specific pathway converting S-adenosyl-L-methionine (SAM) into the unique intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC-synthase (ACS). ACC is subsequently converted into ethylene by ACC-oxidase (ACO). Both ACS and ACO are two enzymes solely dedicated to the ethylene biosynthesis pathway

(2) the nonenzymatic conversion of 2-keto-4-methylthiobutyric acid (KMBA) into ethylene (Mansouri and Bunch 1989; Nagahama et al. 1992; Ladygina et al. 2006). KMBA is a transaminated derivative of methionine that is formed by an NADH:Fe (III)EDTA oxidoreductase, which is enhanced under limited ammonia (Shipston and Bunch 1989). This route of ethylene synthesis is known to occur in *Escherichia coli* and other bacteria, and the fungus *Cryptococcus albidus* (Mansouri and Bunch 1989; Eckert et al. 2014); and (3) the two-step enzymatic conversion of *S*-adenosyl-L-methionine (SAM) to ethylene via the intermediate 1-amino-cyclopropane-1-carboxylic acid (ACC) (Adams and Yang 1977 and 1979).

The third type of pathway requires the action of ACC synthase (ACS) and ACC oxidase (ACO) enzymes (Boller et al. 1979; Ververidis and John 1991; Hamilton et al. 1991), and is considered to be the ethylene biosynthesis pathway of land plants. ACC is not known to participate in any other biochemical pathway, and consequently is a plant-specific hallmark of ethylene biosynthesis. Ethylene biosynthesis using ACC has been reported in many land plants including more deeply branching lineages, such as Marchantiophyta (liverworts), Bryophyta (mosses), lycophytes (spikemosses, clubmosses and quillworts), and monilophytes (ferns) (e.g., Thomas and Harrison 1983; Rohwer and Bopp 1985; Law et al. 1985; Chernys and Kende 1996). Despite some controversy over the actual usage of ACC for ethylene production in certain liverworts, mosses, and ferns (e.g., Chernys and Kende 1996; Kwa et al. 1995; Osborne et al. 1996), the conservation of both *ACS* and *ACO* genes in all the sequenced embryophyte genomes indicates that these are essential enzymes, and provides circumstantial evidence that ethylene is produced via ACC in land plants.

Although considered to be land plant specific, the ethylene biosynthesis route via ACC has also been demonstrated in cyanobacteria (Huang and Chow 1984), red algae (García-Jiménez and Robaina 2012; Garcia-Jimenez et al. 2013), chlorophyte green algae (Vanden Driessche et al. 1988; Maillard et al. 1993; Plettner et al. 2005), some fungi (Meng et al. 2014), and several slime molds (Amagai and Maeda 1992). The phylogenetic distribution of this pathway would be consistent with an ancient origin in the Archaeplastida, possibly derived via endosymbiotic gene transfer from the cyanobacterial ancestor of the plastid. However, there is mixed evidence for ethylene production by cyanobacteria. We are aware of only a single study reporting detection of ethylene production in one strain of *Hapalosiphon* but not in tested strains of *Anabaena*, *Nostoc*, *Cylindrospermum*, *Calothrix*, and *Scytonema* (Huang and Chow 1984). It is not clear whether the lack of detectable ethylene indicates an inability to synthesize ethylene by these other strains, or merely reflects the limits of the detection method. Indeed, ethylene production in *Hapalosiphon* was enhanced by adding methionine or ACC (Huang and Chow 1984), supporting the hypothesis that plant-like ethylene biosynthesis occurs in cyanobacteria. Additional research is required to determine whether ethylene biosynthesis is widespread in cyanobacteria or restricted to *Hapalosiphon*, and to test the hypothesis that a plant-like ethylene biosynthesis pathway was present in the cyanobacterial ancestor of the plastid.

There is strong evidence that both red and green algae can synthesize ethylene via ACC. It has been shown that the red algae *Pterocladiella capillacea* and *Gelidium arbuscula* produce ethylene and show specific ACC synthase and ACC oxidase activities (García-Jiménez and Robaina 2012; Garcia-Jimenez et al. 2013). Ethylene production has also been reported in field samples of *Chondrus crispus* and *Asparagopsis armata* (Broadgate et al. 2004), but evidence for a specific biosynthetic pathway was not gathered. The giant unicellular green alga *Acetabularia mediterranea* produces ethylene in a developmental and circadian dependent manner in response to exogenously supplied ACC (Vanden Driessche et al. 1988). Furthermore, *A. mediterranea* extracts contain ACC and malonyl-ACC (Vanden Driessche et al. 1988), supporting the conclusion that it produces ethylene via ACC using a plant-like pathway. In the relatively closely related species *Ulva intestinalis*, ethylene production is weakly stimulated by IAA, which promotes ACC synthase activity in plants, and is strongly stimulated by ACC (Plettner et al. 2005). In the more distantly related *Haematococcus pluvialis*, addition of precursors L-methionine and SAM increases ethylene production, as does the addition of ACC (Maillard et al. 1993). Interestingly, in *H. pluvialis*, Co^{2+} stimulates ACO activity, whereas in land plants, Co^{2+} inhibits ACO activity, but as expected, the metal chelator salicylhydroxamic acid (SHAM) inhibits ethylene production (Maillard et al. 1993). Clearly, red and green algae synthesize ethylene via ACC using a pathway that is very similar, if not identical, to that of land plants. Taken together, this suggests that land plants acquired the ethylene biosynthesis pathway using ACC from their algal ancestors. Perhaps the unique role of ACC, which is a very specific compound not participating in any other biochemical pathway, allowed plants to more precisely regulate the production of ethylene.

Besides algae and land plants, there are three isolated cases for bacteria, fungi, and slime molds where there is evidence for an ACC route of ethylene production. The fungus *Agaricus bisporus* was shown to have a functional ACO homolog (Meng et al. 2014), so does the slime mold *Dictyostelium mucoroides* (Amagai 2011). The cyanobacterium *Hapalosiphon* produced ethylene when supplemented with ACC (Huang and Chow 1984). All other known ethylene producing bacteria and fungi use an alternative route for ethylene production, suggesting that the ethylene biosynthesis pathway originated multiple times during evolution, or that the ethylene biosynthesis pathway was fine-tuned several times by different groups of organisms, which led to different pathways of ethylene production to fit their requirements and provide possible evolutionary benefits.

In addition to the biosynthetic pathway of ethylene production, a nonbiological mode of ethylene production via light-induced lipid peroxidation has been reported in animals (Moeskops et al. 2006), plants (Mattoo et al. 1986), and diatoms (Wilson et al. 1970) among others. However, this route of ethylene production has also been reported in dissolved lipids in sterilized estuary water (Lee and Baker 1992) and is not likely to represent a dedicated biosynthetic pathway.

7.5 Ethylene Perception and Responses

The starting point for an ethylene response is an ability to perceive ethylene. A survey of ethylene-binding activity across the tree of life found evidence for ethylene binding in only cyanobacteria, some fungi, one charophyte green alga, and plants (Table 7.1; Wang et al. 2006), suggesting a somewhat restricted distribution of potential ethylene binding. By contrast, ethylene responses have been reported in bacteria (Kim et al. 2007), animals (Krasko et al. 1999; Seack et al. 2001), fungi (Chagué et al. 2006), slime molds (Amagai and Maeda 1992), red algae (García-Jiménez and Robaina 2012), chlorophyte green algae (Plettner et al. 2005), and land plants, suggesting a wider distribution of ethylene perception. A broad distribution of ethylene responses across the tree of life might reflect an ancient origin for ethylene signal transduction; however, the evidence presented below suggests that the ability to respond to ethylene arose independently multiple times. Interestingly, ethylene responses are not always coupled with endogenous ethylene production and are quite varied in their nature. Besides endogenous ethylene production, ethylene signaling can be triggered by ethylene produced by other organisms, and by environmental ethylene found in the earth and atmosphere.

Although some bacteria can produce ethylene, with the exception of cyanobacteria, they have not been observed to bind ethylene (Wang et al. 2006), consistent with a lack of reports of ethylene responses in bacteria. An interesting exception is the reported chemotactic response to ethylene in *Pseudomonas* species (Kim et al. 2007). Bacterial responses toward external ethylene (produced by plants, fungi, or just environmental ethylene) could activate specific bacterial responses. It is also possible that pathogenic or symbiotic *Pseudomonas* species use plant ethylene emissions to locate a host. Indeed pathogenic *Pseudomonas* strains express an ACC deaminase gene that catabolizes plant ACC, thereby suppressing host plant immunity (Blaha et al. 2006; Groen and Whiteman 2014), while mutualistic *Pseudomonas* strains use this ACC deaminase to establish a symbiosis with the rhizosphere that will result in the promotion of plant growth (Glick et al. 2014). Mutualistic bacteria also use this ACC deaminase to suppress host immune responses during establishment of symbiosis (Groen and Whiteman 2014). Such bacterial interactions with the plant ethylene system clearly evolved in response to the plant ethylene system and are uninformative for reconstructing the evolutionary history of ethylene.

Interestingly, a few species of α- and β-proteobacteria carry sequences that are homologous to the ethylene-binding domain (EBD) of the plant ethylene receptor (Wang et al. 2006). In these cases, the EBD sequences are fused with other types of bacterial signaling domains (Caren Chang's lab, unpub. data), perhaps demonstrating the interchangeability of signaling modules to create new types of ethylene receptors. The fact that there are very few of these alternative ethylene receptors in prokaryotes might suggest that the EBD was acquired by these proteobacteria via horizontal gene transfer.

Table 7.1 Ethylene-binding activity of organisms in various kingdoms (reprinted from Wang et al. 2006)

Species	Ethylene binding	Species	Ethylene binding
Archaea		Fungi	
Halobacterium salinarium	−	*Aspergillus flavus*	−
Eubacteria		*Neurospora crassa*	+
Agrobacterium tumefaciens	−	*Penicillium chrysogenum*	−
Bacillus luteus	−	*Rhizopus stolonifer*	+
Deinococcus radiodurans	−	*Saccharomyces cerevisiae*	−
Escherichia coli	−	*Schizophyllum commune*	−
Flavobacterium sp	−	Metazoan	
Streptomyces coelicolor	−	*Caenorhabditis elegans*	−
Protists		*Drosophila melanogaster*	−
Dictyostelium discoideum	−	Green algae	
Pythium torulosom	−	*Acetabularia acetabulum*	−
Rhodomonas sp	−	*Chara* sp	++
Tetrahymena thermophile	−	*Chlamydomonas reinhardtii*	−
Cyanobacteria		Plants	
Anabaena PCC 7122	+++	*Amblystegium* sp	++
Chamaesiphon PCC 7430	−	*Arabidopsis thaliana*	++
Fischerella PCC 7414	+++	*Elodea canadensis*	++
Lyngbia PCC 7419	++	*Gingko biloba*	++
Nodularia PCC 73104	++	*Juniperus chinensis*	++
Nostoc PCC 7120	++	*Lycopodium lucidulum*	++
Oscillatoria PCC 7105	+++	*Marchantia polymorpha*	++
Plectonema PCC 73110	−	*Marsilea drummondii*	++
Pseudanabaena PCC 6903	−	*Nephrolepsis exaltata*	++
Spirulina PCC 6313	+++	*Nicotiana tobacum*	++
Synechococcus PCC 6301	−	*Physcomitrella patens*	++
Synechococcus PCC 6908	−	*Polytrichum* sp	++
Synechococcus PCC 7942	−	*Psilotum nudum*	++
Synechocystis PCC 6803	++	*Sphagnum* sp	++
		Vallisneria sp	++

Binding levels +++ 10-fold or more binding over background; ++ 3- to 10-fold binding over background; + 1.5- to 3-fold binding over background; − no measurable binding

While ethylene responses are quite well studied in flowering plants, there are also numerous reports of ethylene responses in evolutionarily more distant lineages of land plants and a few reports of ethylene responses in the even more distant lineages of the aquatic relatives of the red or green algae. In the red alga, *P. capillacea*, ethylene promotes maturation of tetrasporangial branches (García-Jiménez and Robaina 2012), whereas in the green alga *U. intestinalis,* ethylene appears to be involved in regulating chlorophyll concentrations (Plettner et al. 2005). That ethylene is involved in chlorophyll regulation is interesting given the influence of light and circadian rhythms on ethylene production in some green algae (Vanden Driessche et al. 1988; Kreslavsky et al. 1997; Plettner et al. 2005). The abiotic production of ethylene in water bodies by UV light (Lee and Baker 1992) suggests an association between ethylene signaling in green algae and environmental light sensing. That an association between light sensing and ethylene signaling is also known in land plants (Vandenbussche et al. 2012), suggests a possible ancestral role for ethylene signaling in the green lineage. However, in conjunction with nitric oxide, ethylene is involved in regulating programmed cell death in *C. reinhardtii* (Yordanova et al. 2010). It has also been proposed that ethylene and nitric oxide might play a role in transmitting a stress response to neighboring cells in this same species (Zuo et al. 2012). As ethylene is known to play an important role in stress signaling in plants, these findings in *Chlamydomonas* suggest an alternative ancestral role for ethylene in stress responses in the green lineage. At the present time, however, there is too little information on ethylene responses in green algae for firm conclusions to be drawn. Nevertheless, a system of ethylene perception and response is present in some green and red algae, suggesting that ethylene signaling evolved early in the Archaeplastida.

In animals, biosynthesis of ethylene is unknown, but a stress-like survival response at the level of calcium signaling was reported in the sponge *Suberites domuncular* in response to exogenous ethylene (Krasko et al. 1999; Müller et al. 2006). It is tempting to speculate that ethylene induction of a stress response pathway in this sponge is linked to exogenous UV-light-dependent ethylene production in surface water as an environmental signal. A similar calcium signaling-related response to ethylene appears to occur in mammalian cells (Perovic et al. 2001; Seack et al. 2001; Müller et al. 2006), and detection of ethylene produced by lipid peroxidation has been proposed as a diagnostic measure of oxidative stress in humans (Zusterzeel et al. 2002). Given the lack of ethylene biosynthesis in animals plus the absence of ethylene receptors, it seems likely that ethylene responses, where they occur, evolved as a response to either ethylene as an environmental signal or ethylene as a product of oxidative stress. As such, it is unlikely that these responses are evolutionarily related to the plant ethylene system.

In fungi, which are more closely related to animals than they are to plants (Fig. 7.1), ethylene responses are frequently associated with their interactions with plants. For example, *Colletotichum* species respond to the ethylene produced by climacteric fruits (Flaishman and Kolattukudy 1994). Timing of the responses, which include stimulation of germination, branching of the germ tube, and formation of appressoria, to coincide with fruit ripening has obvious benefits for the

fungus. A similar response has also been demonstrated in *Botrytis cinerea* (Zhu et al. 2012), and it has been shown that ethylene enhances expression of pathogenicity genes in this species during infection of *Nicotiana benthamiana* (Chagué et al. 2006). Thus, some plant pathogenic fungi appear to have evolved mechanisms for exploiting plant ethylene signaling for their own purposes. Ethylene signaling also appears to be important in mediating symbiotic relationships, for example, between the endosymbiotic fungus *Piriformospora indica* and *A. thaliana* (Camehl et al. 2010). Illustrating another form of fungal-plant mutualism, ethylene is involved in control of thallus morphogenesis in the lichen *Cladonia sulphurina* (Ott et al. 2000). By contrast, in other fungi, ethylene responses independent of fungal-plant interaction have been described. For example, ethylene appears to mediate autochemotropism (barrier avoidance) in *Phycomyces* (Russo et al. 1977) but this mechanism has been questioned (Métraux and Kende 1983). However, it is unclear how widespread plant-independent fungal responses to ethylene are.

One of the most interesting and perplexing reports of ethylene responses is the ethylene system found in the slime mold *Dictyostelium mucoroides* (Amagai 2011). Slime molds are enigmatic members of the Amoebozoa that alternate between single-cellular and aggregative multicellular states. The Amoebozoa are more closely related to animals and fungi than plants, and yet *Dictyostelium* uses a plant-like ethylene biosynthetic pathway and an ethylene signal transduction pathway, to regulate its unusual life cycle (Amagai and Maeda 1992; Amagai et al. 2007; Amagai 2011). This is perhaps the most complete ethylene hormone system described outside of land plants, consisting of both a specific ethylene biosynthesis pathway and an ethylene sensitive transcriptional response that regulates organismal development. The phylogenetic distance between slime molds and plants makes it extremely unlikely that the seemingly plant-like ethylene system in *Dictyostelium* shares an evolutionary origin with the plant ethylene system. However, the origin of the *Dictyostelium* ethylene system is an open question.

Together, the scattered occurrence of ethylene responses across the tree of life supports the hypothesis that ethylene perception and signaling evolved independently multiple times. Ethylene responses are not always coupled with ethylene biosynthesis, and are often responses to abiotic ethylene sources or ethylene emanating from plants. The plant ethylene system is likely to have evolved uniquely in plants, or earlier in the evolutionary history of the Archaeplastida. In order to more precisely identify the origins of the plant ethylene system, we need to consider its specific genetic components.

7.6 Origins of the Plant Ethylene Hormone System

Surveying the individual components of the ethylene system indicates that ethylene biosynthesis originated very deep in the Arachaeplastida and possibly even earlier. Ethylene signaling, on the other hand, was assembled from a mixture of prokaryotic, eukaryotic, and plant-specific elements.

7.6.1 Ethylene Biosynthesis

Examination of the distribution and mechanisms of ethylene biosynthesis across the tree of life suggests that ethylene biosynthesis from SAM via ACC, as found in land plants, evolved deep in the Archaeplastida (see above) and is still shared by land plants and algae. However, recent phylogenetic study of the 2-oxoglutarate-dependent dioxygenase (2OGD) superfamily, to which the plant ACC-oxidases belong, found that the plant ACO family is restricted to seed plants (Fig. 7.5; Kawai et al. 2014). There have been extensive duplications of *ACO* genes in angiosperms (Clouse and Carraro 2014) and duplication and diversification of 2OGDs in land plants (Kawai et al. 2014). Given that there is strong experimental evidence for ethylene production via ACC in red and green algae, and nonvascular plants, it

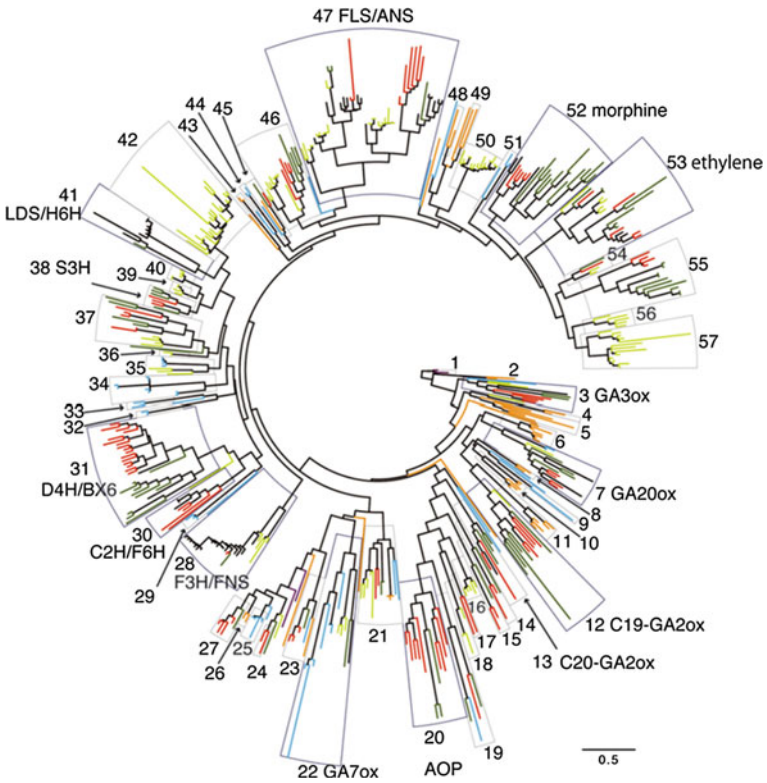

Fig. 7.5 Phylogenetic tree of 2ODG genes in plants. 2ODG that are functionally characterized are indicated by their enzymatic names behind the branch. The colored branches depict different species: *Arabidopsis thaliana* (*red*), *Oryza sativa*, (*green*) *Picea* (*light green*), *Selaginella moelendorfii* (*blue*), *P. patens* (*orange*), and *C. reinhardtii* (*purple*). *Black* branches represent functionally characterized 2DOG from other species. There are 53 genes identified that are related to ethylene biosynthesis. Figure reproduced from Kawai et al. (2014)

seems likely that the phylogeny is not providing a completely accurate prediction of function in this gene superfamily. Indeed, the genome of the chlorophyte green alga *C. reinhardtii* encodes two 2OGDs that could not be placed in any clade (Kawai et al. 2014), and the function of these enzymes is unknown. Furthermore, phylogenetic analysis of a gene family over the entire timescale of green plant evolution (>1.8 billion years) is likely to be inaccurate due to inadequate modeling of the substitution process (Cooper 2014). Therefore, although further experimental evidence will be required to confirm the precise origins of the plant ethylene biosynthetic pathway, we hypothesize that this pathway evolved early in the Archaeplastida and is shared by land plants and red and green algae.

7.6.2 The Ethylene Receptor

The plant ethylene receptor is found only in plants, charophyte green algae, and cyanobacteria. The receptor is unambiguously homologous to a cyanobacterial ethylene-binding protein (Mount and Chang 2002), and a number of cyanobacteria strongly bind ethylene (Table 7.1) (Wang et al. 2006). Figure 7.6 shows the protein sequence alignment of the five *Arabidopsis* ethylene receptor isoforms and the ethylene-binding protein of the cyanobacterium *Synechocystis* slr1212, illustrating the strong sequence conservation. These observations have led to the hypothesis

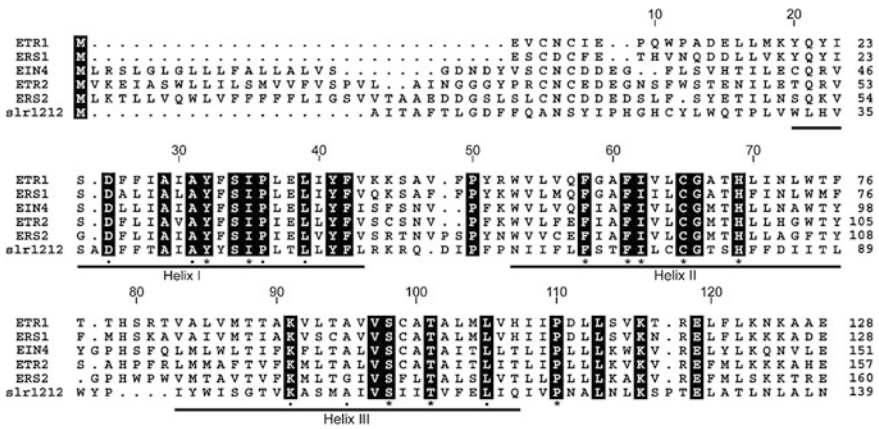

Fig. 7.6 Protein sequence comparison of the transmembrane region of the *Arabidopsis* ethylene receptors and *Synechocystis* slr1212. The three predicated hydrophobic segments are *underlined*. The amino acid residues of each protein are numbered at the *right*. The numbers on *top* of each alignment block indicate the residue numbers of ETR1. The completely conserved residues among the six known EBDs are *shaded*. The *asterisks* below the alignments indicate amino acid residues completely conserved among the six known ethylene-binding domains and the putative ethylene-binding domains from bacteria. Figure reproduced from Wang et al. (2006)

that the ethylene receptor was acquired by plants via horizontal gene transfer from the cyanobacterial ancestor of the plastid (Mount and Chang 2002).

Consistent with ethylene-binding activity reported for the charophyte green alga, *Chara sp.* (Wang et al. 2006), plant ethylene receptor homologs have been reported for expressed sequence tags (ESTs) of the charophytes *Spirogyra pratensis* (Timme and Delwiche 2010) and *Klebosormidium flaccidum* (Hori et al. 2014). No homolog of the plant ethylene receptor is encoded in the available chlorophyte and red algae genomes (Matsuzaki et al. 2004; Derelle et al. 2006; Merchant et al. 2007), but these genomes are not from the same species of red algae and chlorophytes that have been found to respond to ethylene. This distribution of plant ethylene receptor homologs suggests that the receptor gene is derived from the cyanobacterial ancestor of the plastid and was subsequently lost from species of chlorophyte green algae and red algae but maintained in charophyte green algae and land plants.

Interestingly, two proteins that are important for ethylene receptor function in land plants are also conserved in metazoans (animals). One of these, the copper transporter RAN1 provides the copper ion cofactor to the ethylene receptor for ethylene binding, and is homologous to the P-type ATPase transporter associated with Menkes/Wilson disease in humans (Hirayama et al. 1999; Woeste and Kieber 2000). The other protein, whose biochemical function has not been elucidated in either plants or animals, is called RTE1 in *Arabidopsis* or Green-Ripe (GR) in tomato (Resnick et al. 2006; Barry and Giovannoni 2006). RTE1 specifically regulates only one of the five ethylene receptor isoforms in *Arabidopsis* (Resnick et al. 2006; Rivarola et al. 2009). RAN1 and RTE1/GR appear to have ancient eukaryotic origins with functions in metazoans that are unrelated to ethylene perception, and have evolved to play important regulatory functions in ethylene perception in land plants.

7.6.3 The CTR1 Protein Kinase

Acting just downstream from the receptor is the serine/threonine protein kinase, CTR1, a member of the RAF-like kinase family (Kieber et al. 1993). RAF-like protein kinases are widespread in eukaryotes, but generally unknown in bacteria (Kieber et al. 1993). Homologs of CTR1 have been reported in the charophytes *Coleochaete orbicularis*, *Spirogyra pratensis,* and *Klebsormidium flaccidum* (Timme and Delwiche 2010; Hori et al. 2014), suggesting that the role of CTR1 in ethylene signal transduction evolved in ancestral charophytes. However, confirmation of a role for charophyte CTR1 homologs in ethylene signaling awaits experimental evidence.

7.6.4 The Central Regulator EIN2

Although CTR1 is a kinase with sequence similarity to RAF-like kinases that initiate a MAP-kinase cascade, no MAPKKK pathway definitively connecting CTR1 to the downstream component EIN2 has been found. Instead, CTR1 is known to directly phosphorylate the downstream target EIN2 (Ju et al. 2012). EIN2 has sequence similarity to Nramp metal ion transporters but has a unique carboxy-terminal domain (Alonso et al. 1999). While Nramps are found in almost every branch of the tree of life (Nevo and Nelson 2006), no homolog of the EIN2 C-terminal domain has been found outside land plants. A possible EIN2 homolog was reported for *Coleochaete orbicularis* (Timme and Delwiche 2010), but it is unclear whether or not the *Coleochaete* sequence includes the EIN2-specific C-terminal domain. No EIN2 homolog was found in the *K. flaccidum* draft genome (Hori et al. 2014). A possible evolutionary explanation for the unique domain structure of EIN2 is that it evolved from an ancestral Nramp after fusion of an additional domain to its carboxy-terminus, providing it with the unique properties to function as the central regulator of ethylene signaling. Exactly when during the evolution of plants EIN2 acquired its unique combination of domains remains an open question, but its structure is key to its function in ethylene signal transduction. In the absence of ethylene, the CTR1 kinase phosphorylates the C-terminal domain of EIN2, thereby maintaining it in an inactive state (Ju et al. 2012). When ethylene is perceived, CTR1 is inactivated and the unphosphorylated EIN2 C-terminal domain is proteolytically cleaved from the membrane bound Nramp domain, allowing it to migrate into the nucleus where it activates the ethylene response via the downstream EIN3/EIL transcription factors (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012). This mechanism is a core component of the plant ethylene signal transduction pathway, and identifying the origin of EIN2 is fundamental to understanding precisely when during the evolutionary history of plants this central regulator of ethylene signaling was assembled.

7.6.5 Transcription Factors EIN3/EIL

It is widely reported in the literature that the EIN3/EIL family of transcription factors is plant specific. This conclusion can be traced back to the original work on the function of EIN3 (Chao et al. 1997). However, at that time the sequence database was relatively limited, and since then the quantity and phylogenetic diversity of sequence data in reference databases has grown enormously. More recently, homologs of EIN3 have been found in ESTs from two charophyte green algae (Timme and Delwiche 2010) and the draft genome of a third (Hori et al. 2014). The genome of *C. reinhardtii* lacks an EIN3/EIL homolog, suggesting that EIN3/EIL might have evolved sometime during charophyte divergence.

A critical regulatory mechanism in ethylene signaling is EIN3/EIL1 protein accumulation and turnover (by the 26S proteasome), which is regulated by two F-box proteins, EBF1 and EBF2 (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004; An et al. 2010). EBF homologs are retrieved in the genomes of *Selaginella* and *Physcomitrella* (Merchant et al. 2007; Banks et al. 2011) and have been found in several charophyte algae, although it is unclear if they are specific for EIN3/EIL (Timme and Delwiche 2010; Hori et al. 2014). EBFs show sequence conservation in their F-box motif with numerous F-box proteins of other eukaryotes (Guo and Ecker 2003). EIN3 activates ethylene-responsive gene expression, and one of its target genes is *ERF1*, which encodes a transcription factor that activates additional ethylene-responsive genes in a transcriptional cascade with EIN3 (Solano et al. 1998). ERF1 has an AP2/ERF DNA-binding domain belonging to a large family of transcription factors in land plants (Riechmann and Meyerowitz 1998) and some charophyte algal species (Timme and Delwiche 2010). The AP2/ERF domain is considered to be plant specific (Riechmann and Meyerowitz 1998), although homologous AP2 domains have been found in a cyanobacterium, a ciliate, and viruses (Magnani et al. 2004).

Altogether, it is clear that the ethylene signaling pathway in land plants was assembled through a combination of elements that are derived from an ancestral cyanobacterium (ethylene receptors), widespread among eukaryotes (CTR1, RAN1, RTE1), or plant specific (EIN2, EIN3, ERF1) (Bleecker 1999). Given that homologs of the ethylene receptor, ETR1, and all of the major ethylene signaling components, from CTR1 to EIN3, have now been found in one or more species of charophyte green algae, the hypothesis that the plant ethylene system was first assembled in an ancestral charophyte seems increasingly likely, although functional evidence for the signaling components and well-identified physiological responses are missing for the charophyte green algae.

7.7 Evolution of Ethylene Complexity in Plants

Whether or not an ancestral charophyte possessed the complete plant ethylene system, all extant plants seem to have one, so presumably the most recent common ancestor of land plants did as well. The nonvascular liverworts and mosses are two of the earliest diverging lineages of land plants still extant, and consequently hold a key phylogenetic position in the study of plant evolution (Rensing et al. 2008; Banks et al. 2011). Focusing first on liverworts, the genome of the model liverwort *Marchantia polymorpha*, is currently being sequenced, and encodes homologs of each component of the plant ethylene system (Bowman J., personal communication). Ethylene responses have been reported in both the sporophyte (diploid) and gametophyte (haploid) generations of liverworts. In *Plagiochila arctica*, treatment with ethylene disrupts regulation of phyllid (i.e., "leaf") and branch development in the gametophyte (Basile and Basile 1983). Under normal conditions, *P. arctica* produces leaves from only two of the three files of cells derived from its single

shoot apical meristematic cell, and it produces a limited range of branch types, but ethylene treated plants produce leaves from all three cell files and additional branch types (Basile and Basile 1983; Law et al. 1985). In *Pellia epiphylla* sporophytes, the seta (stalk) elongates in response to auxin (Thomas and Harrison 1983). However, ethylene treatment inhibits this auxin-stimulated elongation (Thomas and Harrison 1983). Besides liverworts, there are also compelling reports on ethylene responses in mosses. The genome of the model moss *Physcomitrella patens* encodes homologs of the entire ethylene system (Rensing et al. 2008) including a functional ethylene receptor (Ishida et al. 2010). When *P. patens* is exposed to ABA and salt stress, it shows an upregulation of an ethylene-response factor AP2/EREBP homolog, consistent with the hormonal crosstalk known in angiosperms (Richardt et al. 2010). In this same species, ethylene induces protonemal growth and regulates the submergence response, whereas overexpression of an ethylene-binding site mutant of the ethylene receptor causes ethylene insensitivity and perturbs the submergence response (Yasumura et al. 2012). Thus, the combined evidence from mosses and liverworts suggests that the plant ethylene system was already present and controlling developmental and stress responses in the most recent common ancestor of all land plants.

In early diverging vascular plants (i.e., lycophytes, ferns, and their "allies"), the roles for ethylene include induction of megasporangia formation in the lycophyte *Selaginella* (Brooks 1973), stimulation of cell elongation in the rachis of the fern *Regnellidium diphyllum* (Musgrave and Walters 1974; Cookson and Osborne 1979), induction of apogamous embryos in *Pteridium* (Elmore and Whittier 1975), stimulation of germination and protonemal growth in the fern *Oncolea sensibilis* (Edwards and Miller 1972; Fisher and Miller 1978), and mediation of drought-induced leaflet abscission in the fern *Nephrolepis cordifolia* (Banthoengsuk et al. 2011). In seed plants, ethylene is involved in processes from seed germination (Abeles and Lonski 1969) to programmed senescence (Grbić and Bleecker 1995), and seemingly almost everything in between. For a review of the diversity of roles for ethylene in seed plants see Abeles et al. (1992). How the plant ethylene system evolved such diverse roles from what must have been a far simpler starting point remains an important evolutionary question.

To understand the characteristics of the earliest ancestors of land plants, it is important to focus not only on early diverging land plants, but also on their relatives among the green algae (as well as more distant relatives). It is now evident that at least some charophyte green algae have homologs of the ethylene system (see above), and consequently these organisms are potentially extremely important in developing a better understanding of the evolution of ethylene as a plant hormone. Originally, the role of ethylene in ancestral algae or ancestors of land plants was presumably relatively simple, both because the ethylene system was an evolutionary novelty, and because the organisms in which the system was first functional were probably less structurally complex than typical modern angiosperms. By contrast, in modern land plants ethylene signaling is involved in an enormous variety of processes. As described above, ethylene signaling controls several processes even in nonvascular plants.

Table 7.2 Gene number of ethylene pathway gene families in 12 angiosperm genomes

	Monocots						Eudicots					
Gene family	*Ma*	*Os*	*Bd*	*Sb*	*Zm*	*Pd*	*Tp*	*At*	*Vv*	*Sl*	*Pp*	*Fv*
ACS	11	5	3	3	4	5	9	10	7	11	6	8
ACO	12	8	6	8	12	9	5	5	3	7	5	5
ETR1/ ERS1/ EIN4	7	5	6	6	5	6	6	5	4	8	4	4
RAN1	2	2	2	2	2	1	1	1	1	1	1	1
RTE1	2	3	2	3	4	1	2	2	2	3	2	2
CTR1	3	2	2	2	1	1	1	1	1	3	1	1
EIN2	3	2	3	3	3	2	1	1	1	3	1	1
EBF1	5	2	2	2	4	2	2	2	2	6	2	2
EIN3/ EIL	5	7	6	6	9	6	6	6	4	9	4	6
ERF1	122	82	NA	53	84	NA	NA	65	82	68	59	NA

Reproduced from Jourda et al. (2014)

Ma Musa acuminata; *Os Oryza sativa*; *Bd Brachypodium distachyon*; *Sb Sorgum bicolor*; *Zm Zea mays*; *Pd Phoenix dactylifera*; *Tp Thellungiella parvula*; *At Arabidopsis. thaliana*; *Vv Vitis vinifera*; *Sl Solanum lycopersicum*; *Pp Prunus persica*; *Fv Fragaria vesca*; *NA* not available. *RAN* RESPONSIVE-TO-ANTAGONIST1; *RTE1* REVERSION-TO-ETHYLENE SENSITIVITY1; *EBF1* EIN3-BINDING F BOX PROTEIN1; *ERF1* ETHYLENE RESPONSE FACTOR1

This leads then to the question of how complexity arises in the course of evolution. If we define complexity as referring to the number of distinct cell types, developmental states, and metabolic modes that the plant can achieve in the course of its life cycle, then it presumably tracks rather closely with the number of genes and regulatory elements within the genome. In this context, gene duplications are a likely source of diversity in the processes regulated by plant systems, including ethylene (Taylor and Raes 2004). In principle, duplication and neo- or subfunctionalization of any component of the plant ethylene system could allow increased diversity of the roles that ethylene signaling performs. Indeed, there is evidence for duplication of all components of the plant ethylene system (Table 7.2; Jourda et al. 2014), and probably such diversification involved both whole genome duplication and duplication of individual components of the system. For example, duplication of the biosynthetic pathway genes has resulted in up to 11 *ACS* and 12 *ACO* genes in *Musa acuminata* (banana), and there are up to eight copies of the receptor in *Solanum lycopersicum* (tomato). Also, the number of ethylene receptor genes increased after whole genome duplication, and so did the number of EIN2 genes. Regulatory changes can lead to rapid diversification of function, and the most extensive gene family expansions have occurred in the transcription factors, with up to 17 copies (in banana) of the primary transcription factors of the EIN3/EIL family, and 122 copies (in banana) of the downstream ethylene response factor (ERF)

family of transcription factors (Jourda et al. 2014). However, the functional significance of these gene family expansions remains largely unknown, and the members of a gene family might not all have unique functions resulting in redundancy. Some work on the ethylene receptor of *Arabidopsis* and tomato has made it clear that there are both overlapping and nonoverlapping roles for each receptor (Shakeel et al. 2013). The elucidation of gene-specific functions or redundancy for the other ethylene signaling components remains to be investigated. Studying evolutionary more distant species like algae, might uncover the role of individual ethylene genes and possibly uncover an ancient function of the ethylene hormone system.

7.8 Conclusions

It is almost certain that ethylene is a functional hormone in all land plants. More distant species such as algae seem to produce ethylene and respond to ethylene, although more experimental confirmation of a functional ethylene hormone system is required. The ethylene biosynthesis pathway using ACC as a specific intermediate precursor to produce ethylene is characteristic of land plants, and most likely arose early during Archaeplastida evolution. More distantly related organisms, including bacteria and fungi also have the capacity to produce ethylene, but do so via a different biochemical route, suggesting that ethylene biosynthesis arose independently several times during the course of evolution. The core ethylene signaling pathway of plants was assembled from a combination of proteins having prokaryotic, broadly eukaryotic, and plant-specific origins. It is also clear that the ethylene hormone system is most likely to have been assembled during the course of evolution of the charophyte lineage of green algae that ultimately gave rise to land plants. It is likely that in its earliest forms, the ethylene hormone system served a very simple role, and that hormone signaling complexity increased in concert with the evolution of complexity of land plants, permitting complex multicellularity, cell and tissue diversity, and phenotypic variation. Gene and genome duplication events probably played a key role in the evolution of such complexity. Much additional work is needed to reconstruct the sequence of events in the evolution of the ethylene hormone system. It will require genomic and genetic studies of diverse plants and algae, complemented by physiological and phenotypic studies of these same organisms. The rewards of such study are potentially large, because it can lead to novel insights into the mechanisms underlying ethylene signaling, identify previously underutilized model systems, and provide functional diversity of components of the system that could potentially have useful properties.

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Chapter 8
Interactions of Ethylene and Other Signals

Ziqiang Zhu and Hongwei Guo

Abstract As sessile organisms, plants utilize a wise strategy for adapting to the environment. The strategy involves the integration of its internal hormone signaling variations with exogenous environmental changes to coordinate plant growth and development. Like other phytohormones, ethylene plays a key role in these integration processes. In this chapter, we will discuss the interactions of ethylene with environmental signal (light) and several internal hormones (auxin, jasmonate, and gibberellins) in the regulation of plant growth, and highlight the recent advances in understanding their associated molecular mechanisms.

Keywords Ethylene · Light · Auxin · Jasmonate · Gibberellins · EIN3

8.1 Interaction with Light

Light is more than a source of energy for plants. It is the most important environmental cue for plants because it tells plants time, geographic location, and growth directions. Seedlings grown in dark exhibit long hypocotyls and closed yellow cotyledons (skotomorphogenesis); while seedlings grown in light exhibit short hypocotyls and opened green cotyledons (photomorphogenesis) (Nemhauser and Chory 2002). Consistent with these massive morphological differences, light

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regulates nearly 30 % of the global mRNA transcripts in the genomes of both monocots and dicots (Jiao et al. 2005; Ma et al. 2001).

As described in the previous chapter, all the typical ethylene seedling triple responses (i.e., a short/swollen root and hypocotyl, and an exaggerated apical hook) are observed under dark conditions (Guzman and Ecker 1990); however, light reverses the ethylene-triggered exaggerated hook and ethylene promotes hypocotyl growth instead of inhibition in light. According to a very recent study, germinated seeds sense the soil cover conditions (soil depth and texture) by producing different concentrations of ethylene (Zhong et al. 2014). In other words, germinated seeds can "see" their surrounding environment by sensing different ethylene quantities. It is hypothesized that ethylene promotes the exaggerated hook formation to protect the shoot apical meristem that resides between the two cotyledons when protruding through the soil. After seedlings penetrate the soil cover, light rapidly reverses the apical hook (reducing the hook angle) and induces chlorophyll synthesis and cotyledon opening. This dramatic morphological change during the transition from dark to light prepares cotyledons for photosynthesis.

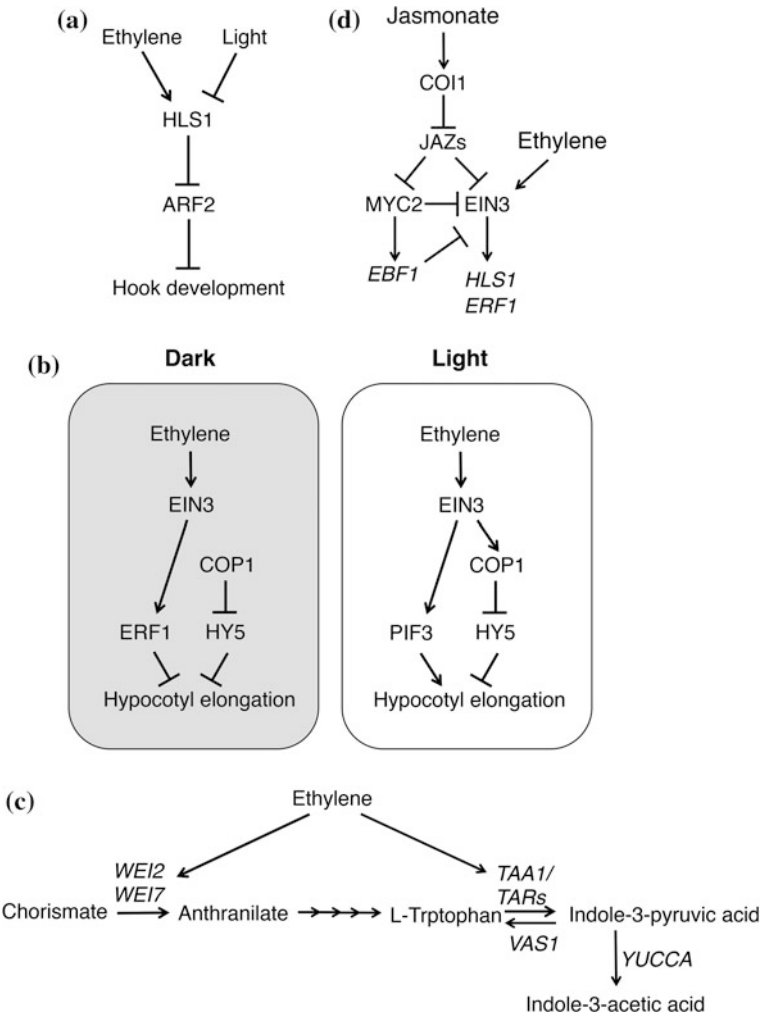
Hook development is caused by the differential elongation of hypocotyl cells (Silk and Erickson 1978), which is governed by another crucial phytohormone auxin. Auxin is synthesized in meristematic tissues and transported in a basipetal direction to fulfill its physiological functions. Blocking auxin transport by N-1-naphthylphthalamic acid (NPA) treatment or treating seedlings with exogenous auxin prohibits hook formation (Lehman et al. 1996). In the early screening for ethylene-insensitive mutants, dozens of *hookless* mutants were identified and the mutated genes were further cloned (Guzman and Ecker 1990; Lehman et al. 1996). Among them, *HOOKLESS1*, which encodes a protein similar to N-acetyltransferase, is a major player in determining apical hook angle. The strong allele of *hookless1* mutant (i.e., *hls1-1*) shows opened cotyledons in dark (no hook angles in the apical area) regardless in the presence or absence of ethylene. On the other hand, weak *hookless1* allele (i.e., *hls1-10*) does not form hook angles in the absence of ethylene and could bend to some extent (average hook angle is 6.5°) in the presence of ethylene, but this response is quite mild comparing with the wild-type controls (average hook angle is 298°). Further observations show that the *hls1* mutation suppresses the constitutively exaggerated-hook phenotype in *ctr1* or *EIN3ox* (a transgenic plant overexpressing EIN3), both of which exhibit constitutive ethylene responses, suggesting that HLS1 acts downstream of CTR1 and EIN3. Ethylene-activated EIN3 directly binds to the *HLS1* promoter to induce *HLS1* expression (An et al. 2012; Chang et al. 2013; Lehman et al. 1996). Expression profiles of several auxin-regulated genes are altered in *hls1* mutant, implying that HLS1 controls auxin activities (Lehman et al. 1996). It is further reported that one auxin responsive transcription factor AUXIN RESPONSE FACTOR2 (ARF2) acts downstream of HLS1, as *arf2* mutation suppresses *hls1* (Li et al. 2004). Overexpression of *ARF2* plants is less sensitive to ethylene in hook development, which indicates that ARF2 is a negative regulator in the hook development process. In contrast with the increase of HLS1 protein, ethylene destabilizes the ARF2 protein in a HLS1 dependent manner (Li et al. 2004). Inhibiting the 26S proteasome by treatment with chemical

MG132 can suppress ARF2 degradation, suggesting that the ubiquitin-mediated protein degradation pathway facilitates the degradation of ARF2 (Li et al. 2004).

Although for decades, it had been documented that light is able to reverse the apical hook in many plant species even in the presence of ethylene (Liscum and Hangarter 1993; Rubinstein 1971), the underlying molecular basis was uncovered only after the characterization of HLS1 and ARF2. Light treatment dramatically reduces HLS1 protein levels but increases ARF2 protein accumulation, wherein the modulation of ARF2 protein level is dependent on the presence of HLS1 (Li et al. 2004). This opposite regulation of HLS1 and ARF2 protein accumulation by light and ethylene explains how light antagonizes ethylene's physiological function with regard to hook development (Fig. 8.1a).

Although a molecular framework of light–ethylene antagonistic regulation on hook development has been established, there are still some questions that need to be explored in the future: (1) The biochemical nature of HLS1 is a mystery, especially whether or how its putative N-acetyltransferase activity regulates ARF2 and controls its downstream event; (2) Which E3 ubiquitin ligase control HLS1 or ARF2 protein degradation as their protein degradation is 26S proteasome-dependent? And (3) what is the link between light-activated photoreceptors to the downstream protein degradation events?

The regulation of hypocotyls elongation presents another interesting observation for light and ethylene interaction. Ethylene inhibits hypocotyl elongation in dark, but promotes it in light (Alonso et al. 1999; Guzman and Ecker 1990; Smalle et al. 1997). The elongation of hypocotyl cells causes ethylene-stimulated hypocotyl elongation in light. It is reported that one basic-helix-loop-helix (bHLH) transcription factor PHYTOCHROME-INTERACTING FACTROR3 (PIF3) is required for this process. Loss-of-function mutant *pif3* is insensitive to the ethylene-stimulated hypocotyl elongation upon light exposure. Given the evidence that *pif3* suppresses the long hypocotyl phenotype of *ctr1* or *EIN3ox*, and that overexpression of *PIF3* rescues the short hypocotyl phenotype of *ein3 eil1*, it is concluded that PIF3 acts downstream of EIN3/EIL1 in the regulation of hypocotyl elongation in light (Zhong et al. 2012). However, *pif3* mutant does not show any remarkable ethylene-related hypocotyl phenotype in dark, suggesting that PIF3 functions specifically in light. Further results show that no matter in light or dark, ethylene treatment induces *PIF3* expression and EIN3 directly associates with *PIF3* promoter to activate its transcription. Although *PIF3* transcript is induced by ethylene in both light and dark conditions, light rapidly promotes PIF3 protein degradation through LIGHT-RESPONSE BRIC-A-BRACK/TRAMTRACK/BROAD (LRB) E3 ubiquitin ligases (Al-Sady et al. 2006; Ni et al. 2013, 2014; Zhong et al. 2012). In other words, PIF3 protein accumulation is abundant in dark but limited in light. It is thus hypothesized that ethylene-induced *PIF3* transcription cannot exert an additive effect in dark but can only contribute to the hypocotyl elongation in light. On the other hand, ethylene-induced AP2-type transcription factor ETHYLENE RESPONSE FACTOR1 (ERF1) controls the ethylene-promoted hypocotyl growth inhibition in dark. Similar to PIF3, *ERF1* is another EIN3/EIL1 target gene.


(a)
Ethylene
Light
HLS1
ARF2
Hook development
(d)
Jasmonate
COI1
JAZs
Ethylene
MYC2
EIN3
EBF1
HLS1
ERF1
(b)
Dark
Light
Ethylene
EIN3
COP1
ERF1
HY5
Hypocotyl elongation
Ethylene
EIN3
COP1
PIF3
HY5
Hypocotyl elongation
(c)
Ethylene
WEI2
WEI7
Chorismate
Anthranilate
L-Trptophan
TAA1/
TARs
VAS1
Indole-3-pyruvic acid
YUCCA
Indole-3-acetic acid

◀ **Fig. 8.1** **a** Light antagonizes ethylene-induced apical hook formation. Ethylene promotes HLS1 expression and ARF2 degradation to induce hook formation, while light represses HLS1 expression and accumulates ARF2 to inhibit hook development. **b** A simplified illustration depicting the opposite ethylene responses under *dark* and *light* conditions. In *dark*, ethylene-activated EIN3 induces ERF1 expression, which inhibits hypocotyl elongation. Although COP1 targets HY5 for degradation and promotes hypocotyl elongation, enhanced ERF1 expression overrides the loss of HY5 effect to ultimately suppress hypocotyl elongation. In *light*, EIN3-induced PIF3 expression plays a predominant role and COP1 is restrained in the nucleus wherein degrading HY5, so the combination of these two pathways leads to hypocotyl elongation. **c** Ethylene induces auxin biosynthesis. WEI2 and WEI7 catalyze the production of anthranilate, which is further converted to L-tryptophan by several steps. TAA1/TARs convert L-tryptophan to indole-3-pyruvic acid, while VAS1 converts indole-3-pyruvic acid to L-tryptophan. Indole-3-pyruvic acid is then converted to indole-3-acetic acid by YUCCA enzymes. Ethylene induces WEI2, WEI7, and TAA1 expression for promoting auxin synthesis. **d** Jasmonate–ethylene interactions. Jasmonate activates MYC2 and EIN3 transcription factors through the removal of JAZ repressors by promoting COI1–JAZ interactions, while ethylene activates EIN3 via enhancing EIN3 protein accumulation. EIN3 induces HLS1 or ERF1 expression for diverse responses. MYC2 induces EBF1 expression to promote EIN3 turn over and physically interacts with EIN3 to further inhibit its activity

Ethylene induces *ERF1* expression in both light and dark conditions as well. Overexpression of *ERF1* causes short hypocotyl phenotype in dark but not in light (Solano et al. 1998; Zhong et al. 2012). In contrast to PIF3, light stabilizes ERF1 protein, while dark triggers its degradation, which makes ERF1 protein level limited in dark but abundant in light. The effect of ethylene-induced *ERF1* expression is masked in light condition but prominent in dark (Zhong et al. 2012). Taken together, ethylene induces *ERF1* and *PIF3* expression in both dark and light; however, the fate of their encoded proteins is totally opposite under different light conditions. In dark, PIF3 is stabilized and saturated, while ERF1 is degraded and limited, so the ethylene-induced *ERF1* expression contributes to the observed phenotype (short hypocotyl). In contrast to dark, ERF1 is saturated and PIF3 is limited in light, so the consequence of ethylene-induced *PIF3* expression is evident (long hypocotyl). These PIF3-ERF1 and HLS1-ARF2 modules suggest that protein degradation plays a vital role in the control of light–ethylene interactions (Lorrain and Fankhauser 2012; Wang et al. 2013).

In addition to this PIF3-ERF1 module in the controlling of hypocotyl elongation in light and dark conditions, it is reported that one basic leucine zipper (bZIP) transcription factor LONG HYPOCOTYL5 (HY5) is also necessary for ethylene-promoted hypocotyl elongation in light (Yu et al. 2013). HY5 positively regulates plant photomorphogenesis through directly controlling expression of thousands of genes (Lee et al. 2007; Zhang et al. 2011). E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) interacts with HY5 and promotes its degradation in dark, whereas light triggers COP1 movement from the nucleus to cytoplasm to relieve its inhibition on HY5 (Ang et al. 1998; Hardtke et al. 2000; Osterlund et al. 2000; von Arnim and Deng 1994).

It is shown that *hy5* mutant is insensitive to ethylene-promoted hypocotyl elongation in light, but its response in dark is indistinguishable to the wild type,

which implies that HY5 is involved in the ethylene-promoted hypocotyl elongation in light. Immunoblot results show that ethylene promotes HY5 protein degradation specifically in light but not in dark and this degradation process is dependent on EIN3 and COP1. Further analysis demonstrates that COP1 acts downstream of EIN3 in control of HY5 stability. Moreover, ethylene stimulates the nuclear localization of COP1 in light but not in dark, and in the absence of EIN3, this effect is absent, suggesting that ethylene facilitates COP1 movement in an EIN3 dependent manner. This ethylene-promoted COP1 nuclear localization causes the degradation of HY5, which ultimately induces the hypocotyl elongation (Fig. 8.1b) (Yu et al. 2013).

These two separate mechanical studies shed new light on the understanding of how light regulates ethylene-triggered hypocotyl elongation and lead to a new question: how can these two seemly unrelated mechanisms be reconciled? One possible explanation is that both of them may regulate auxin biosynthesis or distribution to eventually regulate hypocotyl cell elongation, given the evidence that auxin determines the hypocotyl cell elongation (Chapman et al. 2012). It is reported that auxin biosynthesis mutants or signaling mutants are insensitive to the ethylene-promoted hypocotyl elongation in light and that HY5 regulates auxin synthesis gene expression and auxin transporter localization (Cluis et al. 2004; Liang et al. 2012). Because *PIF3* related genes (*PIF4*, *PIF5* and *PIF7*) directly regulate auxin biosynthesis (Franklin et al. 2011; Hornitschek et al. 2012; Li et al. 2012; Nozue et al. 2011; Sun et al. 2012), it is speculated that *PIF3* may also contribute to affect auxin levels.

Besides regulating hook development and hypocotyl elongation, ethylene coordinates with light to properly synthesize chlorophyll. In etiolated seedlings, chlorophyll precursors (protochlorophyllide) accumulate in the etioplasts, while upon light exposure three isoforms of protochlorophyllide oxidoreductase (POR) are photoactivated and further catalyze the conversion from protochlorophyllide to chlorophyll. However, if the activity of PORs is inadequate upon light exposure, accumulated protochlorophyllide will produce large amounts of reactive oxygen species (ROS) that will cause cellular damage. The amount of protochlorophyllide is positively correlated with the dark incubation time. When seedlings are incubated in dark for 3–4 days and then exposed to light for 2 days, the average greening rate in wild type is more than 90 %. However, when seedlings are incubated in dark for an extended number of days, such as 9 days, followed by light illumination for 2 days, their greening rate decreases to less than 20 %. Ethylene supplement greatly rescues this reduction of greening rate. In the presence of ethylene, although seedlings are still kept in dark for 9 days and then exposed to light for 2 days, the greening rate is improved from 20 to 80 %. Further results show that EIN3/EIL1 are required for this regulation and they directly bind to the *PORA* and *PORB* (two major isoforms of POR genes) promoters to induce their mRNA expression and catalyze the protochlorophyllide conversion (Zhong et al. 2009). In addition, ethylene-induced PIF3 binds to the promoters of *HEMA1*, *GUN4* and *GUN5* genes and represses their expression. HEMA1, GUN4, and GUN5 proteins are essential for the production of protochlorophyllide (Zhong et al. 2014). In conclusion, ethylene-activated EIN3/

EIL1 directly induce *PORA*/*PORB* expression to promote protochlorophyllide conversion and induce *PIF3* expression to further block protochlorophyllide accumulation to prevent light-induced photo-oxidative damage.

8.2 Interaction with Auxin

Without a doubt, auxin is one of the most extensively studied plant hormone in the phytohormone field during the past several decades. The interactions between ethylene and auxin are broadly investigated from earlier physiological studies to the current genomic studies. Due to limited space in this chapter, we could not include most of the early literature regarding exciting discoveries in the field. Instead, we will mainly point out the molecular mechanisms of ethylene–auxin interactions, particularly the interesting discoveries reported recently. There are several specific detailed reviews discussing the findings in the ethylene–auxin interactions (Lee and Cho 2013; Robles et al. 2013; Vanstraelen and Benkova 2012; Zhao and Guo 2011), readers who want to know more are encouraged to further refer to those reviews. Details about the integration of ethylene and auxin signaling can be found in Chap. 10.

Apical hook formation of etiolated seedlings by ethylene is a result of asymmetric auxin distribution, which causes the differential cell elongation. Auxin and ethylene act synergistically to inhibit root elongation and promote root hair initiation, but antagonistically in other growth events, such as lateral root development and root gravitropism. The general concept for the underlying mechanism is that ethylene modulates auxin biosynthesis and/or polar transport, especially in a cell-type-specific manner, which eventually causes the redistribution of auxin in different cells and alters the growth patterns. On the other hand, auxin also regulates ethylene biosynthesis through inducing the expression of several key enzymes in the ethylene biosynthesis pathway. In order to illustrate the molecular mechanisms more clearly, we first introduce the basic auxin biology in the following paragraphs.

Auxin synthesis is complicated and has been studied for a relatively long time. Only recently has the main auxin synthesis route been convincingly elucidated through comprehensive genetic analysis and chemical quantification methods. Indole-3-acetic acid (IAA) is the most common form of auxin in planta. L-tryptophan (Trp) is recognized as the main precursor for auxin synthesis, which is then converted to indole-3-pyruvic acid (3-IPA) by a family of tryptophan aminotransferase named TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) and TAA1-related 1–4 (TARs). TAA1 is identified by several individual research groups, and also named WEAK ETHYLENE INSENSITIVE8 (WEI8), SHADE AVOIDANCE3 (SAV3), or TRANSPORTER INHIBITOR RESISTANT2 (TIR2) in their reports (Stepanova et al. 2008; Tao et al. 2008; Yamada et al. 2009). Flavin-containing monooxygenase family proteins (called YUCCA) convert 3-IPA to IAA (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011). Auxin transporters are required for auxin distribution into different cells.

AUXIN RESISTANT1 (AUX1) and LIKE AUX1 (LAX1) are auxin influx carriers, whereas PIN-FORMED (PIN) proteins and ATP BINDING CASSETTE TYPE B/P-GLYCOPROTEIN/MULTIDRUG RESISTANCE (ABCB/PGP/MDR) proteins are auxin efflux carriers (Benjamins and Scheres 2008).

Auxin signaling comprises two different pathways, one acts inside the nucleus and the other one acts on the cell surface. TRANSPROT INHIBITOR1 (TIR1) and its interacting AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins are the co-receptors for auxin perception in the nucleus (Dharmasiri et al. 2005; Kepinski and Leyser 2005). Crystallography studies show that auxin functions like "molecular glue" to promote TIR1-AUX/IAA protein–protein interaction (Tan et al. 2007). TIR1 is an F-box protein, which forms an SCF^{TIR1} E3 ubiquitin ligase and directly targets AUX/IAA degradation through 26S proteasome pathway (Gray et al. 2001). In the absence of auxin, AUX/IAA proteins recruit the Groucho/Tup1 co-repressor protein TOPLESS and interact with the downstream AUXIN RESPONSE FACTOR (ARFs) transcription factors to repress their transcriptional activities (Szemenyei et al. 2008). After auxin perception, AUX/IAA proteins are degraded by SCF^{TIR1} to release their repression of ARFs. ARFs then elicit the downstream gene expression and regulate a variety of auxin responses. Besides these TIR1-AUX/IAA receptors, cell-surface localized AUXIN BINDING PROTEIN1 (ABP1) was proposed to be an auxin receptor for a long time. ABP1 was found to be involved in cell morphogenesis, root development, and the endocytosis regulation of PIN efflux carriers (Robert et al. 2010; Xu et al. 2010). Further studies reveal that cytoplasm membrane localized ABP1 activates ROPs (Rho-like guanosine triphosphatases from plants) in an auxin-dependent manner to regulate cytoskeleton reorientation and PIN protein endocytosis (Robert et al. 2010; Xu et al. 2010). Very recently, it was reported that auxin promotes the membrane localized TRANSMEMBRANE KINASE (TMK) members of the receptor-like kinase family proteins interact with ABP1, which are required for the activation of ROPs (Xu et al. 2014). The auxin-promoted ABP1–TMK interaction on the cell surface implies that auxin tends to function as "molecular glue" regardless its cellular localizations.

Returning to the ethylene–auxin interactions, ethylene was reported to modulate the auxin action via the regulation of its biosynthesis. It is shown that ethylene promotes auxin biosynthesis through upregulation of *WEAK ETHYLENE INSENSITIVE2* (*WEI2*) and *WEI7* transcription. *WEI2* and *WEI7* each encode a subunit of anthranilate synthase, which is a rate-limiting enzyme for the auxin precursor Trp synthesis (Stepanova et al. 2005). Both *wei2* and *wei7* mutants are insensitive to ethylene in the aspect of root growth inhibition but not in hypocotyl or hook, which suggests that auxin is necessary for the ethylene-triggered root growth inhibition. Ethylene induces the auxin reporter *DR5*::*GUS* expression in root tips, while this induction is diminished in *wei2* or *wei7* mutants, further supporting that ethylene induces auxin synthesis through WEI2 and WEI7. Similar to WEI2 and WEI7, ethylene also induces *WEI8* (*TAA1*) expression and promotes auxin production to inhibit root elongation (Stepanova et al. 2008). Taken together, these two studies demonstrate that ethylene upregulates at least two consecutive catalytic steps to promote auxin biosynthesis for root growth inhibition (Fig. 8.1c).

In an attempt to screen for *sav3*/*wei8*/*taa1* suppressors, *vas1* (for reversal of *sav3* phenotype) mutant was identified and further investigated. *VAS1* encodes a pyridoxal-phosphate-dependent aminotransferase, which converts 3-IPA to Trp (Zheng et al. 2013). More interesting, this reaction relies on methionine (Met) as an amino donor and 3-IPA as an amino acceptor. Of note, Met is the precursor for ACC synthesis. That is to say, VAS1 suppresses both Met and 3-IPA accumulation by catalyzing their conversion to Trp and 2-oxo-4-methylthiobutyric acid. Experimental evidence indicates that both IAA and ACC concentrations in *vas1* mutant are higher than the wild-type control, suggesting that auxin biosynthesis and ethylene biosynthesis are linked by VAS1, a metabolic enzyme (Fig. 8.1c). This study provides a new view on the understanding of the complex interactions between auxin and ethylene.

Another level of ethylene–auxin interactions is ethylene's modulation of auxin distribution because polar auxin transport is necessary for generating proper ethylene responses. Several independent ethylene-insensitive mutant screening experiments (including our unpublished results) have demonstrated that *aux1* and *pin2,* both of which are defective in polar auxin transport, are insensitive to ethylene in root growth inhibition or *DR5*::*GFP* reporter expression (Luschnig et al. 1998; Negi et al. 2008; Pickett et al. 1990). It is further reported that ethylene promotes *AUX1* or *PIN2* transcription to elevate the auxin transport (Lewis et al. 2011; Ruzicka et al. 2007).

The auxin- and ethylene-regulated gene expression profiles at the genomic scale have also been investigated. Including *aux1* (impaired in auxin transport in certain cell types) and *ein2* (blocking ethylene signaling) mutants in their experimental design, Jose Alonso's group found that nearly a quarter of auxin-regulated gene expression is dependent on ethylene and vice versa, suggesting that auxin and ethylene regulate gene expression in a largely independent manner (Stepanova et al. 2007). Revealing the direct gene targets for EIN3, the recent EIN3 chromatin immunoprecipitation sequencing (ChIP-seq) results show that EIN3 can directly target *AUX1*, *IAA29* and several *ARF* genes to modulate auxin responses (Chang et al. 2013), supporting the complicated ethylene-triggered transcriptional control of auxin signaling.

Additional level of the interactions between ethylene and auxin occurs at the protein level, among proteins involved in their signaling. A number of studies demonstrate that ethylene stimulates AUX1 protein degradation on the inner side of apical hook and auxin stabilizes EIN3 protein through $SCF^{EBF1/2}$ in root tips (He et al. 2011; Vandenbussche et al. 2010). Although the E3 ubiquitin ligase that targets AUX1 and how auxin modulates $SCF^{EBF1/2}$ functions are unknown, these reports indicate that the interactions of auxin and ethylene occur both at the transcriptional and posttranscriptional levels.

It can be concluded that the crosstalk between ethylene and auxin exist almost everywhere from their biosynthesis to their posttranscriptional events. With further studies and the advance of research tools, it can be expected that the studies of ethylene and auxin interactions will be more fruitful in the future, especially for the

purposes of identifying different action modes in different cell types and investigating whether ethylene modulates the recently identified ABP1-TMK-ROP cell-surface auxin signaling.

8.3 Interaction with Jasmonate

Ethylene and jasmonate are recognized as two types of crucial defense hormones for against necrotrophic fungi infections (Dong 1998). After pathogen infection, a subset of pathogen responsive genes (such as *ERF1*, *ORA59*, and *PDF1.2*) are quickly induced and to help plants resistant to infections. Both intact ethylene and jasmonate signaling are required for upregulating those gene expressions and enhancing plant resistance to infections. It has been reported that ethylene and jasmonate signaling mutants are more susceptible to *Botrytis cinerea* infections. Both jasmonate and ethylene treatment induce those gene expressions in a synergistic manner. Because jasmonate treatment cannot induce those gene expressions in ethylene insensitive mutants and similarly ethylene cannot induce them in jasmonate insensitive mutants, it suggests that ethylene and jasmonate act interdependently in controlling those gene expressions. (Lorenzo et al. 2003; Penninckx et al. 1996). In addition to their coactions in plant defense, ethylene and jasmonate are also reported to be two necessary hormones for regulating plant root hair development (Zhu et al. 2006). However, signal interactions in plants are more complicated and elaborate than one can imagine. Besides those synergistic interactions, ethylene and jasmonate act antagonistically in many other aspects. Jasmonate inhibits the ethylene-promoted apical hook formation, while ethylene represses the jasmonate-induced wounding responses (Memelink 2009; Turner et al. 2002). Here, we will focus on the recent progresses in understanding those two facets of ethylene and jasmonate interactions.

Without jasmonate perception, bHLH transcription factor MYC2 is repressed by the direct interaction with a family of proteins, named JASMONATE ZIM-DOMAIN (JAZ) proteins (Chini et al. 2007; Thines et al. 2007). JAZ proteins associate with NOVEL INTERACTOR of JAZ (NINJA) adapter protein, which interacts with TOPLESS co-repressors to fulfill the repression on MYC2 (Pauwels et al. 2010). After jasmonate synthesis is induced by pathogen infection or wounding, the bioactive form of jasmonate facilitates the interaction between JAZs and an F-box protein CORONATINE INSENSITIVE1 (COI1), which assembles in SCF^{COI1} E3 ligase and directly targets JAZs degradation (Chini et al. 2007; Devoto et al. 2002; Katsir et al. 2008; Sheard et al. 2010; Thines et al. 2007; Xie et al. 1998; Xu et al. 2002). The degradation of JAZs relieves their repression on MYC2 or other JAZ-interacting transcription factors to activate jasmonate signaling.

As described before, *ERF1* is a direct target gene of EIN3/EIL1 and ERF1, then activates its target genes like *PDF1.2* (Solano et al. 1998). It is thus speculated that EIN3/EIL1 are integration nodes for the interdependent coaction of ethylene and jasmonate. Ethylene activates EIN3/EIL1 by stabilizing EIN3/EIL1 protein

abundance, while jasmonate activates EIN3/EIL1 in an unknown manner. Further experimental results show that JAZ proteins directly interact with EIN3/EIL1 and repress EIN3/EIL1 functions. It is also reported that JAZ proteins interact with HISTONE DEACETYLASE6 (HDA6) as a co-repressor to suppress EIN3/EIL1 functions through histone deacetylation, which suggest that there is an alternative mechanism for JAZ repression other than the NINJA-TOPLESS model. Jasmonate treatment weakens the interaction between HDA6 and EIN3/EIL1 due to the degradation of JAZs, suggesting that jasmonate activates EIN3/EIL1 through a derepression mechanism. In support of this conclusion, *hda6* mutants are hypersensitive to jasmonate treatment (Zhu et al. 2011). Taken together, EIN3/EIL1 integrate jasmonate and ethylene signaling synergy through different mechanisms. Jasmonate activates EIN3/EIL1 through promoting JAZ degradation to release their repression on EIN3/EIL1, while ethylene stabilizes EIN3/EIL1 via the repression on $SCF^{EBF1/2}$ (Fig. 8.1d) (An et al. 2010).

In addition to the synergistic and interdependent interactions, jasmonate antagonizes ethylene-induced hook formation. It is recently reported that jasmonate reduces *HLS1* expression even in the presence of ethylene. Further studies show that jasmonate-activated transcription factor MYC2 directly binds *EBF1* promoter and induces *EBF1* expression. The induction of *EBF1* then promotes EIN3/EIL1 turnover, which suppresses EIN3/EIL1 functions and their downstream *HLS1* expression to fulfill this inhibition effect (Zhang et al. 2014). Moreover, MYC2 physically interacts with EIN3/EIL1 and abrogates EIN3/EIL1 DNA binding ability, which provides another mechanistic illustration for jasmonate-ethylene antagonism (Fig. 8.1d) (Song et al. 2014; Zhang et al. 2014).

The antagonistic interaction between jasmonate and ethylene ensures that their biological effects can be fine-tuned. Because jasmonate- and ethylene-activated downstream defense genes are usually associated with cell death, how to shut down their signal transduction is equivalently important as how to turn it on. Ethylene-stabilized EIN3/EIL1 directly induces *EBF2* expression as a negative feedback regulation (Konishi and Yanagisawa 2008), while jasmonate activates MYC2 to suppress EIN3/EIL1 DNA binding ability and also induce *EBF1* to promote EIN3/EIL1 degradation.

8.4 Interaction with Gibberellins

Gibberellins are a type of growth-promoting hormones, which positively regulate seed germination, apical hook development, hypocotyl elongation, flowering time, and root elongation (Daviere and Achard 2013; Fu and Harberd 2003). It has been reported that ethylene coordinates with gibberellins at least in regulating apical hook development, root elongation, and flowering initiation.

Ethylene promotes apical hook development. However, blocking gibberellins biosynthesis by paclobutrazol (PAC) inhibits hook formation, while treatment with gibberellins induce hook formation (An et al. 2012). It suggests that adequate

gibberellins are required for ethylene function in the regulation of hook development. DELLA proteins are crucial GRAS (for GAI, RGA, SCARECROW) family transcriptional repressors in gibberellin signaling, which include REPRESSOR OF *ga1-3* (RGA), GA-INSENSITIVE (GAI), RGA-LIKE1 (RGL1), RGL2, and RGL3. In the absence of gibberellins, DELLA proteins directly interact with their downstream transcription factors and inhibit or modulate their transcriptional activity. In the presence of gibberellins, gibberellins bind with its receptor GA-INSENSITIVE DWARF1 (GID1) to promote GID1-DELLA interactions, which in turn facilitate the binding of an F-box protein SLEEPY1 (SLY1) to DELLA proteins and then cause DELLA proteins degradation to relieve repressions (Hauvermale et al. 2012; Sun 2008; Sun and Gubler 2004). Further genetic studies demonstrate that *della* mutant (with all five DELLA gene mutations) shows exaggerate hook, but *ein3 eil1* or *ein2* can fully suppress *della* hook phenotype (An et al. 2012). In addition to the phenotypic observations, *HLS1* mRNA expression is also examined under gibberellins treatment. Gibberellins induce *HLS1* expression in the wild type but not in *ein3 eil1*. These results imply that gibberellins positively regulate hook development through ethylene-activated EIN3/EIL1 pathway. Two members of DELLA proteins, GAI and RGA physically interact with EIN3/EIL1. Although there seems to be a lack of biochemical experiments to determine how DELLA-EIN3/EIL1 interactions affect EIN3/EIL1 functions, it is deduced that DELLAs inhibit EIN3/EIL1 transcriptional activities with the help of a reporter line, which carries EIN3-Binding Site driven glucuronidase (*EBS*::*GUS*) to specifically reflect EIN3/EIL1 functions. Ethylene stimulates *GUS* expression, as a consequence of the activation of EIN3/EIL1, but PAC treatment strongly represses *GUS* expression even in the presence of ethylene. PAC treatment is a commonly used experimental strategy for upregulating in vivo DELLA protein levels because blocking gibberellin synthesis inhibits DELLA degradation (Feng et al. 2008; Wang et al. 2009). In conclusion, DELLA proteins directly interact with EIN3/EIL1 and inhibit *HLS1* expression for repressing hook development. Gibberellin treatment leads to the degradation of DELLA and releases their repression of EIN3/EIL1, which activates *HLS1* expression and promotes hook formation.

Ethylene inhibits root elongation, which is largely dependent on auxin as discussed in Sect. 8.2. However, it is reported that gibberellin signaling is also involved in ethylene-induced root growth inhibition. DELLA mutants are less sensitive to ethylene in the aspect of root growth inhibition, while application of gibberellins suppresses ethylene's effect on the root growth inhibition (Achard et al. 2003). These results suggest that gibberellins antagonize ethylene in root growth. Previous report has shown that the degradation of DELLA is required for root elongation (Fu and Harberd 2003). Gibberellin-triggered nuclear GFP-RGA disappearance degradation is slower when ethylene is applied simultaneously, which suggests that ethylene inhibits DELLA degradation. Consistent with this ethylene treatment, GFP-RGA is more stable in the constitutive ethylene responsive mutant *ctr1* than in wild-type background (Achard et al. 2003). Taken together, it is concluded that ethylene modulates DELLA protein stabilities for the regulation of root elongation.

Gibberellins are positive regulators in flowering time control, but how ethylene regulates flowering initiation is not fully understood. It is shown that application of ethylene or activation of ethylene signaling (*ctr1* mutants or *ebf1 ebf2* mutants) delays flowering time (Achard et al. 2007). Consistent with this observation, the bioactive gibberellin contents in *ctr1* are much lower than in wild-type controls. Furthermore, DELLA mutants suppress *ctr1* late flowering phenotype, suggesting that DELLA proteins are downstream factors in the ethylene-regulated flowering time control. Experimental results show that lower bioactive gibberellin contents cause the accumulation of DELLA proteins in *ctr1*, which inhibits floral meristem identity genes like *LEAFY* (*LFY*) and *SUPRESSOR OF OVEREXPRESSION CONSTANS1* (*SOC1*) and causes late flowering (Achard et al. 2007; Blazquez and Weigel 2000).

The promotion of hypocotyl elongation is one of the most remarkable functions of gibberellin. It has been demonstrated that DELLA proteins interact with PIF3 and PIF4 to repress their DNA binding abilities. Gibberellin treatment induces DELLA degradation so as to activate PIF3 and PIF4 and then promotes cell elongation (de Lucas et al. 2008; Feng et al. 2008). Since ethylene promoted cell elongation in light is archived by EIN3-activated *PIF3* mRNA expression and EIN3 physically interacts with DELLA proteins (An et al. 2012; Zhong et al. 2012), it is plausible to further investigate if EIN3 affects PIF3 DNA binding ability by modulating DELLA-PIF3 interactions.

8.5 Concluding Remarks

In conclusion, interactions between ethylene and other plant growth signals exist, from the biosynthesis pathway to the signal transduction pathway. Notably, protein–protein interactions and protein–DNA associations are major connections for integrating different signal interactions. Although there are plenty of achievements in the field of ethylene, the current understanding is still just the tip of iceberg. With the improvement of single cell detection methods and next-generation sequencing techniques, it will be very intriguing to dissect signal interactions in different cell types and take advantage of systems biology approaches to generate signal networks to fully understand signal interactions not only at the static whole plant level, but also at the dynamic single cell level.

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Chapter 9
Integration of Ethylene and Gibberellin Signaling

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Abstract The phytohormones ethylene and gibberellin (GA) act synergistically to regulate a diversity of plant growth and development processes. In the presence of ethylene, the signaling mediated by ethylene receptors and CTR1 (CONSTITUTIVE TRIPLE RESPONSE1) is switched off, while EIN2 (ETHYLENE INSENSITIVE2) and EIN3 (ETHYLENE INSENSITIVE3) together mediate ethylene signaling. GA promotes plant growth by facilitating the degradation of the DELLA proteins, a family of nuclear growth repressors. Although the existence of crosstalk between ethylene and GA in the context of growth and development has long been known, its molecular basis is only now beginning to be understood. Both the synthesis and the signaling pathways controlled by ethylene and GA are reciprocally regulated. In this chapter, recent advances in the understanding of how they regulate germination, root and hypocotyl growth, apical hook development, and flowering initiation are reviewed. The significance of ethylene–GA crosstalk in the plant response to abiotic stress is described.

Keywords Crosstalk · Germination · Root development · Hypocotyl elongation · Apical hook formation · Flowering time · Abiotic stress

9.1 Introduction

The gibberellins (GAs) form a family of tetracylic diterpenoid plant hormones which impinge on various aspects of plant growth and development, from germination through stem, hypocotyl and root growth to the switch from vegetative to

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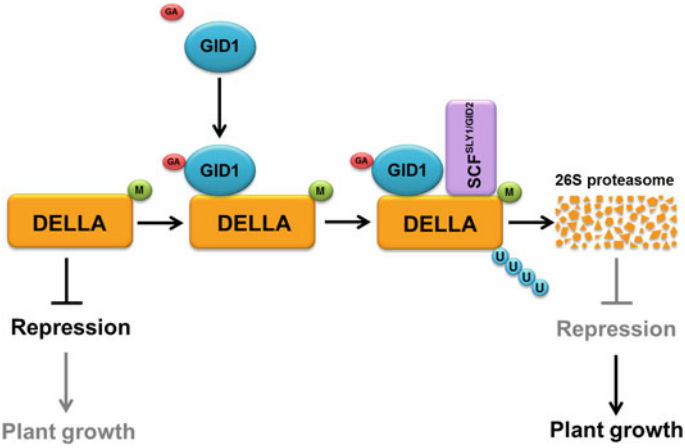

Fig. 9.1 The GA signaling pathway involving DELLA proteins. In the absence of GA, the DELLA proteins repress plant growth. The binding of GA to its receptor GID1 permits the interaction between GID1 and DELLA. The formation of the GID1-GA-DELLA complex enhances the interaction between DELLA and the $SCF^{SLY1/GID2}$ F-box component, leading to the polyubiquitination of DELLA and their targeting for degradation via the 26S proteasome pathway. *M*, post-translationally modified DELLA; *U*, ubiquitinated DELLA

reproductive growth (Sun and Gubler 2004; Jiang and Fu 2007; Gao et al. 2008, 2011). The analysis of GA-insensitive mutants in both *Arabidopsis thaliana* and rice has identified a number of GA signaling components, in particular, the GA receptor GID1 (GIBBERELLIN INSENSITIVE DWARF1), the DELLA proteins (the major repressors of GA signaling), and F-box-containing proteins such as SLY1 [SLEEPY1] in *A. thaliana* and GID2 [GIBBERELLIN INSENSITIVE DWARF2] in rice (Dill et al. 2004; Fu et al. 2004; Ueguchi-Tanaka et al. 2007; Itoh et al. 2008; Shimada et al. 2008). On this basis, a derepression model for GA signaling has been elaborated, according to which once bioactive GA has been perceived and bound by GID1, the resulting complex can drive the formation of a GA-GID1-DELLA protein complex capable of efficiently binding the E3 ubiquitin ligase $SCF^{SLY1/GID2}$ complex (Griffiths et al. 2006). This binding results in the polyubiquitination of the DELLA proteins, thereby directing their degradation via the 26S proteasome, and so relieving the growth suppression exerted by them (Fig. 9.1) (Gao et al. 2011; Daviere and Achard 2013).

The gas ethylene also regulates a wide range of growth and developmental processes. It is perceived by the ETR1 (ETHYLENE RESPONSE1) family of ethylene receptors (Hua et al. 1995, 1998; Hua and Meyerowitz 1998; Sakai et al. 1998; Guo and Ecker 2004). In its absence, ETR1 activates CTR1 (CONSTITUTIVE TRIPLE RESPONSE1), a Raf-like Ser/Thr protein kinase which suppresses ethylene signaling (Kieber et al. 1993; Huang et al. 2003; Mayerhofer et al. 2012). EIN2 (ETHYLENE INSENSITIVE2) acts downstream of CTR1 and represents a critical component of the ethylene signaling pathway (Alonso et al. 1999). In the presence of ethylene, its receptors become inactivated, in turn switching off CTR1

and permitting EIN2 to function. The basis for the EIN2-mediated transduction of the ethylene signal from the ethylene receptors associated with the endoplasmic reticulum to its downstream transcription factors EIN3 and EIL1 (EIN3-LIKE1) has been described independently by three research groups (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012). EIN3 and EIL1 mediate a wide array of the plant responses to ethylene (Chao et al. 1997; Solano et al. 1998; Stepanova and Alonso 2009). The levels of EIN3 and EIL1 in the nucleus are finely tuned by the Skp1-Cullin-F-box protein (SCF) E3 ubiquitin ligase isoforms EBF1 and EBF2 (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004).

The existence of crosstalk between ethylene and GA signaling has been recognized for a long time, but its molecular basis remains to be determined. Both ethylene and GA affect one another at the level of synthesis, signaling and gene expression (De Grauwe et al. 2007, 2008; Dugardeyn et al. 2008). In this chapter, we focus on the interaction between ethylene and GA signaling in the context of the regulation of germination, root growth, hypocotyl elongation, apical hook development, and floral induction (Fig. 9.2). In addition, we also describe their role in the plant response to abiotic stress (Fig. 9.3).

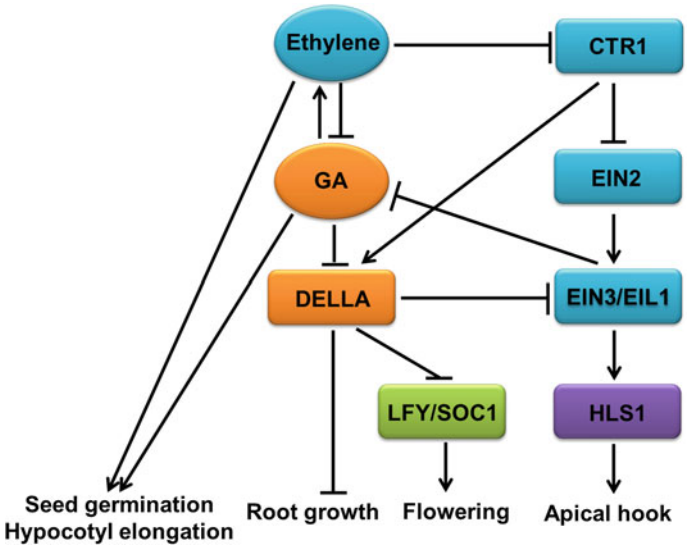

Fig. 9.2 The modulation of *A. thaliana* growth and development, as controlled by ethylene and GA (modified from Weiss and Ori (2007)). Ethylene and GA in concert promote germination and induce the elongation of the hypocotyl in the presence of light. Ethylene and GA also together regulate apical hook development in the absence of light by up-regulating *HLS1*. Ethylene stabilizes EIN3/EIL1, while GA relieves the DELLA-imposed repression on EIN3/EIL1. EIN3/EIL1 links the ethylene and GA pathways to activate *HLS1* transcription. Ethylene inhibits root growth by controlling the abundance/stability of DELLA. Activation of ethylene signaling reduces the level of bioactive GA, thereby promoting the accumulation of DELLA, which in turn delays flowering via the regulation of *LFY* and *SOC1* transcription. *CTR1* CONSTITUTIVE TRIPLE RESPONSE1; *EIN2* and *EIN3*, ETHYLENE INSENSITIVE2 and 3; *EIL1*, EIN3-LIKE1; *HLS1* HOOKLESS1; *LFY* LEAFY; *SOC1* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1

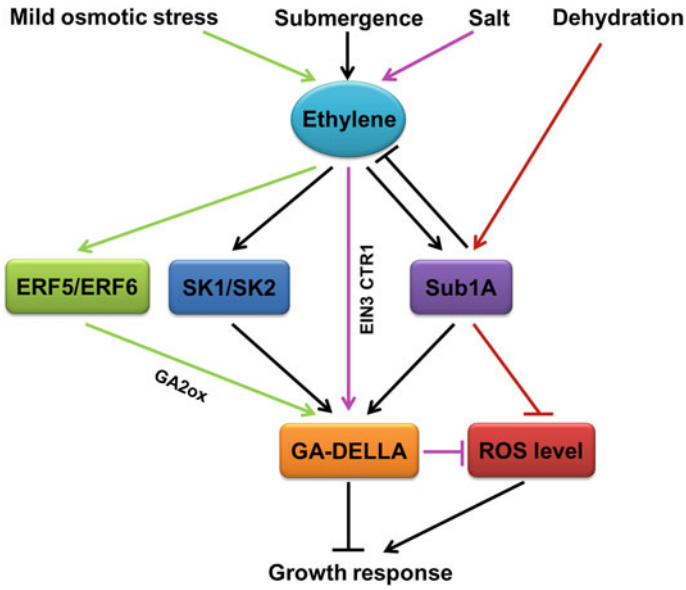

Fig. 9.3 The rice ethylene and GA regulatory network during an episode of abiotic stress (modified from Bailey-Serres and Voesenek (2010)). In deepwater rice, ethylene up-regulates *SK1* and *SK2* and elevates the level of GA, thereby inducing a rapid elongation of the stem internode, allowing the canopy to rise above the water's surface. In submergence-tolerant rice, ethylene activates *Sub1A*, which promotes DELLA accumulation, enhancing survival by limiting stem elongation and restricting assimilate consumption. *Sub1A* also promotes the tolerance of mild osmotic stress by preventing the build-up of damaging levels of reactive oxygen species. Mild osmotic and salinity stress activates ethylene signaling and stabilizes the DELLA proteins, which act to limit plant growth. *ERF1* ETHYLENE-RESPONSIVE FACTOR; *ROS* reactive oxygen species; *SK1* and *2* SNORKEL1 and 2; *Sub1A* Submergence 1A

9.2 Ethylene and GA Coordinately Promote Seed Germination

Seed of the *A. thaliana* GA-deficient mutant *ga1-3* (which synthesizes little or no *ent*-kaurene because it lacks a functional copy of *GA1*) is unable to germinate without the provision of exogenous GA. Ethylene influences germination in *A. thaliana* (Kepczynski and Kepczynska 1997), since the ethylene synthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC) can compensate for the absence of GA in the *ga1-3* seed, allowing it to germinate without GA supplementation in the light; its promotive effect is less marked in the dark (Karssen et al. 1989; Vriezen et al. 2004). Several ethylene response genes, including *ACO* (encoding *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE*), are up-regulated in imbibed *ga1-3* seed following the exogenous supply of GA_4 (Ogawa et al. 2003). The transcription of both the ethylene-inducible gene *HOOKLESS1*

(*HLS1*) (Lehman et al. 1996) and the ethylene receptor gene family's *ERS1* (*ETHYLENE RESPONSE SENSOR1*) (Hua et al. 1998) is similarly increased by the exogenous supply of GA_4 (Ogawa et al. 2003). The implication is that GA may activate ethylene synthesis and/or production, thereby promoting the ethylene responses in the imbibed *A. thaliana* seed.

The dominant *A. thaliana etr1-1* mutant is ethylene insensitive. Freshly harvested *etr1-1* seed germinates less readily than does wild-type (WT) seed (Beaudoin et al. 2000). Only bioactive GA_4 is present in mature WT seed at a concentration of 25 ng/g dry weight (DW), while the concentrations of GA_1, GA_4 and GA_7 are all higher in the *etr1-2* mutant seed than in WT seed. WT seed accumulates GA_1 over the first 18 h of germination, but thereafter its concentration declines, with a concomitant modest rise in the concentration of GA_4. Germinating and germinated WT seed contains 7–12 ng/g DW GAs, whereas *etr1-2* seed contains more GA_1 and GA_4 (Chiwocha et al. 2005). Thus, it appears that while GA may promote ethylene synthesis in imbibed *ga1-3* seed, the enhanced level of GA present in *etr1* seed indicates that ethylene may suppress GA synthesis. The indication is that a feedback mechanism regulates GA and ethylene synthesis, and that these two phytohormones synergistically influence germination (Fig. 9.2).

9.3 Ethylene Inhibits *A. thaliana* Root Growth by Interacting with DELLA

The DELLA proteins are members of the plant-specific GRAS family [named from the first three members of the family to be identified, namely GAI (GIBBERELLIN INSENSITIVE), RGA (REPRESSOR OF GA1-3), and SCARECROW] and are thought to represent transcriptional regulators. The *A. thaliana* DELLA proteins include GAI, RGA, RGL1 (RGA-LIKE1), RGL2, and RGL3 (Jiang and Fu 2007), all of which act to repress GA signaling (Dill et al. 2001; Silverstone et al. 2001; Fu and Harberd 2003). The root is a key organ because it is responsible for the acquisition of water and nutrients. The *ga1-3* mutant develops a shortened primary root, which can be made to grow to a WT length either by providing the plant with exogenous GA or by knocking out DELLA. These observations show that the GA-DELLA regulatory system operates in the context of seedling root growth (Fu and Harberd 2003). Ethylene also inhibits root growth. When *A. thaliana* seedlings are exposed to either ethylene or ACC, primary root growth is inhibited (Abeles et al. 1992), and this inhibition involves the DELLA proteins GAI and RGA (Achard et al. 2003) (Fig. 9.2). When ACC is not supplied to seedlings of DELLA protein-lacking mutants (*gai-t6*, *rga-24*, and *gai-t6 rga-24*), the root length is identical to that of the WT. Conversely, in the presence of ACC treatment, the WT roots are shorter than those of *rga-24* seedlings, and the double mutant *gai-t6 rga-24* roots grow even longer than those of seedlings of the two single mutants. These data suggest that GAI and RGA together mediate ethylene-induced root growth inhibition. In addition, GA treatment can overcome the ACC-induced inhibition of seedling root growth (Fig. 9.2).

GFP (green fluorescent protein)-RGA fusion protein is detectable in root cell nuclei, but rapidly disappears in response to GA treatment (Silverstone et al. 2001). Ethylene appears to inhibit root growth in *A. thaliana* by delaying the GA-mediated degradation of GFP-RGA (Achard et al. 2003) (Fig. 9.2). When *pRGA*::*GFP-RGA* containing transgenic plants are treated with GA, the GFP signal is markedly attenuated within 90 min at both the root tip and the root elongation zone and disappears completely by 3 h. However, when the same seedlings are grown in an atmosphere containing ethylene gas, the fusion protein becomes more stable, remaining readily detectable even 3 h after the GA treatment. Ethylene promotes many responses which are antagonized by CTR1, and the GFP-RGA fusion product is less readily degraded as a result of GA treatment in the mutant than in WT plants. The implication is that ethylene delays the GA-mediated degradation of GFP-RGA via a CTR1-dependent signaling pathway (Achard et al. 2003) (Fig. 9.2).

9.4 Ethylene and GA Synergistically Induce the Elongation of the *A. thaliana* Hypocotyl

In *A. thaliana*, hypocotyl elongation is achieved predominantly via cell elongation and is tightly regulated by various phytohormones. GA promotes the process, whereas ethylene inhibits it (Cowling and Harberd 1999; Collett et al. 2000). Ethylene has been shown to stimulate hypocotyl elongation when seedlings are grown in a low nutrient medium (LNM) (Smalle et al. 1997). In this situation, most of the elongation occurs over the first three days following imbibition. Treatment with ACC extends the period of rapid growth for an additional day. In contrast, treatment with GA_3 has no effect on the duration of rapid growth, but does increase the growth rate between days two and three (Saibo et al. 2003). The effect of a combined application of ACC and GA_3 is at the very least additive, and in most cases synergistic, thereby resulting in the highest increase in hypocotyl length (Saibo et al. 2003; vandenBussche et al. 2007) (Fig. 9.2). Furthermore, the hypocotyl elongation in the *gai etr1-3* double mutant plant is completely insensitive to either phytohormone (De Grauwe et al. 2007).

The ethylene-mediated regulation is now known to depending on blue light and cryptochrome signaling, and that GA is required for the ethylene-stimulated hypocotyl elongation to occur (vandenBussche et al. 2007). Treatment with ACC in the presence of blue light up-regulates the transcription of the GA synthesis genes *GA20ox1* (*GA 20-oxidase*) and *GA3ox1* (*GA 3-oxidase*) and down-regulates that of the GA metabolism genes *GA2ox1* (*GA 2-oxidase*) and *GA2ox7*, as well as that of the GA synthesis gene *GA3ox2* (Vriezen et al. 2004; Vandenbussche et al. 2007). There is no substantial effect on the abundance of transcript of genes encoding either GA receptors or the DELLA proteins, although the accumulation of GFP-RGA fusion protein is enhanced in ACC-treated *pRGA*::*GFP-RGA* plants (Vandenbussche et al. 2007).

GA and ethylene have an effect on endoreduplication frequency (Traas et al. 1998; Gendreau et al. 1999). Ethylene induces endoreduplication in both light- and dark-grown hypocotyl, and the process involves the ethylene signaling pathway genes *CTR1* and *EIN2* (Gendreau et al. 1999). Hypocotyl cells in LNM-grown seedlings contain nuclei with three ploidy levels: 2C, 4C, and 8C (C is the DNA content of the haploid genome in the G1 phase). ACC treatment increases the 8C:4C ratio and a small fraction of cells undergo an additional round of endoreduplication to deliver about 2 % 16C nuclei. Similarly, GA_3 treatment increases the 8C:4C ratio, but no 16C nuclei are produced. A combined ACC and GA_3 treatment has the greatest enhancing effect on the 8C:4C ratio and on the representation of 4 % 16C nuclei (Saibo et al. 2003).

Although cell elongation is mainly responsible for hypocotyl growth, cell division also should be considered. Treatment with either ACC or GA_3 on their own increases cortical cell number by, respectively, 1.4 and 1.3, while in combination, they enhance cortical cell number by 2.5 (Saibo et al. 2003). However, the measurements of GUS activity generated by *CycB1;1*::*GUS* transgenics (the *CycB1;1* product is involved in cell division) imply that cell division occurs solely in the hypocotyl epidermal layers, which contributes to the development of stomata. Treatment with either ACC or GA increases stomata number in the hypercotyl by, respectively, 33 % and 21 %, while the combined treatment increases it by 55 % (Saibo et al. 2003).

9.5 Ethylene-Induced Apical Hook Development Is Dependent on GA

The apical hook is a transient curvature of the hypocotyl tip assumed to protect the cotyledons and the shoot apical meristem from mechanical damage as the seedling grows through the soil. The curvature is generated by asymmetric growth, specifically via differential cell division and elongation of the inner and outer sides of the hypocotyl (Lehman et al. 1996; Raz and Ecker 1999; Raz and Koornneef 2001). The formation of the apical hook involves three developmental phases: formation, maintenance, and opening (Raz and Ecker 1999; Abbas et al. 2013). Ethylene is known to be an important regulator of apical hook development. Dark-grown seedlings treated with exogenous ethylene or ACC produce a shortened root, a shortened and radially expanded hypocotyl, and an exaggerated apical hook (Abeles et al. 1992; Ecker 1995). GA is also involved in apical hook development. The *ga1-3* mutant does not form an apical hook in three-day-old etiolated seedlings, whereas in the absence of either GAI and/or RGA, a hook is formed by this mutant. These data indicate that GA opposes DELLA proteins repression and can promote the apical hook formation (Achard et al. 2003) (Fig. 9.2). In addition, the GA-DELLA regulatory system also affects apical hook maintenance, involving both ethylene and auxin signaling.

In the presence of either ethylene or ACC, the size of the apical hook is exaggerated in WT seedlings, but this is not the case in the *ga1-3* mutant (Achard et al. 2003). The *ctr1-1* mutant does not form a hook in the presence of inhibitor of GA synthesis paclobutrazol (PAC), consistent with the notion that GA is involved in apical hook formation, even in a constitutive ethylene signaling status (Vriezen et al. 2004). The development of the apical hook requires differential cell division and elongation at the hypocotyl apex (Raz and Koornneef 2001). Although GA and ethylene have little effect on cortical cell division in the hypocotyl, the influence of both ACC and GA on cell division during apical hook development is clear. PAC almost completely inhibits cell division in two- or three-day-old dark-grown seedlings, and *CycB1*;*1*::*GUS* transgenic seedlings harbor almost no GUS-stained cells. Thus, GA_3 counteracts the PAC effect and stimulates cell division. The combined application of ACC and GA_3 has the strongest stimulatory effect on cell division; but when compared with the effect of GA_3 on its own, the extent of the enhancement is not significant. The conclusion is that ethylene acts to enhance the effect of GA on cell division (Vriezen et al. 2004).

Similar to the effect on GFP-RGA level in the root and hypocotyl, ethylene also induces RGA accumulation in the nucleus within the apical hook. In addition, ACC treatment clearly up-regulates the transcription in the apical region of the GA-responsive gene *GASA1* (Herzog et al. 1995). GA homeostasis is regulated by feedback mechanisms, triggered by changes in GA-signaled GA synthesis and metabolism (Hedden and Phillips 2000). For example, the transcription of *GA1* is strongly up-regulated in PAC-treated seedlings. ACC treatment also markedly up-regulates *GA1* in the apical hook endodermis, which suggests that ACC, like PAC, attenuates the GA response and modulates the level of feedback over GA synthesis (Vriezen et al. 2004).

Gallego-Bartolome et al. (2011) have described a kinematic analysis of GA action during apical hook development. A comparison between the impact of PAC, ACC, and GA on WT (*A. thaliana* ecotype Landsberg *erecta*), the *gai-1* (*GA* signaling pathway gain-of-function mutant), *della* (a complex mutant which does not produce any of the five DELLA proteins), and the *ein2-1* (ethylene-insensitive mutant) (Guzman and Ecker 1990; Peng et al. 1997; Feng et al. 2008) demonstrates that GA plays a prominent role during the formation and opening the apical hook. PAC-treated WT seedlings fail to form a hook and gradually enter the opening phase, whereas the *della* mutant develops an exaggerated apical hook which is not induced to open by ACC treatment. Seedlings of *ein2-1* form no hook, although this failure can be partially reversed by GA treatment. The conclusion is that GA and ethylene probably act independently of one another during hook formation, whereas the two phytohormones cooperate to prevent hook opening. In further experiments, the same authors have established that GA activity in the endodermis is essential for normal hook development, at least during the late formation phase, whereas epidermal GA activity is non-essential.

The *gai-1* mutant displays a reduced level of transcription of the ethylene synthesis genes *ACC SYNTHASE8* (*ACS8*) and *ACS5/ETO2* (Vogel et al. 1998; Yamagami et al. 2003), as well as of the ethylene-inducible gene *HLS1* (Lehman

et al. 1996), which all contribute to ethylene-induced hook development. Given that GAI and PIF5 (PHYTOCHROME INTERACTING FACTOR5) interact with one another in vivo, and that PIF5 binds the *ACS8* promoter in a GA-dependent manner, a possibility is that the DELLA proteins repress *ACS8* expression by inhibiting PIF5 activity. Moreover, GA-regulated *ACS* expression is associated with ethylene production in etiolated seedlings, and the *della* mutant produces more ethylene than does the WT plant (Gallego-Bartolome et al. 2011). GA also up-regulates *HLS1* expression (Gallego-Bartolome et al. 2011; Abbas et al. 2013). Analysis has shown that there is a temporal coincidence in the requirement of GA and HLS1 activity during hook development (Gallego-Bartolome et al. 2011).

EIN3/EIL1 are the primary transcription factors in the ethylene signal transduction pathway (Alonso et al. 2003), and overexpression of either *EIN3* or *EIL1* results in exaggerated hook curvature (Chao et al. 1997; An et al. 2010). An and her colleagues find that GA_3 enhances, whereas PAC represses, ethylene- and EIN3-overexpression-induced hook curvature in six-day-old etiolated seedlings, and *della* mutant exhibits exaggerated hook curvature, which requires an intact ethylene signaling pathway (An et al. 2012). *HLS1* encodes a protein with sequence similarity to N-acetyltransferase, and mutation in *HLS1* has no effect on the growth of the hypocotyl apex in the presence of exogenous ethylene (Lehman et al. 1996). HLS1 is also required for GA to have any effect on hook development (Gallego-Bartolome et al. 2011). The *hls1* mutation overrides the exaggerated hook curvature phenotype shown by *della*, and the *hls1 della* sextuple mutant forms no hook, which has been taken to imply that GA- and DELLA-regulated hook development is dependent on *HLS1* (An et al. 2012) (Fig. 9.2). Furthermore, ethylene and GA induce *HLS1* transcription in an EIN3/EIL1-dependent manner and neither can induce *HLS1* transcription in the *ein3 eil1* mutant. Other experiments have shown that EIN3 binds directly with the *HLS1* promoter to induce its expression (An et al. 2012). The DELLA proteins RGA and GAI may interact with the DNA-binding domains of EIN3/EIL1 in vivo and repress EIN3/EIL1-regulated *HLS1* expression (An et al. 2012) (Fig. 9.2).

9.6 Ethylene-Induced Flowering May Result in Part by a Modulation of DELLA Activity

A number of mechanisms have evolved to take account of endogenous signals and environmental cues to time the plant's transition from vegetative to reproductive growth, a critical moment in the life cycle of the flowering plant (Achard et al. 2007). That ethylene modulates the vegetative growth of *A. thaliana* in response to changes in the environment has been amply demonstrated (Abeles et al. 1992; Wang et al. 2002; Achard et al. 2006). However, its participation in the regulation of the switch from vegetative to reproductive growth is less clear (Thomas and Vince Prue 1997; Achard et al. 2007). On the other hand, GA is clearly important for controlling this

transition in *A. thaliana* (Mutasa-Gottgens and Hedden 2009; Srikanth and Schmid 2011). The *ga1-3* mutant is unable to flower under short-day (SD) conditions without exogenously supplied GA, and the flowering of the *gai* mutant is substantially delayed under SD conditions, a phenotype which cannot be rescued by GA_3 treatment (Wilson et al. 1992). GA promotes flowering in *A. thaliana* by activating floral meristem identity genes such as *LEAFY* (*LFY*) and *SUPPRESSOR OF OVEREXPRESSION CONSTANS1* (*SOC1*). The DELLA proteins act to delay flowering under SD conditions by repressing *LFY* and *SOC1* transcription (Moon et al. 2003; Achard et al. 2004, 2007; Mutasa-Gottgens and Hedden 2009).

Ethylene delays flowering in a DELLA-dependent manner (Fig. 9.2). In the presence of ACC or in an ethylene-rich atmosphere, flowering is delayed in WT *A. thaliana,* although the effect is less marked in both the *gai-t6 rga-24* double mutant and the quadruple DELLA mutant *gai-t6 rga-t2 rgl1-1 rgl2-1* (Achard et al. 2006). Floral transition is also delayed in the *gai eto2-1* double mutant, which overproduces ethylene, and even more strongly than in WT plants exposed to ethylene (De Grauwe et al. 2008). The loss-of-function *ctr1-1* mutant is late flowering under both long-day (LD) and SD conditions. Compared with WT plants, the level of GA_1 and GA_4 is significantly reduced in LD-grown *ctr1-1* mutant plants, while the content of intermediate GA species (such as GA_{24} and GA_{53}), which act as substrates for GA 20-oxidase in the synthesis of bioactive GA (Hedden and Phillips 2000), is significantly enhanced. Both *AtGA3ox1* and *AtGA20ox1* transcript abundance is higher in the *ctr1-1* mutant than in WT plants, but in the triple mutant *ctr1-1 gai-t6 rga-24*, the levels of transcript revert to those seen in the WT, thereby implicating DELLA function in the up-regulation of the two GA oxidase genes in *ctr1-1* (Achard et al. 2007).

The abundance of both *LFY* and *SOC1* transcript is lower in the *ctr1-1* mutant than in WT plants, but GA-treated *ctr1-1* plants or *ctr1-1* plants lacking both *GAI* and *RGA* exhibit a relatively normal *LFY* and *SOC1* transcript level. The inference is that the ethylene-mediated inhibition of CTR1 activity reduces the level of bioactive GA, resulting in a down-regulation of *LFY* and *SOC1*, and hence a delay in the switch to reproductive growth (Achard et al. 2007) (Fig. 9.2). The *gai-t6 rga-24* double mutant, however, rescues the late-flowering phenotype expressed by *ctr1-1* plants exposed to SD conditions. Similarly, the delayed flowering of *ctr1-1* grown under LD conditions can be negated either by GA treatment or by deleting *GAI* and *RGA*. SPINDLY (SPY), an *O*-linked *N*-acetylglucosamine transferase, acts as a negative regulator of GA signaling, and this has been demonstrated by showing that the loss-of-function *spy* mutation partially suppresses the late-flowering phenotype of the GA-deficient *ga1-2* mutant exposed to SD (Jacobsen and Olszewski 1993). However, the *ctr1-1 spy-5* double mutant flowers much earlier than the *ctr1-1* single mutant. Thus, the greater sensitivity to GA brought about by the lack of SPY at least partially suppresses the delay to floral transition conferred by the *ctr1-1* mutation (Achard et al. 2007). Ethylene activates ethylene responses by inhibiting $SCF^{EBF1/EBF2}$ activity, which in turn increases the stability of EIN3 and EIN3-LIKE proteins (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004). The constitutive ethylene response phenotype shown by the loss-of-function *ebf1-1 ebf2-1* mutant is

stronger than that of *ctr1-1*, and the flowering delay can be overridden by exogenously supplied GA. The level of EIN3 present is unaffected both by GA treatment of the *ebf1-1 ebf2-1* mutant, and by the absence of GAI and RGA (as in the *ctr1-1 gai-t6 rga-24* mutant), indicating that the DELLA proteins act downstream of CTR1/EIN3 in the ethylene-dependent regulation of flowering (Achard et al. 2007) (Fig. 9.2).

9.7 The Interaction of Ethylene and GA Signaling in Plant Response to Abiotic Stress

Environmental stresses, such as drought, soil salinity, and flooding, can all depress plant growth and seed production, and a suite of protective mechanisms, many of which involve phytohormones, have been evolved to cope with these stresses. By modifying the level, distribution, and/or signal transduction activity of these phytohormones, a plant is able to adjust its physiology and biochemistry quite rapidly, a critical requirement for its survival (Colebrook et al. 2014). Abscisic acid (ABA) and ethylene are the two most frequently encountered phytohormones associated with the abiotic stress response. In *A. thaliana*, the response to salinity is triggered (at least in part) by an accumulation of ABA, which activates a variety of ABA signaling pathways (Zhu 2002; Shinozaki et al. 2003). Salinity inhibits growth (at least in part) via the ABI1-dependent, ABA-mediated enhancement of DELLA (Achard et al. 2006). In deepwater rice, submergence stimulates the degradation of ABA, thereby enhancing the plant's responsiveness to GA and promoting the elongation of the internode (Hoffmann-Benning and Kende 1992). A brief account follows describing what is known regarding the interaction between ethylene and GA signaling during the abiotic stress response.

Prolonged flooding results in the development of hypoxic conditions in the soil, which has a dramatic impact on levels of respiration and photosynthesis, on redox homeostasis, and on intracellular pH. Paddy rice, and particularly deepwater rice, has developed various strategies to prevent the build-up of anoxia in their tissue (Xu et al. 2006; Fukao et al. 2006). Deepwater rice plants respond rapidly to a rising water level by elongating their stem internodes to maintain the upper canopy above the water's surface. This process is strongly regulated by ethylene, the rapid accumulation of which in submerged tissue (via physical entrapment and active synthesis) triggers shoot elongation, adventitious root formation, and alterations in carbohydrate metabolism (Steffens et al. 2006; Xu et al. 2006; Hattori et al. 2009). GA appears to be involved in some of these ethylene-mediated responses (Steffens et al. 2006; Xu et al. 2006). Ethylene also coordinates the balance of GA and ABA content during submergence (Xu et al. 2006). In deepwater rice, adventitious roots are induced to grow from a node, and their development in time replaces or at least supports the main root system which will have become increasingly dysfunctional as a result of prolonged hypoxia (Steffens et al. 2006). The growth of these

secondary roots is mediated by ethylene (Suge 1985; Lorbiecke and Sauter 1999), while GA has little or no effect on their development. Nevertheless, a combined treatment of ethylene and GA results in a synergistic promotive effect, while the application of a competitive inhibitor of ethylene abolishes root growth. The inference is that ethylene perception is required for adventitious root growth, and that the resulting ethylene-induced growth of adventitious roots is strongly promoted by GA (Steffens et al. 2006).

GA's involvement in the regulation of the ethylene-mediated growth response of deepwater rice lies not only in inducing adventitious root growth but also in promoting shoot growth. Hattori et al. (2009) have identified that the genes *SNORKEL1* (*SK1*) and *SK2* are responsible for allowing the rapid elongation of the stem internode. These genes are specific to deepwater rice germplasm, and are up-regulated by flooding. The constitutive expression of *SK1* and *SK2* in non-deepwater rice has a positive effect on stem internode elongation even under non-flooded conditions. SK1 and SK2 both possess a single APETALA2/ethylene response factor (AP2/ERF) domain. Their transcription is inducible by exogenously supplied ethylene, but not by any other phytohormone. The *SK1* and *SK2* promoter regions harbor core sequences for EIN3-binding site and can bind to the rice EIN3-like protein (OsEIL1b). The suggestion is that *SK1* and *SK2* serve as ethylene response factors and form part of an ethylene signaling pathway which positively regulates stem internode elongation in deepwater rice (Fig. 9.3).

Physiological experiments show that ethylene, ABA, and GA are all involved in the submergence response (Kende et al. 1998). Ethylene has been identified as an initiation factor for internode elongation (Raskin and Kende 1984; Kende et al. 1998). When plants are submerged, although ethylene accumulates in both deepwater and non-deepwater rice, it only induces stem internode elongation in the former type. The exogenous supply of GA induces stem internode elongation in deepwater rice plants even when they are not submerged, while in flooded plants, the level of GA_1 at the nodes is heightened in deepwater rice, but not in non-deepwater rice. Stem internode elongation is repressed in deepwater rice by treatment with the GA synthesis inhibitor uniconazole, and this repression can be lifted by supplying the plant with GA. The indications are that GA positively regulates stem internode elongation in deepwater rice, operating through the action of *SK1* and *SK2* (Hattori et al. 2009) (Fig. 9.3). The probable scenario therefore is that under submergence, the response of deepwater rice is to rapidly accumulate ethylene, which has the effect of up-regulating *SK1* and *SK2*; additionally, this pair of genes may, directly or indirectly, promote GA synthesis and/or GA signal transduction, in order to promote stem internode elongation, thereby allowing the apex of the plant to remain above the water's surface where it can access the oxygen necessary to escape hypoxic stress (Hattori et al. 2009, 2011) (Fig. 9.3).

Flash flooding also has the effect of submerging the plant, but in this case, the water subsides after a period of some days. Submergence-tolerant rice cultivars respond to temporary submergence by restricting the growth of their shoot and limiting its respiration, recommencing normal growth once the water has subsided (Singh et al. 2001; Das et al. 2005; Fukao et al. 2006; Xu et al. 2006). A major

quantitative trait locus designated *Submergence 1* (*Sub1*) has been identified as harboring three distinct genes (*Sub1A*, *Sub1B*, and *Sub1C*) responsible for this mode of submergence tolerance (Fukao et al. 2006; Xu et al. 2006). Each of the *Sub1* genes encodes a protein featuring an ERF-like DNA-binding domain. Genotypic variation at *Sub1* locus confers distinctions in submergence tolerance. *Sub1B* and *Sub1C* are present in both tolerant and non-tolerant paddy cultivars, whereas *Sub1A* is only found in tolerant ones. Under submergence, the level of ethylene rises rapidly in each of a pair of near isogenic paddy rice lines, one carrying *Sub1B* and *Sub1C* and the other carrying *Sub1A, Sub1B*, and *Sub1C*, but it rises to a higher level in the former (non-tolerant) line. The constitutive expression of *Sub1A* in a non-tolerant cultivar results in a marked improvement in submergence tolerance (Xu et al. 2006). There is an indication that the abundance of *Sub1A* transcript in node and internode regions in some rice accessions containing *Sub1A* is positively correlated with the degree of submergence tolerance. *Sub1A* transcription is inducible by low levels of ethylene (up to 100 ppm) but this treatment has the effect of reducing endogenous ethylene production in submerged plants via negative feedback regulation (Singh et al. 2010). The inference is that the presence of *Sub1A* is necessary and sufficient to confer submergence tolerance (Xu et al. 2006) (Fig. 9.3).

Under normal conditions, the non-tolerant and tolerant isogenic lines develop at a similar rate and their stem elongation response to GA_3 treatment is similar. In submerged plants, the tolerant line responds positively to GA_3 supply with negative implication for its survival. The exposure of the non-tolerant line to PAC reduces its stem elongation, thereby enhancing its survival. The suggestion is that GA-induced stem elongation has a negative impact on tolerance to prolonged submergence (Setter and Laureles 1996; Das et al. 2005). Rice plants engineered to constitutively express *Sub1A* display a classic GA-insensitive phenotype: germination is delayed, the plants are semi-dwarfed, flowering is late, maturity is slow, and grain set is reduced. The seedling GA_3 dose response curve indicates that the ectopic expression of *Sub1A* compromises the plant's response to GA, thereby having a negative effect on GA-dependent processes. SLR1 and SLR1 LIKE1 (SLRL1) are both nuclear-localized GRAS proteins which function as suppressors of GA signaling in rice. The transcription of both *SLR1* and *SLRL1* is submergence-inducible in both submergence-tolerant and non-tolerant plants, but the level of inducibility is greater and its onset is more rapid in the tolerant ones. Engineered constitutive *Sub1A* expressors display an elevated basal level of *SLR1* and *SLRL1* transcription under normal growing conditions, but higher levels when the plants are submerged. These observations confirm that the submergence-induced expression of *Sub1A* enhances the transcription and translation of *SLR1* and *SLRL1*, thereby restraining GA-mediated underwater elongation, prolonging submergence endurance, and sustaining the plant's capacity to regrow once the flooding has subsided (Fukao and Bailey-Serres 2008) (Fig. 9.3).

The abundance of neither *SLR1* nor *SLRL1* transcript is increased by the ethylene treatment of a submergence non-tolerant plant, but not so for a tolerant one. At the protein level, the treatment suppresses the production of SLR1 in the non-tolerant plant but not in the tolerant one, while the abundance of SLRL1 is little changed in the former, but markedly increased in the latter. The indication is therefore that

ethylene induces *Sub1A* expression, which has the effect of up-regulating *SLR1* and *SLRL1* transcription and translation (Fukao and Bailey-Serres 2008). The ethylene does not affect the growth of non-submerged tissue, whether or not *Sub1A* is present. However, a combined ethylene plus GA_3 treatment promotes the GA-mediated elongation of the aerial part of the non-tolerant plant, but significantly attenuates the response in the tolerant one. The interpretation is that in the absence of *Sub1A*, ethylene enhances GA responsiveness by lowering the level of both SLR1 and SLRL1. On the other hand, ethylene-induced *Sub1A* expression stimulates the transcription and translation of *SLR1* and *SLRL1*, which results in the suppression of GA-mediated growth (Fukao and Bailey-Serres 2008) (Fig. 9.3). The overall picture is that submergence tolerance in paddy rice is modulated by *Sub1A*, the product of which dampens ethylene production and responsiveness, resulting in a restriction in ethylene-promoted GA responsiveness achieved via the accumulation of SLR1 and SLRL1; the ultimate result is to limit stem internode elongation and assimilate consumption during a submergence episode.

It is notable that both the *SK* genes and *Sub1A* encode ERFs (ETHYLENE-RESPONSIVE FACTORs) and are associated with GA and ethylene action; however, they confer opposite functions in regulating stem internode elongation in response to flooding: while *Sub1A* restricts shoot elongation during submergence, the *SK* genes stimulate it (Fig. 9.3). In addition, a novel rice ERF gene *OsEATB* (for ERF protein associated with tillering and panicle branching) also mediates crosstalk between ethylene and GA to regulate rice internode elongation. Ethylene treatment sharply down-regulates *OsEATB* expression while increases *OsCPS2* expression (a key GA synthesis gene encodes ent-kaurene synthase A). *OsEATB* suppresses the internode elongation process through the restriction of GA biosynthesis, specifically down-regulating the expression of *OsCPS2* (Qi et al. 2011). *OsEATB* expression is also negatively regulated by ABA and abiotic stress, but further investigations of the molecular crosstalk of the related signaling pathways are still required.

At the end of a submergence episode, rice plants often experience dehydration stress due to reduced hydraulic conductivity in leaf sheaths (Setter et al. 2010). Individuals which harbor *Sub1A* recover by developing new leaves once the water has subsided (Fukao and Bailey-Serres 2008), and also show markedly enhanced recovery from drought at the vegetative stage (Fukao et al. 2011). During a drought episode, *Sub1A* contributes to limiting the extent of water loss, and also promotes the recommencement of leaf growth and development once the stress has been lifted. The abundance of *Sub1A* transcript is markedly enhanced by drought stress, which increases expression of genes associated with acclimation to dehydration. The presence of *Sub1A* during a period of drought stress is associated with a reduced accumulation of ROS (reactive oxygen species), which limits the stress-induced damage to a range of cellular components, and so aids in survival (Fukao et al. 2011) (Fig. 9.3). Therefore, in addition to providing robust submergence tolerance, *Sub1A* improves survival of rapid dehydration following submergence subsidence and water deficit during drought (Fukao et al. 2011) (Fig. 9.3).

The GA and ethylene associated brake on growth imposed by abiotic stress is mirrored in *A. thaliana* (Achard et al. 2006, 2008; Dubois et al. 2013). In response

to water deprivation, the growth of the leaf is halted via the rapid up-regulation of the two transcription factors *ERF5* and *ERF6* (Hruz et al. 2008; Dubois et al. 2013). The growth under osmotic stress of *erf5 erf6* mutant leaves is less restricted than that of WT leaves, but the mutant shows no added tolerance to mild salinity stress. The transcription of either *ERF5* or *ERF6* in WT plants is up-regulated by mild osmotic stress and several other stresses including drought (Hruz et al. 2008; Dubois et al. 2013), but it is not up-regulated by mild salinity stress. *ERF6* over-expressors are dwarfed in stature and form dark green leaves and stunted rosettes, and they are highly sensitive to mild osmotic stress. The inference is that *ERF6* negatively regulates leaf growth under mild osmotic stress. The *GA2ox6* (which encodes an enzyme involved in GA inactivation) activated by mild osmotic stress is rapidly induced in *ERF6* overexpressors; meanwhile, in the *erf5 erf6* double mutant, the induction of *GA2ox6* is delayed (Dubois et al. 2013), which indicates that *ERF6* negatively regulates the level of GA by inducing *GA2ox6* expression. Overexpressing *GA20ox1* (which encodes the rate-limiting GA synthesis enzyme) suppresses the retarded growth phenotype conferred by *ERF6* overexpression. In addition, *ERF6* stabilizes RGA (the major DELLA protein species expressed in the developing leaf). These data confirm that, under mild osmotic stress, an elevated level of *ERF6* reduces the level of endogenous GA through the suppression of *GA2ox6*. The resulting stabilizing effect on the DELLA proteins eventually helps to negatively regulate leaf growth (Dubois et al. 2013). As mild osmotic stress triggers the accumulation of ACC, it is likely that *ERF5* and *ERF6* integrate ethylene and GA signaling to regulate plant growth and the mild osmotic stress response (Dubois et al. 2013) (Fig. 9.3).

High levels of soil salinity restrict the ability of the root to take up water; once the salt has entered the root, it compromises many aspects of cell physiology and slows growth (Hasegawa et al. 2000; Munns and Tester 2008). Achard et al. (2006) have shown that the seedlings of the *A. thaliana gai-t6 rga-t2 rgl1-1 rgl2-1* quadruple DELLA mutant are less inhibited by salinity (100 mM NaCl) than WT seedlings. WT plants respond to salinity by reducing the endogenous level of GA_1 and GA_4, and salt treatment promotes the accumulation of GFP-RGA in *pRGA*::*GFP-RGA* transgenic plants. Thus, one way in which salinity stress (as well as other forms of stress) can slow growth is by reducing the endogenous level of GA, which consequently favors the accumulation of DELLA proteins. Extreme salt concentrations kill plants, compared with WT and the quadruple DELLA mutant, the *ga1-3* and *gai* mutants show increased tolerance of extreme salt concentration (200 mM NaCl). Thus, DELLA proteins can contribute the survival of salt toxicity. Treatment with ethylene allows the WT plant to withstand a higher level of salinity stress (Wang et al. 2002), while the *ctr1-1* mutant shows an enhanced rate of survival when challenged by salinity stress, possibly because of the constitutive activation of ethylene signaling pathways (Guo and Ecker 2003; Potuschak et al. 2003); their increased tolerance is overridden in mutants which also lack *GAI* and *RGA*. Thus, salinity slows growth through the activation of ethylene signaling, the effects of which are at least partly integrated with GA signaling at the level of DELLA function (Fig. 9.3). Meanwhile, the accumulation of DELLAs up-regulates a suite of

genes encoding ROS detoxification enzymes, thereby limiting ROS-induced damage and enhancing salinity tolerance (Achard et al. 2006). The restraint imposed on growth and respiration allows the plant to ration its carbohydrate reserves during the stress period, so that energy is available when growth can resume (Fukao and Bailey-Serres 2008). A common regulatory mechanism mediating both the restraint on growth and survival of adverse conditions is likely to exist. Phytohormonal signaling pathways (ethylene, GA) may be involved in this mechanism, integrated at the level of DELLA function.

9.8 Conclusions and Perspectives

Ethylene and GA clearly participate in the plant growth, development, and the response to abiotic stress, but many aspects remain to be uncovered. For example, the molecular basis of the interaction between ethylene and GA during germination still needs elucidation. A number of questions are outstanding, such as: do ethylene and GA affect root cell division, cell differentiation, or stem cell fate? Where and how is signaling transduced? Other than GA, are there other hormones involved in ethylene-mediated flowering? How are developmentally separated hormone-mediated responses integrated with DELLA function to bring together several signaling pathways in the response to abiotic stress? Characterization of the many interactions involved and the identification of the roles played by the various phytohormones and gene products will require an integration of cell biological and system biology approaches. The goal is to gain an understanding of how plant growth and development is quantitatively modulated in the face of abiotic stress.

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Chapter 10
Integration of Ethylene and Auxin Signaling and the Developmental Consequences of Their Crosstalk

Gloria K. Muday, Gregory S. Maloney and Daniel R. Lewis

Abstract Ethylene and auxin have overlapping effects on growth and development of young seedlings, with either synergistic or antagonistic actions depending on the developmental process. This chapter introduces the growth and developmental processes that are regulated by these two hormones and explores recent studies that provide insight into the mechanistic basis for the regulation of these processes. Ethylene and auxin both inhibit root elongation and stimulate root hair elongation, while acting in opposition on lateral root development and hypocotyl elongation. The interplay between the hormones is even more complex in differential growth processes, such as gravitropism, nutation, and apical hook opening. The presence of well characterized mutants with altered ethylene and auxin signaling and synthesis, as well as auxin transport, has been essential to demonstrate the mechanistic basis of crosstalk between these hormones. As both of these hormones lead to profound changes in gene expression, genome-wide transcript abundance data sets are identifying additional genes that are induced or repressed through this hormonal crosstalk. Experimental tests of predicted regulatory networks involving these genes will likely yield the next set of new insights into the mechanisms by which these hormones control plant growth and development.

Keywords Auxin · Ethylene · Auxin transport · Root development · Lateral root · Apical hook · Root hair · Gravitropism · Elongation

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10.1 Ethylene and Auxin Regulate Root Growth and Development

10.1.1 Primary Root Elongation

The complex interactions between ethylene and auxin on growth and development have been studied in greatest detail in roots. The ability of auxin to regulate primary root elongation and gravitropism, as well as the initiation, emergence, and elongation of lateral and adventitious roots has been studied for decades (Woodward and Bartel 2005; Overvoorde et al. 2010; Bellini et al. 2014). The inhibition of root elongation by auxin has been used as the screen to isolate auxin insensitive mutants, including *aux1* and *axr1-axr6*, revealing important features of the auxin signaling pathways that control a diversity of growth and developmental processes (Mockaitis and Estelle 2008). Like auxin, ethylene, and its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) inhibit root elongation, as shown in Fig. 10.1. The root elongation inhibition by both hormones is rapid, within 5 min of treatment (Le et al. 2001; Rahman et al. 2007) and occurs predominantly in the central root elongation zone (Swarup et al. 2007; Ruzicka et al. 2007; Strader et al. 2010; Rahman et al. 2007; Alarcon et al. 2013), consistent with these two hormones acting to regulate growth through converging signaling pathways. Genetic approaches have demonstrated that ethylene regulates elongation through the canonical signaling pathways that mediate the triple response in dark grown seedlings and fruit ripening (Muday et al. 2012). Mutants with reduced sensitivity to ethylene, including *etr1*, *ein2*, *ein3*, and *eil1* in *Arabidopsis* and *never ripe* (*nr*) and *green ripe* (*gr*) in tomato have elevated primary root growth (Barry and Giovannoni 2006; Ruzicka et al. 2007; Stepanova et al. 2007; Swarup et al. 2007; Negi et al. 2010). In contrast, roots of seedlings with enhanced ethylene signaling or synthesis, *ctr1* and *eto1*, respectively, exhibit reductions in the rate of root elongation (Kieber et al. 1993).

10.1.2 Lateral Root Development

In contrast to primary root elongation, lateral root development is oppositely regulated by auxin and ethylene. The role of auxin in most aspects of lateral root initiation has been well studied in *Arabidopsis*, including priming pericycle cells in the basal meristem, initiating cell cycle progression and asymmetric division and driving the emergence and elongation of lateral roots (Overvoorde et al. 2010; Lavenus et al. 2013; van Norman et al. 2013). Recent genetic studies in *Arabidopsis* and tomato have shown that ethylene negatively regulates lateral root formation (Ivanchenko et al. 2008; Negi et al. 2008, 2010). Elevated levels of ethylene

Fig. 10.1 Auxin and ethylene alter root growth and development. Seedlings were transferred to medium containing indole 3-acetic acid or the ethylene precursor ACC and the tip of roots at time of transfer was marked by a *black dot*. When roots were imaged two days later, both auxin and ethylene decreased *Arabidopsis* root elongation relative to an untreated control as judged by the length of root that forms below the *black dots*. In contrast, auxin treatment enhances lateral root formation and elongation while ethylene treatment inhibits both of these processes

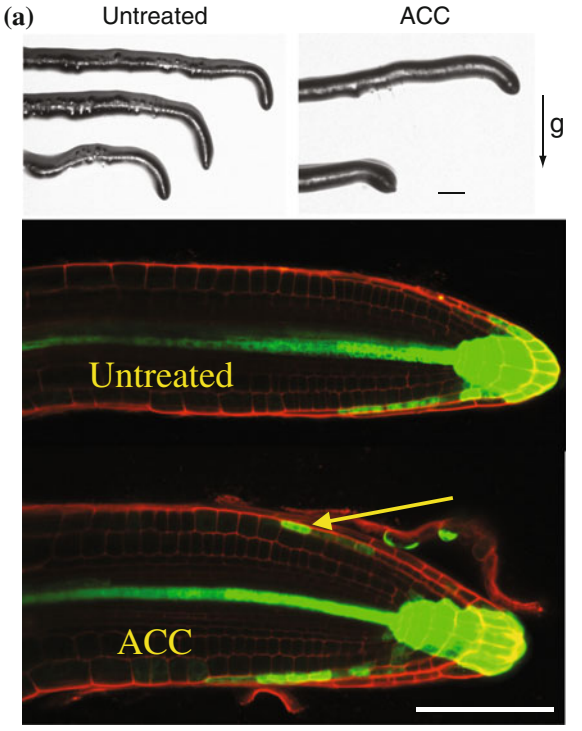
through treatments with ethylene or ACC inhibits lateral root formation at early stages of root initiation (Ivanchenko et al. 2008). *Arabidopsis* and tomato ethylene insensitive mutants exhibit elevated numbers of lateral roots (Negi et al. 2008, 2010; Strader et al. 2010). Although most of these studies examined root growth on agar medium, the increase in lateral root formation seen in *Nr* is even more striking in seedlings grown in soil (Negi et al. 2010), suggesting that ethylene may have even more profound effects on roots during standard cultivation. These opposite effects of auxin and ethylene on lateral roots are evident in Fig. 10.1. This image also illustrates that the stimulation of auxin on lateral root development is greatest in mature regions of the primary root near the root shoot junction, while ethylene inhibits lateral root formation near the root tip in regions of the primary root formed after exposure to ethylene.

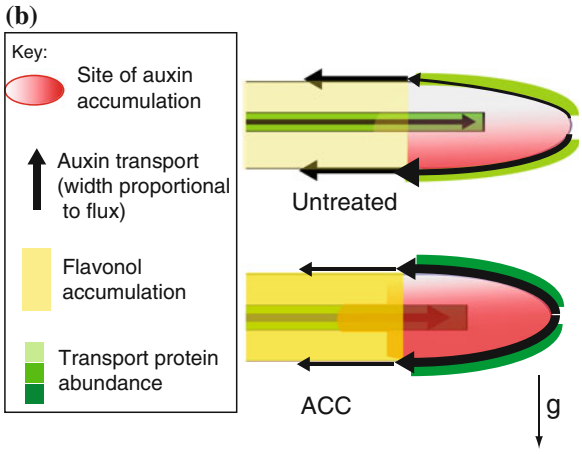
10.1.3 Adventitious Root Development

The reported effects of ethylene on adventitious root formation are more complex, as ethylene increases formation in some species (reviewed in De-Klerk et al. 1999; Bellini et al. 2014), while decreasing adventitious root formation in others (Coleman et al. 1980; Nordstrom and Eliasson 1984). The effect of ethylene on adventitious root formation has only been examined in genetic models in a small number of cases (Clark et al. 1999; Kim et al. 2008; Negi et al. 2010; Sukumar 2010). In tomato, elevated endogenous or exogenous ethylene levels increase adventitious root formation, while ethylene insensitive *nr* produces fewer adventitious roots (Clark et al. 1999; Kim et al. 2008; Negi et al. 2010). In contrast in *Arabidopsis*, ACC treatment, as well as the *eto1* and *ctr1* mutations, results in reduced adventitious root formation (Sukumar 2010). A recent study looking for suppressors of the *sur2* (*superroot2*) mutation, which has elevated auxin synthesis and proliferation of adventitious roots, identified genes linked to both auxin and ethylene synthesis, consistent with roles of both of these hormones in this process (Pacurar et al. 2014).

10.1.4 Root Gravitropism and Waving

Auxin's role in regulating differential growth processes, such as root gravitropism and waving has been well studied (Muday and Rahman 2008; Baldwin et al. 2013). These processes are readily observed when plants are grown on agar surfaces. Gravitropism is observed after root reorientation relative to the gravity vector, and waving is observed when seedlings are grown on impenetrable agar surfaces. When roots are reoriented relative to gravity, auxin is redistributed across the root tip, accumulating on the lower side, where it inhibits root elongation (Baldwin et al. 2013). The resulting auxin gradients across the root have been observed with numerous approaches (Geisler et al. 2014), with the asymmetric expression of the auxin responsive *DR5* promoter driving GFP as illustrated in Fig. 10.2a. Inhibition of auxin transport with inhibitors or by mutations in genes encoding auxin transport proteins blocks the gravity response (Muday and Rahman 2008; Geisler et al. 2014). The differential growth required for root gravitropism and root waving is also altered by ethylene. Ethylene treatment inhibits the gravitropic response in maize and *Arabidopsis* and increases the amplitude and frequency of the root waving response (Lee et al. 1990; Chang et al. 2004; Buer et al. 2003, 2006; Lewis et al. 2011b). The inhibition of gravitropism by ethylene is lost in *ein2* and *etr1* mutants (Buer et al. 2006), suggesting an intact ethylene signaling pathway is necessary for the effect of ethylene on differential growth.


(a)
Untreated
ACC
g
Untreated
ACC


(b)
Key:
Site of auxin accumulation
Auxin transport (width proportional to flux)
Flavonol accumulation
Transport protein abundance
Untreated
ACC
g

◀ **Fig. 10.2** Ethylene negatively regulates gravity response. Treatment of roots with either ethylene or its biosynthetic precursor, ACC, reduces root gravitropic curvature. **a** The images of roots include both root tip angles at low magnification and confocal images of roots at 8 h after reorientation 90° relative to the gravity vector. The angle of gravity is indicated by an *arrow*. The confocal image shows individual cells after propidium iodide staining (*red*) and the asymmetric expression of the auxin responsive DR5-GFP reporter across the untreated root (*green*). In contrast, ACC treatment leads to DR5-GFP fluorescence on the *upper side*, which then minimizes the auxin gradient across the root reducing gravitropic curvature. *Scale bars* = 100 μm. **b** The model indicates the presence of elevated auxin transport protein synthesis and enhanced auxin accumulation in the root tip. The increases in auxin transport are accompanied by increases in flavonol accumulation in the elongation zone of ethylene treated seedlings, which combine to prevent auxin export from the root tip, reducing formation of the gradient of auxin across a gravity stimulated root tip that is needed for gravitropic curvature. Reprinted with permission from Trends in Plant Science (Muday et al. 2012)

10.1.5 Root Hair Initiation and Elongation

In contrast to the negative effects of ethylene on root elongation and lateral root formation, root hair initiation and elongation are synergistically increased by ethylene and auxin (Rahman et al. 2002; Pitts et al. 1998; Kieber et al. 1993; Schiefelbein 2000). Auxin-insensitive signaling mutants (*axr2/iaa7*, *axr3/iaa17*, *slr1/iaa14*, and *iaa28*) develop fewer and shorter root hairs (Wilson et al. 1990; Leyser et al. 1996; Rogg et al. 2001; Fukaki et al. 2002), as do ethylene insensitive mutants, such as *ein2* (Rahman et al. 2002). Root hair elongation is enhanced by auxin treatment (Rahman et al. 2002) and in mutants that have elevated auxin synthesis, including *sur1* and *yucca* (Boerjan et al. 1995; Zhao et al. 2001). The constitutive activation of ethylene signaling or synthesis by *eto1 or ctr1*, treatment with exogenous ethylene, or ACC also promotes root hair elongation (Pitts et al. 1998; Dolan 2001; Strader et al. 2010). In contrast, root hairs are significantly shorter in ethylene insensitive mutants (Masucci and Schiefelbein 1994; Pitts et al. 1998; Rahman et al. 2002) and seedlings treated with inhibitors of ethylene synthesis or signaling (Dolan 2001; Zhang et al. 2003). Auxin is able to rescue root hair elongation defects in ethylene insensitive mutants, and inhibition of auxin influx exacerbates the *ein2* root hair phenotype (Rahman et al. 2002). Application of ACC or IAA to the root hair deficient mutant, *rhd6*, restores root hair initiation (Masucci and Schiefelbein 1994). Collectively, these results indicate that ethylene and auxin responses are both required for maximal root hair initiation and elongation and that these two hormones act in concert on this process.

10.2 Auxin and Ethylene Regulate Hypocotyl Growth and Development

10.2.1 Hypocotyl Elongation

In hypocotyls, the ability of ethylene to control elongation has received considerable study, while the role of auxin in controlling the maintenance of the apical hook and control of hypocotyl elongation has more recently been linked to modulation of ethylene responses. Ethylene inhibits hypocotyl growth as part of the triple response, while auxin is best known for its ability to enhance hypocotyl growth. The effects of these hormones on shoot growth also differ by environmental conditions and species. In the dark, ethylene causes growth inhibition of *Arabidopsis* hypocotyls (Bleecker et al. 1988; Guzman and Ecker 1990), while in the light it stimulates growth (Smalle et al. 1997; Collett et al. 2000). Auxin is more effective in stimulating growth and auxin transport levels in light-grown, than in dark-grown seedlings (Sargent et al. 1974; Yang and Hoffman 1984; Hall et al. 1985; Jensen et al. 1998; Rashotte et al. 2003; Poupart et al. 2005; Liu et al. 2011).

10.2.2 Apical Hook Formation

Most research examining auxin-ethylene crosstalk in the hypocotyl has focused on apical hook development. There are three phases to hook development (formation, maintenance, and opening) with hook formation and maintenance involving asymmetrical growth caused by both altered cell division and elongation (Raz and Ecker 1999; Gupta et al. 2012). Evidence that auxin is involved in hook development comes from the observation that seedlings with auxin transport blocked by auxin transport inhibitors or in mutants with altered auxin transport have a reduced apical hook (Garbers et al. 1996; Lehman et al. 1996; Friml and Palme 2002; de Grauwe et al. 2005; Muday et al. 2006; Abbas et al. 2013). However, in at least one case, the defect in auxin transport may be tied to elevated ethylene synthesis (Skottke et al. 2011). Similarly, wild type seedlings in the presence of high levels of exogenous auxin or mutants that over-accumulate auxin lack an apical hook (Boerjan et al. 1995; King et al. 1995; Lehman et al. 1996; Zhao et al. 2001), and auxin resistant mutants have reduced or no hook (Lehman et al. 1996; Tian and Reed 1999; Dharmasiri et al. 2005). Application of ethylene to wild-type plants or mutations in *eto1* and *ctr1* lead to exaggerated apical hooks (Guzman and Ecker 1990; Kieber et al. 1993). In contrast, ethylene insensitive mutants fail to form an exaggerated apical hook in the presence of applied ethylene (Roman et al. 1995). Time-lapse imaging of apical hooks shows that ethylene prolongs the formation phase in apical hook development leading to an increased hook angle (Vandenbussche et al. 2010; Zadnikova et al. 2010) during a limited developmental window (Raz and Ecker 1999).

10.2.3 Hypocotyl Nutation and Gravitropism

Auxin-ethylene crosstalk has also been studied in the context of differential growth leading to hypocotyl curvature. Nutational bending and gravitropism of shoots has been linked to alterations in auxin transport and auxin accumulation in the zone of bending (Britz and Galston 1982; Hashiguchi et al. 2013). Ethylene also stimulates nutations in hypocotyls of etiolated *Arabidopsis* seedlings (Binder et al. 2006). These nutations are eliminated by application of NPA suggesting that ethylene-stimulated nutations require normal auxin transport. Interestingly, ETR1 ethylene receptor is both necessary and sufficient for nutations, but is not necessary for apical hook formation (Binder et al. 2006; Kim et al. 2011). Thus ETR1, but not the other receptor isoforms, may be subtly influencing auxin transport or accumulation. Ethylene has been reported to negatively impact shoot gravitropism (Gupta et al. 2012), consistent with complex interactions between tropistic growth and ethylene in this tissue.

10.3 Auxin and Ethylene Signaling Pathways

10.3.1 Auxin Signaling Pathways

To explore the mechanisms by which auxin and ethylene act together or in opposition to control growth and development, it is necessary to summarize the signaling pathways that control these responses. The signaling cascade that drives auxin-regulated gene expression has been well characterized (as reviewed by Chapman and Estelle 2009). Auxin causes profound and rapid increases in expression of genes with changes in the *AUX/IAA*, *GH3*, and *SAUR* families being the best described (Chapman and Estelle 2009). With the advent of genome-wide transcriptional profiling, additional rapidly regulated auxin-dependent transcripts have been identified (Vanneste et al. 2005; Paponov et al. 2008; Lewis et al. 2013). Genetic approaches have provided insight into the function of several transcriptional targets and other proteins that mediate auxin signaling and transcriptional responses. Auxin binds to the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) receptor or another auxin F-box (AFB) protein, which are E3-ubiquitin ligases that add ubiquitin tags to AUX/IAA transcriptional repressor proteins (Chapman and Estelle 2009). This ubiquitination targets AUX/IAA proteins for proteolytic destruction (Dharmasiri et al. 2005; Kepinski and Leyser 2005). Destruction of AUX/IAA repressors release auxin response factors (ARFs), which are transcription factors that mediate auxin-dependent transcription (Abel et al. 1995a; Ulmasov et al. 1999). Mutations in genes encoding ARFs and TIR1/AFBs alter auxin responses, as do mutations that stabilize AUX/IAA proteins, preventing their proteolytic destruction (Dharmasiri et al. 2005; Lee et al. 2009). The core auxin transcriptional response machinery drives the synthesis of proteins that modulate auxin dependent growth processes. The right half of Fig. 10.3 depicts the auxin signaling pathway, culminating in the transcription of auxin-responsive genes.

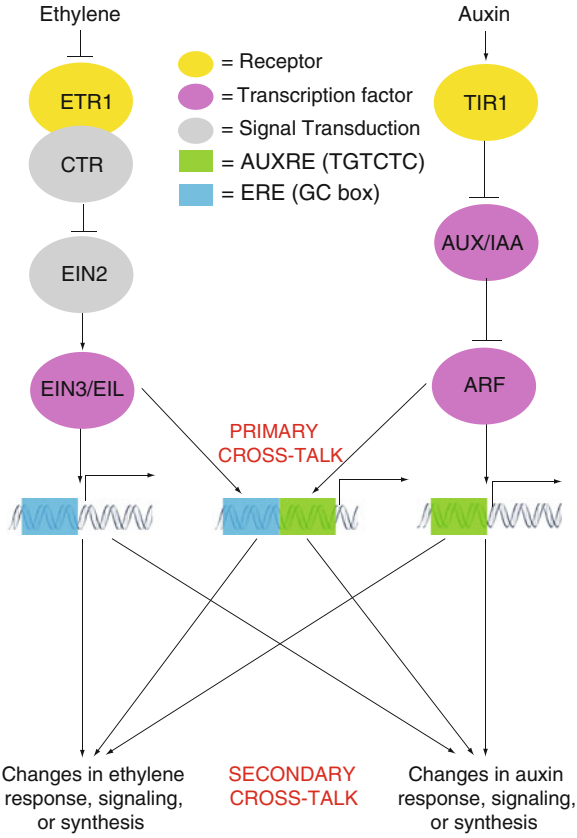


Fig. 10.3 Model of auxin/ethylene crosstalk. In *Arabidopsis*, ethylene, and auxin responses are initiated by binding the ETR1 and TIR1 receptors, respectively. Ethylene binds to and inhibits ETR1 activity, which in turn leads to inhibition of the CTR kinase, a negative regulator of EIN2 activity. EIN2 activates the EIN3 and EIN3-like (EIL) family of transcription factors, which in turn promote transcription of genes containing an ethylene responsive element (ERE) in their promoter region. Auxin signaling is mediated by proteasome-dependent degradation of AUX/IAA transcriptional repressors, which release ARF transcription factors to activate transcription of genes with auxin responsive elements (AUXRE) in their regulatory region. Primary crosstalk occurs by activation of genes that contain both AUXRE and ERE in their promoter region allowing both signaling pathways to directly regulate transcription. Secondary crosstalk occurs through expression of genes that are either auxin or ethylene responsive, but whose activities control expression of genes that regulate the other hormones synthesis, signaling, or response. Reprinted with permission from Trends in Plant Science (Muday et al. 2012)

10.3.2 Auxin Transport

Another mechanism that controls the available auxin for signaling is its redistribution throughout the plant from sites of synthesis. Auxin is transported from cell to cell in a polar fashion. In shoots, indole 3-acetic acid (IAA), the predominant

naturally occurring auxin, moves unidirectionally from the apex to the base (Zazimalova et al. 2010). In roots, auxin transport is more complex, with two distinct polarities. IAA moves acropetally or in the rootward direction through the central cylinder and basipetally (shootward) through the outer layers of root cells. In *Arabidopsis* roots, both polarities of IAA movement control distinct processes and are mediated by unique suites of auxin transporters and their accessory proteins. Rootward (acropetal) movement of IAA from the shoot toward the root apex has been implicated in the control of lateral root formation and elongation (Reed et al. 1998; Casimiro et al. 2001; Bhalerao et al. 2002; Lewis et al. 2011a). AUX1 (Marchant et al. 2002; Negi et al. 2008; Lewis et al. 2011a), ABCB19 (Wu et al. 2007), and PIN 1, 3, and 7 (Benkova et al. 2003; Laskowski et al. 2008; Lewis et al. 2011a) participate in rootward auxin transport and lateral root initiation. Shootward (basipetal) movement of IAA in roots from the root apex is required for gravity response (Rashotte et al. 2000). AUX1 (Bennett et al. 1996; Marchant et al. 1999), PIN2 (Chen et al. 1998; Müller et al. 1998; Rashotte et al. 2000), and ABCB4 (Lewis et al. 2007) mediate this polarity of IAA transport and mutants of these genes exhibit altered gravitropic responses. By using plants with mutations in these genes, it is possible to separate ethylene's effect on auxin transport from its potential effect on auxin signaling and elucidate the mechanisms governing the developmental processes that are influenced by these two hormones.

10.3.3 Ethylene Signaling Pathways

The ethylene signal transduction pathway has been identified through genetic analyses and the details of this pathway have been reviewed elsewhere (Giovannoni 2007; Kendrick and Chang 2008; Stepanova and Alonso 2009; elsewhere in this book). Important proteins in the ethylene signaling and synthesis pathways are briefly summarized here, as plants with mutations in these genes have been used to examine crosstalk with auxin. Physiological and genetic characterization of ethylene mutants has revealed a linear signaling pathway that begins with ethylene binding to and turning off its receptor proteins, including ETR1 and its tomato ortholog, NEVER-RIPE (NR) (Chang et al. 1993; Wilkinson et al. 1995). CTR1, a protein kinase with sequence similarity to the catalytic domain of RAF protein kinase (a mitogen-activated protein kinase kinase kinase) is downstream from ETR1 and functions as a negative regulator of signaling (Kieber et al. 1993; Huang et al. 2003). EIN2 is an essential, positive modulator of ethylene signaling (Alonso et al. 1999) that regulates the activity of transcription factors, including EIN3 and EIN3-like (EIL) proteins, whose targets include *ETHYLENE RESPONSE FACTOR1* (*ERF1*) and *EDF1* though 4 (*ETHYLENE RESPONSE DNA BINDING FACTOR 1* through 4) (Solano et al. 1998; Alonso et al. 2003). As a result of this hierarchical transcriptional cascade, ethylene either positively or negatively regulates diverse genes encoding proteins that mediate the growth response to ethylene (as reviewed by Stepanova et al. 2007; Kendrick and Chang 2008). Figure 10.3

contains a simplified schematic of the *Arabidopsis* ethylene signaling pathway with these proteins indicated. This detailed understanding of ethylene signaling, and the associated genetic resources, provide an excellent framework in which the role of ethylene in root growth and development can be examined.

10.4 Examination of Crosstalk Between Auxin and Ethylene Signaling Pathways

10.4.1 Signaling Crosstalk Regulating Root Elongation

The ability of auxin and ethylene to control root elongation is linked to ethylene enhancing auxin signaling. Auxin-dependent gene expression is strongly enhanced by elevated levels of ethylene. The ability of ethylene to induce auxin signaling is readily observed after ethylene or ACC treatment or in the *eto1* background by increased expression of auxin-inducible reporters in the root elongation zone, including DR5-GUS (Ruzicka et al. 2007; Stepanova et al. 2007; Negi et al. 2008; Strader et al. 2010), DR5-GFP (Ruzicka et al. 2007; Lewis et al. 2011a), DR5-vYFP (Lewis et al. 2011b) and IAA2-GUS (Swarup et al. 2007). An example of DR5-vYFP induction after ACC treatment is shown in Fig. 10.4. Ethylene's ability to regulate root growth depends on the presence of a functional auxin signaling network (Ruzicka et al. 2007; Stepanova et al. 2007; Swarup et al. 2007). The auxin transcriptional machinery that controls this response is not yet clear. Two studies have examined the role of ARF7/ARF19 in ethylene-mediated primary root growth inhibition with contradictory findings (Li et al. 2006; Ruzicka et al. 2007). A recent study indicated that ARF7 or ARF19 function is required for ethylene response in etiolated hypocotyls (Robles et al. 2012).

The evidence is much less clear on whether a fully functional ethylene signaling pathway is required for maximal auxin dependent root growth inhibition. Two groups report different growth effects of exogenous auxin on the ethylene insensitive mutants, *ein2* and *etr1*, with one report of insensitivity to NAA (Ruzicka et al. 2007), and another report of normal response to IAA (Stepanova et al. 2007). Finally, *etr1* and *ein2* mutants exhibit a wild-type response to IAA when transcript abundance of genes encoding auxin transport proteins and flavonol biosynthetic enzymes are examined, even though these mutations block the transcriptional responses to ACC (Lewis et al. 2011a, b). These results suggest that ethylene signaling is not necessary in some cases either for the effect of IAA on transcription of target genes or on growth inhibition. In a genome-wide transcript data set in which IAA and ethylene treated dark grown roots were examined, 28 % of the IAA responsive transcripts showed altered response in the *ein2* mutant (Stepanova et al. 2007), suggesting that the transcriptional response of some IAA responsive genes does require ethylene signaling.

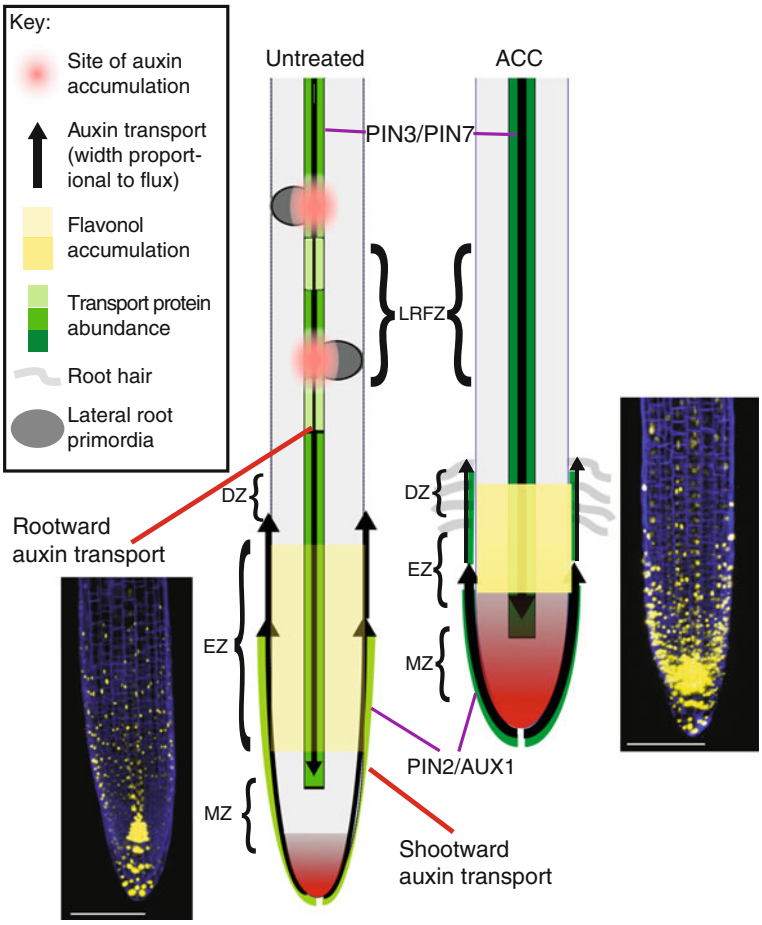


Fig. 10.4 Auxin and ethylene synergistically inhibit root elongation and antagonistically regulate lateral root development. In untreated roots, lateral root formation occurs in the lateral root forming zone (LRFZ) as a result of localized auxin maxima. This accumulation may occur as a result of depletion of PIN3 and PIN7 proteins that prevents auxin movement away from these points, enhancing auxin accumulation. Ethylene increases rootward auxin transport by increasing transcription and translation of PIN3 and PIN7 in the central cylinder, which may then deplete the lateral root-forming region of auxin, while increasing auxin accumulation in the root apex or meristematic zone (MZ). This effect may be responsible for ethylene's negative regulation of lateral root formation. At the root tip, increases in transcription and translation of AUX1 and PIN2 enhance shootward transport of the auxin into the elongation zone (EZ). Accumulation of quercetin and kaempferol, two flavonoids, increases upon ACC treatment (shown in yellow), which limits shootward auxin transport leading to enhanced accumulation at the root tip. *Insets* show confocal micrographs of root tips expressing nuclear localized DR5:vYFP in the presence and absence of ACC treatment. Reprinted and modified with permission from Trends in Plant Science (Muday et al. 2012)

10.4.2 Signaling Crosstalk Regulating Lateral Root Development

The interactions between auxin and ethylene signaling pathways have also been examined in control of lateral root formation, a process in which auxin and ethylene have antagonistic effects. Although the expression of the DR5rev:GFP reporter increases in the root tip in response to ACC treatment, consistent with the synergistic inhibition of growth in this region, in the mature regions of the root where lateral roots form, DR5rev:GFP fluorescence was reduced after ACC treatment with opposite responses in both regions to the ethylene synthesis inhibitor, aminoethoxyvinylglycine (AVG) (Lewis et al. 2011a). The ACC-dependent auxin transport protein transcript changes are lost in ethylene signaling mutants, *ein2* and *etr1*, while IAA-induced changes are not (Lewis et al. 2011a). Similarly, in *tir1*, auxin-induced gene expression changes are lost, but ACC-induced transcription and repression of root branching are maintained (Lewis et al. 2011a). These results are consistent with independent auxin and ethylene signaling pathways controlling transcription of the same target genes.

10.4.3 Signaling Crosstalk Occurs at the Level of Transcriptional Responses

A model depicting the potential layers of crosstalk that may control transcriptional responses to auxin and ethylene is shown in Fig. 10.3. Although auxin and ethylene may both enhance expression of certain genes, these transcriptional effects may operate through independent signaling pathways and lead to primary transcriptional crosstalk, in which both auxin and ethylene dependent transcription factors directly bind to and regulate expression of target genes. Consistent with this possibility, examination of the upstream regulatory region of genes encoding flavonol biosynthetic enzymes, whose transcription is induced by both auxin and ethylene, contained regions with sequence similarity to both AuxRE (auxin-responsive element) and ERE (ethylene-responsive element). AuxRE and ERE are known sites of ARF or EIN3/EIL binding, respectively. In contrast, the upstream regulatory region of one flavonol biosynthesis gene (*TT7*), which was induced by auxin, but not ethylene, had a potential AuxRE, but no identifiable ERE-like sequence (Lewis et al. 2011b).

To dissect the interactions between auxin and ethylene at the level of transcription more globally, a microarray analysis of dark grown *Arabidopsis* roots was performed using wild-type, *ein2*, and the auxin insensitive *aux1* mutant with and without auxin and ethylene treatment (Stepanova et al. 2007). This experiment uncovered numerous genes that were only regulated by auxin or ethylene, but also found that 33 % of ethylene regulated genes and 23 % of auxin regulated genes were in both data sets (Stepanova et al. 2007). Of the transcripts whose expression changed after auxin treatment, 38 % required EIN2 function for this induction, while 28 % of ethylene

altered transcripts required AUX1 (Stepanova et al. 2007). Interpretation of the later result is complex, since AUX1 is an auxin transport protein and mutations in *AUX1* cause auxin insensitivity as a secondary phenotype. When the 5′-UTR of the genes in this large data set were examined, ARF binding sites were found to be enriched in the genes whose transcripts increased after auxin treatment, especially those that were not induced by ethylene. In contrast, EIN3/EIL and AP2/EREBP binding sites were not enriched in any of the populations identified in this study. The authors concluded that other types of transcription factors may be necessary for the ethylene response. They also concluded that ethylene and auxin may mostly act independently to control transcription (Stepanova et al. 2007). This possibility is also indicated in Fig. 10.3 as a secondary crosstalk, in which targets of ethylene and auxin crosstalk are further downstream from primary auxin- or ethylene-responsive genes.

To fully understand the crosstalk between auxin and ethylene in regulation of gene expression additional experimentation is required. The experiment described above (Stepanova et al. 2007) and many others focused on ethylene effects on gene expression used dark grown plants in which root development is minimal. In contrast, experiments focused on auxin induced transcript changes used light grown seedlings and have demonstrated strong interconnected networks of light and auxin signaling (Cluis et al. 2004; Sibout et al. 2006). Furthermore, many of the microarray studies of auxin response were performed with RNA extracted from whole seedlings, rather than root tissue, further complicating interpretation of transcriptional events linked to root growth and development, as is clear from a recent meta-analysis of whole seedling versus root specific auxin transcriptomes (Lewis et al. 2013). In two root specific auxin-transcriptome data sets from light grown seedlings, transcripts linked to ethylene signaling and synthesis have been identified (Vanneste et al. 2005; Lewis et al. 2013). A recent study of the transcriptome of light grown *Arabidopsis* roots identified 240 transcripts that are differentially expressed after ACC treatment, with only 7 of these transcripts linked to auxin signaling, synthesis, or transport (Markakis et al. 2012). It is clear that understanding this crosstalk will require additional genome-wide transcript studies to understand these transcriptional interactions. Together, these studies show that auxin signaling networks are required for ethylene effects on some growth and gene expression responses, but additional experiments provided evidence that crosstalk between auxin and ethylene also occurred at other levels including regulation of the synthesis and transport of auxin.

10.5 Ethylene Modulates Auxin Transport

Ethylene regulates elongation growth, gravitropism, and lateral root development in part by modulating auxin transport. Early investigations into the mechanisms of ethylene action focused predominately on plant shoots, with several implicating auxin transport regulation as part of ethylene's mode of action in a range of plant species (Burg and Burg 1967; Beyer and Morgan 1971; Suttle 1988). More recent studies have focused on roots of genetically tractable species, including *Arabidopsis*

and tomato. These studies have shown that elevated ethylene levels caused by treatment with the ethylene precursor, ACC, or by elevated synthesis in the *eto1* and *epi* mutants positively regulate both shootward and rootward auxin transport (Negi et al. 2008, 2010). The elevated IAA transport after ACC treatment is lost in the *Arabidopsis* and tomato ethylene signaling mutants *etr1* and *ein2*, and *nr*, respectively (Negi et al. 2008, 2010). Plants with mutations in genes encoding auxin transport proteins were used to identify the specific proteins that mediate ethylene regulated auxin transport (Negi et al. 2008; Lewis et al. 2011a).

10.5.1 Ethylene Regulated Auxin Transport Controls Root Elongation

The rapid growth inhibition caused by ethylene or ACC treatment is dependent in part upon regulation of auxin transport within the root tip. Forward genetic screens have identified ethylene insensitive mutant alleles of *PIN2* and *AUX1*, suggesting normal auxin transport capacity is necessary for the full inhibition of growth by ethylene (Pickett et al. 1990; Luschnig et al. 1998). This necessity is specific to this set of transport proteins, as *pin1*, *pin4*, and *pin7* are normally sensitive to ethylene's effect on root elongation (Ruzicka et al. 2007). Ethylene increases the accumulation of *AUX1* and *PIN2* transcripts and promoter or protein fusion reporters (Ruzicka et al. 2007; Lewis et al. 2011b), which is linked to enhanced auxin accumulation at the root tip. Treatment of *aux1* with low levels of NAA, which bypasses AUX/LAX proteins (Yamamoto and Yamamoto 1998), partially reverses ethylene insensitivity (Rahman et al. 2001), while IAA, which requires influx proteins for entry into cells, does not. This result suggests that auxin influx proteins are essential for ethylene inhibition of elongation. Reduced cell expansion may be caused by the enhanced accumulation of auxin in the elongation zone mediated by elevated expression of PIN2 and AUX1 in epidermal cells.

10.5.2 Ethylene Regulates Auxin Transport to Modulate Root Gravitropism

The inhibition of root gravitropism by ethylene may also act through modulation of auxin transport. Root gravitropism is driven by formation of an auxin gradient across a root tip, which requires AUX1 and PIN2 mediated shootward IAA transport. Both decreases and increases in auxin transport can impair formation of this gradient (Muday and Rahman 2008). For instance, the reduction in shootward auxin transport in *eir1/agr1* and *pid-9* or perturbation of transport by auxin transport inhibitors reduces or abolishes root gravitropic curvature (Chen et al. 1998; Rashotte et al. 2000; Sukumar et al. 2009). Similarly, an enhancement in shootward

transport of auxin is accompanied by a reduced rate of gravity response in *rcn1* and *tt4* mutant roots (Rashotte et al. 2001; Buer and Muday 2004). Ethylene increases shootward IAA transport (Negi et al. 2008), upon which gravitropism relies for differential growth (Rashotte et al. 2000), suggesting that ethylene may modulate gravitropism by altering auxin transport. Consistent with ethylene targeting auxin transport, mutants with defects in genes encoding auxin transport proteins have defects in gravitropism and ethylene responses. These mutants include the auxin influx mutant *aux1* (Maher and Martindale 1980) and the auxin efflux mutants *agr1/eir1/pin2/wav6* (Chen et al. 1998; Luschnig et al. 1998; Müller et al. 1998; Utsuno et al. 1998). Similarly, mutations that alter auxin signaling are also associated with reduced ethylene sensitivity and altered gravitropic curvature, including *axr1* (Estelle and Somerville 1987), *tir1-1* (Ruzicka et al. 2007; Shibasaki et al. 2009), *axr2 (*Wilson et al. 1990*)*, *axr3* (Leyser et al. 1996; Ruzicka et al. 2007), and *dgt* of tomato (Zobel 1973; Muday et al. 1995). A recent genetic screen identified the *are1-1* mutant as a second site modifier of the *ctr1-1* mutant, which conveys root specific ethylene insensitive root growth and gravitropism (Shin et al. 2013). This mutant has altered auxin distribution and transport (Shin et al. 2013). Taken together, these results provide evidence that auxin and ethylene may act in concert to regulate the root gravity response.

However, mutants that are ethylene resistant, but auxin sensitive, show more variable gravitropic phenotype. Both *ein2* and *etr1* show wild-type root gravity response in the absence of ethylene (Rahman et al. 2001; Buer et al. 2006). In contrast, in the presence of elevated ethylene, which reduces gravitropic response in wild-type, *etr1*, *ein2*, and the ACC insensitive *alh1* (*ACC related long hypocotyls*), exhibit enhanced root gravity response compared with wild-type (Vandenbussche et al. 2003; Buer et al. 2006). Many ethylene insensitive plants have wild-type root gravitropism when ethylene is limiting such as when plants are grown along the surface of agar plates. However, when plants are grown in soil, ethylene levels are likely to be much higher due to more limited diffusion, yielding greater differences in response between wild-type and ethylene insensitive mutants. In support of this possibility, the root phenotype of the ethylene insensitive *Nr* mutant is enhanced when roots are growing in soil relative to roots grown on agar medium (Negi et al. 2010). Additionally, when *Arabidopsis* plants are grown along the surface of agar media in plates that are wrapped to prevent diffusion of ethylene gas, root waving is impaired relative to plants grown where diffusion is not limited (Buer et al. 2006).

The demonstration that exogenous application of ACC enhances shootward auxin transport (Ruzicka et al. 2007; Negi et al. 2008) could explain the observed ACC inhibition of root gravitropism. Ethylene-elevated shootward transport of auxin would prevent the formation of an auxin gradient required for the early phase of gravitropic response, as shown in the model in Fig. 10.2. In particular, in the presence of ethylene, auxin may be elevated on both sides of the root, rather than simply on the lower side, as shown using DR5-GFP reporter constructs in Fig. 10.2.

10.5.3 Ethylene Regulates Auxin Transport During Mechanical Stimulation

The above model also explains the roles of ethylene and auxin in the altered growth of mechanically impeded roots. During mechanical impedance, root morphology is modulated by enhanced ethylene signaling (Okamoto et al. 2008). Not surprisingly, the change in ethylene response is coupled with a change in root auxin response. Mechanical impedance also induces the expression of *Anthranilate Synthase* (*AS*) *AS-α* and *AS-β* genes, whose gene products catalyze the first committed step of biosynthesis of tryptophan, an auxin precursor (Radwanski et al. 1996; Okamoto et al. 2008). Further analyses of the auxin responsive reporters *DR5-GUS* and *IAA2-GUS* revealed the formation of an auxin gradient with greater accumulation in the lower side of the mechanically impeded roots (Okamoto et al. 2008). Taken together, these results suggest that localized enhancements in ethylene signaling in mechanically impeded roots stimulates auxin production in the root tip, and promotes its asymmetric redistribution, which is an absolute requirement for root gravity response (Muday and Rahman 2008). Consistent with this hypothesis, the *aux1* mutant is insensitive to the mechanical impedance response (Okamoto et al. 2008). Further support to this idea comes from a recent report demonstrating that tomato root penetration in soil was completely blocked by ethylene signaling inhibitors, which also altered auxin dependent gene expression at the root apex (Santisree et al. 2011).

10.5.4 Ethylene Regulates Synthesis of Flavonols, Which Act as Endogenous Inhibitors of Auxin Transport

Ethylene reduces root elongation and gravitropism at least partially through modulation of auxin transport by inducing the synthesis of flavonols, which are negative regulators of auxin transport (Buer et al. 2006; Lewis et al. 2011b). Flavonols have been shown to regulate auxin transport in vivo, by demonstration of elevated auxin transport in the *tt4* mutant, which synthesizes no flavonols (Brown et al. 2001; Buer and Muday 2004; Peer et al. 2004) and in vitro by inhibition of auxin efflux either in tissue segments (Jacobs and Rubery 1988) or through ABCB proteins expressed in a heterologous system (Geisler et al. 2005; Bailly et al. 2008). The full inhibition of gravitropism by ethylene requires the presence of flavonols, with genetic evidence indicating that quercetin is the active molecule (Buer et al. 2006; Lewis et al. 2011b). Treatment of *Arabidopsis* seedlings with ethylene induces flavonol accumulation (Buer et al. 2006; Lewis et al. 2011b), as shown in Fig. 10.4, in an *ETR1* and *EIN2* dependent fashion (Lewis et al. 2011b). Taken together, these data support the model shown in Fig. 10.4 in which the coordinated increases in auxin transport from the root tip to the elongation zone (via PIN2 and AUX1) and inhibition of auxin transport within the elongation zone (by flavonols) together elevate the auxin concentration in expanding root cells to growth inhibiting ranges.

10.5.5 Ethylene Regulates Lateral Root Development by Altering Auxin Transport

A mechanism by which ethylene regulates auxin transport and inhibits root branching have recently been identified. Ethylene increases rootward auxin transport and decreases lateral root number in *Arabidopsis* and tomato (Negi et al. 2008, 2010; Lewis et al. 2011a, b), a surprising result given the generally positive correlation between shoot to root transport of auxin and root branching. Mutants in auxin transport proteins that are less sensitive to the effect of ethylene on lateral root formation and rootward auxin transport include *pin3*, *pin7*, *aux1*, and *lax3*. In contrast, neither *abcb19* nor *pin2* show any difference in ethylene responsiveness in either process. Additionally abundance of transcripts encoding PIN3, PIN7, and AUX1 and fluorescent protein reporters fused to these proteins decreased in the presence of the ethylene synthesis inhibitor, AVG, and increased after ACC treatment, as illustrated in the model in Fig. 10.4. Specifically, local depletions of PIN3 and PIN7 protein below (or on the rootward side) of developing lateral root primordia has been suggested to lead to local auxin maxima that drive lateral root formation (Laskowski et al. 2008). The local reduction in PIN3 and PIN7-GFP fluorescence is abolished after treatment with ACC, replaced by global elevation of PIN3 and PIN7 abundance through the whole root that correlates with enhanced long distance auxin movement (Lewis et al. 2011a). While this enhanced rootward transport depletes auxin in the regions from which lateral roots initiate, it likely contributes to the elevated auxin levels in the root tip, which inhibit root elongation. This effect is also shown schematically in Fig. 10.4.

10.5.6 Ethylene and Auxin Transport Regulate Root Hair Elongation

Recent evidence suggests that an intracellular threshold of auxin, controlled by auxin influx and efflux processes, is required for optimal elongation of root hairs. The auxin influx mutant *aux1* develops shorter root hairs (Okada and Shimura 1994; Pitts et al. 1998; Rahman et al. 2002). The restoration of the root hair length of *aux1* to wild-type level with a minute amount of NAA, which does not affect the intracellular ethylene response, further confirms that besides ethylene, auxin also plays a major role in regulating the root hair elongation process (Rahman et al. 2002). The auxin efflux mutant *eir1/pin2* also shows a short root hair phenotype, which has been attributed to the reduced auxin supply from the root tip to the hair differentiation zone (Cho et al. 2007). The requirement of auxin transport in regulating root hair elongation has been confirmed in several recent studies (Schlicht et al. 2008; Jones et al. 2009; Ganguly et al. 2010), including the report that the long root hair phenotype of ethylene over production mutant *eto1* can be reversed by specifically blocking auxin influx in an *aux1 eto1* double mutant (Strader et al. 2010).

10.6 Ethylene Regulate Auxin Transport, Signaling, and Synthesis in Hypocotyls

10.6.1 Ethylene Modulates Auxin Transport in Hypocotyls

Ethylene-auxin crosstalk in hypocotyls also acts through modulation of auxin transport. Ethylene inhibits auxin transport in excised pea stems (Burg and Burg 1966; Suttle 1988) and high concentrations of IAA increase ethylene production that, in turn, diminishes growth. Application of NPA inhibits hypocotyl growth of light-grown *Arabidopsis* seedlings (Jensen et al. 1998), but has minimal effect on hypocotyl growth of dark-grown seedlings (Garbers et al. 1996; Lehman et al. 1996; Jensen et al. 1998), suggesting that auxin transport is important for hypocotyl elongation in the light, but not in the dark. Consistent with this, auxin transport in hypocotyls of *Arabidopsis* (Rashotte et al. 2003) and tomato (Liu et al. 2011) is lower in dark-grown seedlings compared to light-grown seedlings. However, ethylene-stimulated hypocotyl nutational bending of dark-grown *Arabidopsis* seedlings is blocked by NPA, suggesting that altered auxin transport may have subtle growth effects on dark-grown hypocotyls (Binder et al. 2006). Auxin transport is important for the effects of ethylene since NPA blocks hook formation in *eto1* and *ctr1* mutants (Lehman et al. 1996), while application of auxin restores hook formation in ethylene insensitive mutants (Vandenbussche et al. 2010).

10.6.2 Ethylene Regulates Auxin Signaling and Synthesis in Hypocotyls

Further evidence for ethylene's effect on auxin accumulation or signaling comes from reporter-gene and physiological experiments using auxin-deficient mutants. *DR5-GUS* accumulates on the concave side of the apical hook and ethylene treatment results in higher levels of accumulation (Li et al. 2004; Zadnikova et al. 2010). When the apical hook opens, this expression of *DR5-GUS* becomes more diffuse (Zadnikova et al. 2010). Additionally, application of ethylene enhances *TAR2-GUS* levels on the concave side of the hook and the apical hook is eliminated in *wei8 tar2* double mutants (Stepanova et al. 2008; Vandenbussche et al. 2010). In contrast, ethylene reporter gene analysis shows that ethylene signaling is homogenous across the hook in air (Vandenbussche et al. 2010), although, application of exogenous ethylene leads to an asymmetrical distribution of *ACO2* in the apical hook (Raz and Ecker 1999). Thus, ethylene may accentuate the apical hook by increasing auxin levels on the concave side through both increased synthesis, signaling, and transport.

10.6.3 Ethylene Alters Auxin Transport During Apical Hook Formation

Several auxin transporters were found to play a role in normal hook development. Subtle effects on the apical hook are found in *pin3*, *pin4*, *pin7*, *aux1*, and *lax3* mutants (Vandenbussche et al. 2010; Zadnikova et al. 2010). Many of these transporters have overlapping roles in the apical hook since *pin4 pin7* and *aux1 lax3* double mutants have more severe apical hook phenotypes than the single mutants (Vandenbussche et al. 2010; Zadnikova et al. 2010). ACC increases *DR5-GUS*, *PIN3-GFP*, and *AUX1-GUS* expression on the concave side of the hook during the period of maximal curvature (Vandenbussche et al. 2010; Zadnikova et al. 2010) suggesting that ethylene exerts at least some of its effects on hook curvature through both auxin influx and efflux carriers.

10.6.4 Ethylene Regulates Auxin Signaling in the Apical Hook

In addition to altering auxin levels across the apical hook, it is also likely that ethylene alters molecular components involved in auxin signaling. The expression of *IAA3-GUS*, *IAA12-GUS*, and *IAA13-GUS* are elevated on the concave side of the hook (Zadnikova et al. 2010). The asymmetric distribution of these reporters' signal is enhanced upon application of ACC, while NPA diminishes the asymmetric distribution of *IAA13-GUS* (Zadnikova et al. 2010). It has also been reported that *nph4* mutant has diminished auxin response and asymmetric growth including apical hook formation (Stowe-Evans et al. 1998; Harper et al. 2000). However, these mutants show normal triple responses (including an exaggerated apical hook when ethylene is added) (Stowe-Evans et al. 1998; Harper et al. 2000). Thus, there appears to be complex interactions between ethylene and auxin that affect hook curvature.

The *HLS1* gene was identified in a screen in which it failed to form an apical hook in the presence of exogenous ethylene (Guzman and Ecker 1990) and was used extensively to examine ethylene-auxin crosstalk in the apical hook. Overexpression of *HLS1* causes an exaggerated apical hook (Lehman et al. 1996). Epistasis analysis placed *HLS1* downstream of *CTR1* (Roman et al. 1995; Lehman et al. 1996). The mRNA levels for *HLS1* increase when seedlings are treated with ethylene and decrease in *ein2* mutants (Lehman et al. 1996). In the *hls1* mutant both *SAUR-Ac1* transcripts and *DR5-GUS* signal are reduced in the region where an apical hook should form (Li et al. 2004), suggesting that disrupted auxin distribution leads to a failure to form an apical hook (Lehman et al. 1996). A *hls1* suppressor screen identified *ARF2* as a down-stream component in this regulation where *hls1 arf2* double mutants showed partially restored hook curvature, responsiveness to ethylene, and *DR5-GUS* expression (Li et al. 2004). Application of ethylene causes a decrease of ARF2 protein in wild-type plants, while *hls1* mutants over-accumulate ARF2 (Li

et al. 2004). Thus, HLS1 appears to negatively regulate the levels of ARF2 and may thereby lead to changes in auxin induced gene expression.

10.7 Reciprocal Regulation of Auxin and Ethylene Synthesis

An interesting reciprocal regulation of auxin and ethylene synthesis has been reported. Elevated levels of auxin lead to increased ethylene synthesis (Morgan and Hall 1962), due to increased synthesis of specific members of the ACC synthase (ACS) family, which catalyze the rate limiting step in ethylene synthesis (Abel et al. 1995b; Stepanova et al. 2007). Additionally, ethylene may also positively regulate auxin synthesis (Basu et al. 2011). Free IAA levels increase in the root tip after treatment with 100 µM ACC and this response is lost in *etr1* or after treatment with the ethylene synthesis inhibitor AVG (Ruzicka et al. 2007; Swarup et al. 2007). The rate of IAA synthesis was also measured after ACC treatment and in one case, 10 and 100 µM ACC increased IAA synthesis (Swarup et al. 2007), but in another case, 100 µM ACC did not have a significant effect on auxin synthesis (Ruzicka et al. 2007). Yet, reductions in free IAA were detected by both groups in the presence of AVG (Ruzicka et al. 2007; Swarup et al. 2007) leading to the hypothesis that ethylene increases auxin biosynthesis. The demonstration that constitutive ethylene signaling resulted in a fivefold increase in auxin concentration over wild-type in the *ctr1* root apex (Ikeda et al. 2009) further supports this idea.

10.7.1 Ethylene Increases Auxin Synthesis

Strong evidence that ethylene can induce auxin synthesis can be found in a screen for weak ethylene insensitive (*wei*) mutants that identified several genes encoding proteins that function in ethylene-induced auxin synthesis (Stepanova et al. 2005, 2008). These include the alpha and beta subunits of anthranilate synthase, ASA1/WEI2/TIR7 and ASB1/WEI7, respectively, which catalyze the first step of tryptophan biosynthesis and thereby contribute to IAA precursor availability (Stepanova et al. 2005). The *WEI8* gene encodes a tryptophan aminotransferase (TAA) that functions in the indole-3-pyruvic acid branch of IAA synthesis and its cloning led to identification of the TAR (TAA-related) gene family (Stepanova et al. 2008). A *wei8 tar2* mutant has significant reductions in free IAA, *DR5-GUS* expression at the root tip in the presence and absence of ACC, and root growth inhibition (Stepanova et al. 2008). The identification of a small molecule, L-kynurenin, which inhibits TAA/TAR proteins, further supports the presence of a feedback loop between auxin biosynthesis and ethylene signaling (He et al. 2011). A recent study identified an additional aminotransferase, VAS1, which is involved in both auxin

and ethylene biosynthesis (Zheng et al. 2013). Loss of function of VAS1 causes auxin and ACC levels to increase (Zheng et al. 2013) suggesting this gene regulates the homeostasis of both hormones. The interplay between auxin and ethylene synthesis may vary between organisms, as *Brachypodium* shows different interactions between ethylene and auxin synthesis (Pacheco-Villalobos et al. 2013). Together, these results suggest that ethylene and auxin enhance each other's synthesis as one of the mechanisms by which these two hormones synergistically inhibit root elongation.

10.8 Concluding Remarks

Ethylene alters many features of auxin-dependent seedling growth. In all cases examined to date, these ethylene responses alter some feature of auxin function; either signaling, synthesis, or transport, and in most cases all three. Ethylene mediates these effects on seedling growth by acting through the canonical ethylene signaling pathway. What is most fascinating about these interactions is that although ethylene and auxin synergistically affect many processes, such as root elongation and root hair formation, they act antagonistically in other processes, such as lateral root formation. The interactions are even more complex for processes in which auxin is asymmetrically accumulated to drive differential growth, such as gravitropism or hook opening, where ethylene prevents this asymmetry by either lowering or raising auxin accumulation on both sides of these organs.

The level of understanding of ethylene's modulation of auxin transport, synthesis, and signaling is quite divergent. The evidence for ethylene altering auxin transport by altering the activity or synthesis of auxin transport proteins is now clearly demonstrated, facilitated by the extensive understanding of the proteins that mediate auxin transport. With recent insight into the pathways for auxin synthesis, the mechanisms by which these two hormones regulate each other's synthesis are now being clarified. The areas in which additional insights are likely to emerge in the near future are the identification of the precise transcriptional factor networks that regulate these synergistic and antagonistic activities of ethylene and auxin in controlling growth and developmental responses.

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Chapter 11
Ethylene and Plant Immunity

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Abstract The phytohormone ethylene is implicated in diverse biological processes including resistance against pathogens. Ethylene production in plants is enhanced in response to pathogen infection. Activated ethylene signaling contributes positively or negatively to resistance depending on enemies and environmental conditions. In some cases, reported roles of ethylene during plant immunity are controversial. Although the core ethylene signal transduction pathway from the biosynthesis, perception to transcriptional response is well characterized, it is highly interconnected with other signaling pathways such as those mediated by the phytohormones salicylic acid and jasmonates. This fact could explain the complexity and controversy of findings. Recent advances using molecular genetics, genomics and computational approaches have started untangling the role of ethylene in the complex immune signaling network. In addition, ethylene is emerging as a key modulator of plant-microbe interactions beyond plant immunity. This chapter highlights the significance of and mechanisms underlying the ethylene signaling network in plant-microbe interactions.

Keywords Botrytis-induced kinase 1 (BIK1) · Ethylene · Jasmonates (JA) · Immune signaling network · Map kinase (MAPK) · Phytohormones · Plant immunity · Salicylic acid (SA)

11.1 Introduction

Pathogen challenge of plants in many cases triggers enhanced ethylene production (Broekaert et al. 2006; Erb et al. 2012; Howe and Jander 2008; Lai and Mengiste 2013; Yang et al. 2013). The production of the ethylene precursor 1-aminocyclo-

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propane-1-carboxylic acid (ACC) by ACC synthases (ACS) is the first committed and considered as the rate-limiting step in ethylene biosynthesis (Broekaert et al. 2006). Produced ethylene binds to its receptors such as ETHYLENE RESPONSE1 (ETR1) which predominantly localize to the membrane of the endoplasmic reticulum (Merchante et al. 2013). In the absence of ethylene, the active receptors negatively regulate the key signaling component ETHYLENE INSENSITIVE2 (EIN2) through phosphorylation via the protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Merchante et al. 2013). Upon ethylene perception, the receptors become inactivated, which in turn facilitates EIN2 activation to mediate the ethylene signaling (Merchante et al. 2013). Upon activation, the C-terminal part of EIN2 is cleaved off and moves into the nucleus to mediate the ethylene signaling via the key transcription factors ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1) (Ju et al. 2012; Wen et al. 2012; Qiao et al. 2012). EIN3 and EIL1 regulate expression of ethylene-responsive genes such as the transcription factor *ETHYLENE RESPONSE FACTOR1* (*ERF1*) and *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59* (*ORA59*) (Solano et al. 1998; Zander et al. 2012). Although the core ethylene signaling pathway seems linear, recent discoveries suggest existence of a much more complex pathway with both positive and negative regulatory feedbacks (Merchante et al. 2013). Limited studies show how the ethylene signal transduction pathway eventually contributes to resistance against pathogens (Botanga et al. 2012; Lloyd et al. 2011). For instance, a quantitative metabolomics study suggests that ethylene signaling contributes to resistance against a fungal pathogen, *Botrytis cinerea* through cell wall modifications (Lloyd et al. 2011). However, further research is required to fully understand the molecular mechanisms in ethylene-mediated resistance against pathogens.

The phytohormones ethylene, jasmonates (JA) and salicylic acid (SA) are immune-related hormones. In general, ethylene and JA-mediated signaling play important roles in resistance against necrotrophic pathogens which actively kill plants to get nutrients whereas SA signaling is a major contributor of resistance against biotrophic pathogens which require living hosts for multiplication (Glazebrook 2005). Roles of ethylene signaling in resistance against these different types of pathogens are sometimes controversial. For instance, upon recognition of conserved bacterial signatures such as flagellin, ethylene production is enhanced as a plant immune response (Liu and Zhang 2004; Broekaert et al. 2006). On the other hand, bacterial virulence factors actively trigger ethylene production for virulence (Kenyon and Turner 1992; Xiao et al. 2007). These results raise a question whether ethylene signaling is a positive or negative regulator in immunity against bacterial pathogens. This controversy could be explained by the fact that ethylene signaling is highly interconnected with many signaling pathways including those mediated by JA and SA, which results in the complex ethylene signaling network (Fig. 11.1) (Glazebrook 2005; Pieterse et al. 2012). Furthermore, plant immune responses are affected by many abiotic environmental factors such as temperature and light (Hua 2013). Thus, the outcome of ethylene signaling is influenced by many factors and interlinked signaling pathways. Therefore, the contribution of ethylene signaling in plant immunity is not easy to determine and sometimes controversial. This book

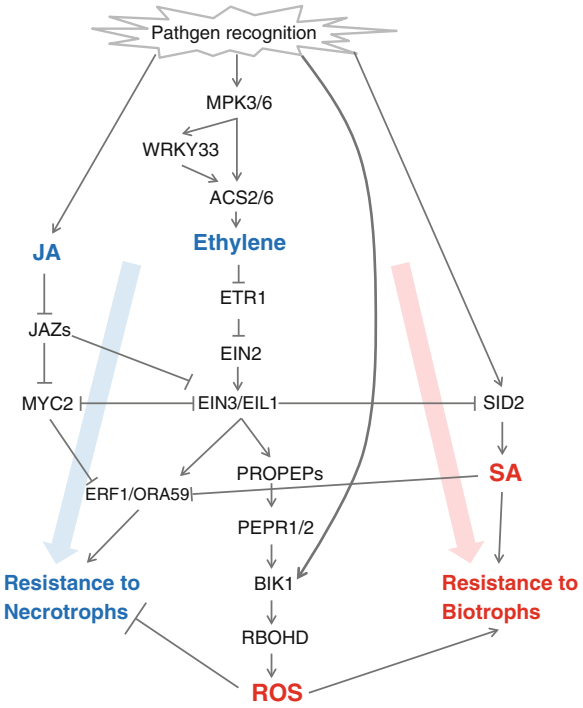

Fig. 11.1 A simplified schematic representation of the ethylene signaling network in plant immunity. *Arrows* and *end-blocked lines* indicate positive and negative regulation, respectively. In general, ethylene and JA signaling contribute to resistance against necrotrophs while SA signaling against biotrophs. MAPK signaling contributes to enhanced ethylene production in plant immunity. BIK1 is important for ROS production through RBOHD. Ethylene-activated PEPR signaling is involved in immune signal amplification through BIK1

chapter focuses on recent discoveries in ethylene and plant immunity, with a special emphasis made on studies that impact our mechanistic understanding of how ethylene signaling is integrated in the complex signaling network in plant immunity against microbial pathogens. Ethylene signaling is also a major signaling cassette for resistance against insect herbivores and rhizobacteria-mediated induced systemic resistance (ISR), which are, however, extensively reviewed elsewhere (Howe and Jander 2008; Erb et al. 2012; Van Wees et al. 2008) and are not discussed here.

11.2 Ethylene and MAP Kinases

Mitogen-activated protein kinase (MAPK) cascades are important signaling pathways that link extracellular stimuli to intracellular responses in eukaryotes through phosphorylation of substrates, and many studies show their implications in plant immunity

(Meng and Zhang 2013). Activation of MAPKs is carried out by their upstream kinases, MAPK kinases (MAPKKs) which are regulated by their upstream kinases, MAPKK kinases (MAPKKKs) (Meng and Zhang 2013). The MAPKs MPK3 and MPK6 are activated and are major regulators during plant immunity (Meng and Zhang 2013). *Arabidopsis* mutants deficient in both *MPK3* and *MPK6* are lethal, suggesting a functional overlap between MPK3 and MPK6 (Wang et al. 2007). Ethylene production is enhanced upon perception of microbe-associated molecular patterns (MAMPs) such as flg22, a part of the bacterial flagellin (Liu and Zhang 2004; Avni et al. 1994; Bar and Avni 2009). MPK6 phosphorylates selected isoforms of ACS, the rate-limiting enzyme of ethylene biosynthesis (Liu and Zhang 2004). Phosphorylation of type I isoforms ACS2 and ACS6 by MPK6 leads to the stabilization of the ACS proteins, resulting in elevated levels of ACS activity and ethylene production (Liu and Zhang 2004). Unphosphorylated ACS6 protein is rapidly degraded by the 26S proteasome pathway, which is blocked through its phosphorylation by MPK6 (Joo et al. 2008). Overexpression of a phospho-mimic ACS6 is sufficient for enhanced ethylene production (Liu and Zhang 2004). MPK3 plays also an important role in phosphorylation of ACS2 and ACS6 proteins. Upon infection with the necrotrophic fungal pathogen *B. cinerea*, ethylene production is greatly compromised in a conditional *mpk3 mpk6* double mutant but no single mutants, indicating overlapping roles for these MAPKs in *Botrytis*-induced ethylene production (Han et al. 2010). Double mutation in *ACS2* and *ACS6* genes greatly reduces *B. cinerea*-induced ethylene production (Han et al. 2010). Involvement of MPK3 and MPK6 for ethylene production is also supported by another study (Xu et al. 2008). Thus, the stabilization of ACS2 and ACS6 proteins through phosphorylation by MPK3 and MPK6 is the critical step for enhanced ethylene production in plant immunity.

Regulation of ACS activity seems more complex. A tomato type I ACS is phosphorylated by a calcium-dependent protein kinase (CDPK) and its phosphorylation stabilizes the enzyme, leading to increased ACS activity and ACC content (Kamiyoshihara et al. 2010; Tatsuki and Mori 2001). ACS6 protein is dephosphorylated by the protein phosphatase 2A ROOTS CURL IN 1-N-NAPHTHYLPHATHALAMIC ACID1 (RCN1). Mutation in *RCN1* leads to ethylene overproduction dependent on *ACS2* and *ACS6* (Skottke et al. 2011) although the role of RCN1 in plant immunity remains to be investigated. A subset of *ACS* genes is transcriptionally induced upon pathogen attack. Recently, it was shown that expression of *ACS* genes is regulated by MPK3 and MPK6. The transcription factor WRKY33 is a substrate of MPK3 and MPK6 (Mao et al. 2011). When MPK3 and MPK6 are activated by expression of the constitutive active form of the upstream MAPKK or *B. cinerea* infection, mutants deficient in *WRKY33* are partially compromised in expression of *ACS2* and *ACS6* and ethylene production (Li et al. 2012). Furthermore, WRKY33 directly binds to the promoters of *ACS2* and *ACS6* genes (Li et al. 2012). These results suggest that WRKY33 is activated by MPK3 and MPK6 and activated WRKY33 directly regulates expression of *ACS2* and *ACS6*, resulting in enhanced ethylene production. Taken together, regulation of ACS activity involves multiple steps with positive and negative outcomes and is the critical step for increased ethylene production during plant immunity.

The controversial involvement of a MAPK cascade in signaling downstream of ethylene perception has been discussed (Yoo et al. 2009; Ji and Guo 2013; Merchante et al. 2013; An et al. 2010; Yoo et al. 2008; Ouaked et al. 2003). For instance, MPK6 is activated by the treatment with ACC. This activation does not occur in *etr1* but does in *ein2* and *ein3*, suggesting that MPK6 is a signaling component between the ethylene receptor ETR1 and signaling component EIN2 (Ouaked et al. 2003). In addition, both MPK3 and MPK6 are activated by ACC through the MAPKK MKK9. The *mkk9* mutant exhibits a wide range of ethylene-insensitive phenotypes (Yoo et al. 2008). Furthermore, MPK3 and MPK6 activated by MKK9 directly phosphorylate EIN3, which contributes to its stabilization and activation of downstream transcriptional reprogramming (Yoo et al. 2008). However, another study shows that EIN2 is absolutely required for ethylene-induced EIN3/EIL1 stabilization whereas MKK9 is not (An et al. 2010). Furthermore, ethylene response phenotypes and gene expression downstream of EIN3 are not compromised in the *mkk9* mutant (An et al. 2010). Thus, the involvement of the MAPK cascade in ethylene signaling after ethylene perception is clearly controversial and requires further studies to clarify the point.

There is another layer of interactions between ethylene and MAPK signaling. MPK6 interacts with and phosphorylates the transcription factor ETHYLENE RESPONSE FACTOR104 (ERF104), and the complex is dissociated in response to flg22 (Bethke et al. 2009). This complex dissociation requires MPK6 activity and ethylene signaling as inactive variants of MPK6 fails to release ERF104 in response to flg22 and the complex dissociation is compromised in the ethylene-insensitive *ein2* and *ein3 eil1* mutants (Bethke et al. 2009). These results suggest that ethylene signaling acts on MPK6 to allow ERF104 to access target genes.

11.3 Ethylene and Pattern-Triggered Immunity

Plants recognize MAMPs derived from microbes through plasma membrane-localized pattern recognition receptors (PRRs) and trigger pattern-triggered immunity (PTI) (Monaghan and Zipfel 2012; Tsuda and Katagiri 2010). The best characterized PRRs include *Arabidopsis* FLAGELLIN SENSING2 (FLS2) for flg22 and ELONGATION FACTOR-TU (EF-Tu) RECEPTOR (EFR) for elf18 (a part of the bacterial EF-Tu) (Zipfel et al. 2004, 2006). BRI1-ASSOCIATED KINASE1 (BAK1) is a part of the receptor complexes for both the MAMPs flg22 and elf18 (Chinchilla et al. 2007; Sun et al. 2013). The receptor complexes interact with and phosphorylate BOTRYTIS-INDUCED KINASE1 (BIK1) for downstream immune responses (Cui et al. 2010; Lu et al. 2010; Kadota et al. 2014; Li et al. 2014; Lin et al. 2014). Ethylene signaling is important for accumulation of the PRR FLS2. Expression of *FLS2* is directly controlled by EIN3 and EIL1 through their bindings on the *FLS2* promoter and is compromised in ethylene signaling mutants (Mersmann et al. 2010; Boutrot et al. 2010). Although ethylene signaling plays a critical role in the basal accumulation of FLS2 before infection, it does not seem to be a major contributor of later

transcriptional induction of *FLS2* during PTI (Tsuda et al. 2009; Mersmann et al. 2010). Nevertheless, the basal FLS2 accumulation controlled by ethylene signaling contributes to early immune responses during flg22-triggered PTI (Boutrot et al. 2010; Mersmann et al. 2010).

The *Arabidopsis* PRRs PEP1 RECEPTOR1 (PEPR1) and PEPR2 recognize the endogenous elicitor-active epitopes conserved in ELICITOR PEPTIDE PRECURSORs (PROPEPs) such as Pep1 and trigger immune responses somewhat similar to those in PTI (Huffaker et al. 2006; Yamaguchi et al. 2006; Huffaker and Ryan 2007; Yamaguchi et al. 2010; Ma et al. 2012). Immune responses such as production of reactive oxygen species (ROS), callose deposition and transcriptional reprogramming including *PROPEP2* induction in response to flg22 and elf18 are compromised in the ethylene-insensitive *ein2* mutants (Tintor et al. 2013). Resistance triggered by elf18 against the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* is compromised in *ein2* and *pepr1 pepr2* mutants (Tintor et al. 2013). As indicated in the name, *BIK1* was isolated as a gene whose mutants showed susceptibility to *B. cinerea* and is implicated in ethylene signaling (Veronese et al. 2006; Laluk et al. 2011). PEPR1 specifically interacts with BIK1 and the related protein PBS1-LIKE1 (PBL1) to trigger Pep1-induced immunity (Liu et al. 2013). PEPR1 directly phosphorylates BIK1 in response to Pep1 as analogous to FLS2-BIK1 in response to flg22 (Liu et al. 2013). Interestingly, mutants deficient in *PEPR1* and *PEPR2* or *BIK1* show reduced sensitivity to ethylene and are compromised in ethylene-induced resistance to *B. cinerea* (Liu et al. 2013). Ethylene treatment induces BIK1 phosphorylation in a PEPR-dependent manner but Pep1-triggered BIK1 phosphorylation is independent of *EIN3* and *EIL1* (Liu et al. 2013). These results illustrate a signaling mechanism by which ethylene and PEPR signaling pathways are coordinated to amplify PTI responses: MAMP recognition triggers BIK1 phosphorylation and ethylene production, enhanced ethylene production is transduced through the core ethylene signal transduction pathway (receptors-EIN2-EIN3/EIL1) to induce expression of *PROPEP* genes, and Pep peptides presumably processed from PROPEP proteins are perceived by PEPRs to further activate BIK1 through phosphorylation.

Recently, two reports showed that BIK1 directly interacts with and phosphorylates the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) to regulate ROS production (Kadota et al. 2014; Li et al. 2014), suggesting regulation of ROS production by ethylene signaling through BIK1. Consistently, biphasic production of ROS is dependent on ethylene signaling during the hemi-biotrophic oomycete pathogen *Phytophthora parasitica* infection in tobacco (Wi et al. 2012). Several studies also support the idea that ethylene signaling enhances ROS production (Desikan et al. 2006; Pogany et al. 2009). Thus, ethylene signaling can contribute to resistance against pathogens through ROS production regulated by BIK1. However, involvement of ethylene signaling on ROS accumulation is more complicated as ethylene-induced flavonols are shown to scavenge ROS (Watkins et al. 2014). ROS positively or negatively regulates ethylene production depending on context (Pogany et al. 2009; Wi et al. 2012), which could be explained by the fact that ROS signaling is highly interconnected with other signaling pathways such as MAPK, JA and SA (Scheler et al. 2013; Torres et al. 2006).

11.4 Ethylene and Other Hormone Signaling Pathways

Roles of ethylene signaling during plant immunity are dependent on infectious agents and sometimes controversial, which could be explained by its intimate interactions with JA and SA signaling (Fig. 11.1). This section highlights studies deciphering molecular mechanisms of interactions between ethylene and JA or SA signaling.

Synergy between ethylene and JA signaling in plant immune responses such as transcriptional reprogramming has been observed in many cases (Lorenzo et al. 2003; Xu et al. 1994; Penninckx et al. 1996, 1998; Thomma et al. 1998, 1999). For instance, expression of *ERF1* is induced by ethylene, JA or synergistically by both hormones and requires both the JA co-receptor CORONATIVE INSENSITIVE1 (COI1) and the ethylene signaling component EIN2 (Lorenzo et al. 2003). Overexpression of *ERF1* rescues immune response defects in *coi1* and *ein2* mutants (Lorenzo et al. 2003). Similar observations were made for another transcription factor, ORA59 (Pre et al. 2008). These results indicate that induced expression of *ERF1* and *ORA59* is the consequence for synergy between ethylene and JA signaling. Perception of JA (JA-isoleucine) by COI1 triggers degradation of negative regulators of JA signaling JASMONATE ZIM-DOMAIN (JAZ) proteins which interact with for instance the key transcription factor of JA signaling MYC2 and its homologs, resulting in repressing their functions (Thines et al. 2007; Chini et al. 2007; Yan et al. 2009; Fernandez-Calvo et al. 2011). A study uncovered that activity of the key transcription factors of ethylene signaling EIN3 and EIL1 are also suppressed by JAZ proteins through the co-repressor HISTONE DEACETYLASE6 (HDA6) (Zhu et al. 2011). Thus, EIN3 and EIL1 are interaction sites for synergy between ethylene and JA signaling; ethylene stabilizes and JA de-represses EIN3 and EIL1, resulting in high expression of *ERF1* and *ORA59*. Ethylene and JA signaling also act antagonistically (Lorenzo et al. 2004). Recently, two reports revealed the molecular mechanism of this antagonism. MYC2 physically interacts with EIN3 to inhibit its DNA binding activity and conversely, EIN3 represses MYC2 function (Song et al. 2014; Zhang et al. 2014). MYC2 also directly binds the promoter of *ORA59* to suppress its expression and *myc2* mutants are resistant to *B. cinerea* infection (Zhai et al. 2013). Thus, coordinated expression of the transcription factors such as *ERF1* and *ORA59* by ethylene and JA is important for resistance against necrotrophic pathogens such as *B. cinerea* and ethylene and JA signaling interlink at the key transcription regulators such as JAZs, MYC2 and EIN3/EIL1 with synergy and antagonism.

As in the interaction between ethylene and JA, ethylene and SA signaling also interact positively and negatively. EIN3 directly binds the promoter of the key SA biosynthesis gene *SALICYLIC ACID INDUCTION-DEFICIENT2* (*SID2*) to repress its expression (Chen et al. 2009). Consistently, the *ein3 eil1* double mutant shows constitutive SA accumulation (Chen et al. 2009). On the other hand, SA signaling suppresses ORA59 protein accumulation (Van der Does et al. 2013). Thus, ethylene and SA signaling can be mutually inhibitory. Positive interactions between ethylene

and SA signaling are also proposed although the molecular mechanisms are not clear (Lawton et al. 1994; Mur et al. 2008).

JA and SA signaling are generally inhibitory to each other (Glazebrook 2005; Spoel et al. 2003), and ethylene signaling modulates this cross talk (Leon-Reyes et al. 2009, 2010). Thus, interactions of ethylene signaling with JA and SA signaling are very complex. A study tackled this complexity through quantitative measurements of immunity levels of a quadruple mutant in which the ethylene, JA, SA and PHYTO-ALEXIN DEFICIENT4 (PAD4, an important regulator of SA accumulation) (Zhou et al. 1998; Jirage et al. 1999) signaling sectors are all disrupted as well as all combinatorial mutants (single, double and triple mutants) (Tsuda et al. 2009). The signaling allocation analysis was used to estimate contributions of each single signaling sector and interactions among the signaling sectors. The analysis revealed that on the contrary to previous ideas, the ethylene signaling sector as well as the JA and SA signaling sectors can contribute positively to both the biotrophic pathogen *P. syringae* and the necrotrophic fungal pathogen *Alternaria brassicicola* as the single signaling sectors (Tsuda et al. 2009). Recently, a dynamic signaling network model using the same mutant set was built to describe signal flows in the network during PTI triggered by different MAMPs against *P. syringae* strains (Kim et al. 2014). The model predicted that the ethylene signaling sector inhibits the JA and PAD4 signaling sectors and is the sole inhibitory sector in the PTI signaling network (Kim et al. 2014). These results clearly point to the importance of multiple and combinatorial mutant analysis to elucidate true functions of signaling sectors in highly interconnected networks. Taken together, ethylene signaling is a critical component for immunity against different types of pathogens as an individual signaling sector and an important modulator of immune responses by influencing JA and SA signaling.

11.5 Emerging Roles of Ethylene

Plants are associated with numerous microbes in natural environments (Bulgarelli et al. 2013). Most associated microbes are non-pathogenic and sometimes beneficial for plant fitness (Bulgarelli et al. 2013). Using a synthetic bacterial community representing the most abundant phyla in the phyllosphere, a recent study found that the *ein2* mutation strongly affects bacterial community composition but mutations in JA and SA biosynthesis had little effect (Bodenhausen et al. 2014). Similarly, a lower bacterial density and an altered community were observed in ethylene-insensitive tobacco plants compared to wild-type plants (Long et al. 2010). Interestingly, some bacteria and fungi produce ethylene to interfere with plant responses (Fukuda et al. 1993; Volksch and Weingart 1998). Some plant root-associated bacteria produce the ACC degradation enzyme ACC deaminase (ACCD) to promote plant root growth (Saleem et al. 2007). Thus, ethylene signaling is a crucial component of plant-microbe interactions beyond plant immunity.

11.6 Ethylene and Practical Applications

As pointed out, ethylene signaling is implicated in many aspects of plant-microbe interactions, which provides great potential for agricultural improvements against diseases. Although manipulation of ethylene levels for regulating fruit ripening is popular, ethylene-mediated strategies to improve disease resistance are in infancy stages. It is speculated based on the analyses of transgenic rice plants defective in ethylene, the phytohormone abscisic acid (ABA) and MAPK pathways that ethylene improves resistance to rice (*Oryza sativa*) blast caused by the hemi-biotrophic fungal pathogen *Magnaporthe oryzae* through its antagonistic interactions with ABA and OsMAPK5 (Bailey et al. 2009). Consistently, a transgenic rice line, which expresses the rice *ACS2* under a pathogen-inducible promoter to overproduce ethylene upon infection, shows increased resistance to both *M. oryzae* and the necrotrophic fungal pathogen *Rhizoctonia solani* that causes sheath blight (Helliwell et al. 2013). Since ethylene production is pathogen-inducible in these plants, it has low or no effect on crop yield under normal circumstances. *Resistance* (*R*)-gene mediated immunity which provides race-specific resistance has been used for rice improvement against *M. oryzae* but is often overcome by emerging virulent strains (Bonman 1992; Dai et al. 2010). Therefore, manipulation of ethylene signaling can be a viable alternative strategy for rice disease management in the field.

In addition to induced ethylene production, reduction of ethylene levels has been also tried. For instance, plant-produced ethylene suppresses *Agrobacterium tumefaciens*-mediated transformation which has been used to generate transgenic plants (Hao et al. 2010; Someya et al. 2013). Low transformation efficiency has been an issue for generation of transgenic plants in some species and the transformation efficiency is increased by expressing the ACC degradation enzyme ACCD, pointing to its great potential for more efficient generation of transgenic plants (Nonaka et al. 2008). In addition, reducing ethylene levels in plants by generating transgenic plants or inoculation of roots with bacteria that produce ACCD improves disease resistance or tolerance against certain pathogens (Gontia-Mishra et al. 2014). For instance in tomato, reduction of pathogen-induced ethylene production by expressing an ACCD improves disease tolerance for *Verticillium* wilt caused by *Verticillium dahlia* (Robison et al. 2001). Similarly, the treatment of tomato plants with the ACCD-producing bacteria reduces the severity of *A. tumefaciens* or *Agrobacterium vitis*-mediated crown gall disease (Toklikishvili et al. 2010). These examples illustrate potential applications in agriculture through manipulation of ethylene effects.

11.7 Conclusions and Perspectives

As described, ethylene signaling is highly interconnected with other signaling with synergism and antagonism. An important question is under what conditions these complex interactions occur. Recently, the dynamic ethylene-induced transcriptional

reprogramming was characterized using genome-wide chromatin immunoprecipitation sequencing and transcript sequencing with samples taken at multiple time points after ethylene treatment (Chang et al. 2013). This study revealed that ethylene-induced transcription occurs in temporal waves controlled by EIN3 which modulate numerous downstream transcriptional cascades, pointing out the importance of time-course experiments to understand the role of ethylene signaling in the dynamic and complex transcriptional network. Moreover, considering the high interconnectivity of ethylene signaling with other signaling pathways, simple genetics using single mutants may not be enough to fully understand the ethylene signaling network and may lead to misinterpretations. Multiple combinatorial mutant analyses were proved to be useful and therefore, should be considered in future studies to understand how ethylene signaling is integrated in the immune signaling network and contributes to plant immunity. In nature, plants are associated with a large number of microbes including pathogens, commensals and mutualists that affect plant fitness. In addition to its significant roles in plant immunity, ethylene signaling is also a critical component for interactions with non-pathogenic microbial communities. It is poorly understood how ethylene signaling affects the outcome of plant-microbe interactions. Metabolomics and proteomics approaches combined with genetics would be helpful to answer this question. Finally, accumulation of knowledge on the ethylene-mediated immune signaling network at the molecular level will certainly contribute to development of agricultural disease management.

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Chapter 12
Research Tools: Biochemical and Biophysical Techniques for Studying Ethylene Signaling

Melanie M.A. Bisson and Georg Groth

Abstract Significant progress has been made in recent years in a molecular understanding of the processes and molecular mechanisms underlying ethylene signaling. Individual elements of the ethylene pathway recognized from genetic studies have been analyzed on the molecular level to identify the structural basis of their interaction with upstream and downstream signaling elements and to resolve the molecular principles and mechanism involved in the signaling of the plant hormone. In this chapter, we will highlight biochemical and biophysical studies on purified proteins, isolated membrane systems, and on intact plant cells that have directly contributed to this knowledge.

Keywords Recombinant protein · Protein–protein interactions · Spectroscopic analysis · Fluorescence imaging · Ethylene-binding domain · Kinase activity

12.1 Introduction

For studying their molecular interactions, mechanism, or architecture the proteins involved in ethylene signaling have to be separated from the rest of the cellular components. To this end, these proteins have to be either produced recombinant in a heterologous host in sufficient amounts or marked in the cell by a sensitive and specific label, as they are usually present only in minor amounts in plant tissues. Bacterial, yeast, and insect cells have been used successfully as a host for the recombinant production of different proteins from the ethylene pathway (Schaller and Bleecker 1995; Gamble et al. 1998; Rodríguez et al. 1999; Huang et al. 2003; Voet van Vor-

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mizeele and Groth 2003; Xie et al. 2003; Zhang et al. 2004; Moussatche and Klee 2004). Receptors from various plant species such as *Arabidopsis*, tomato, tobacco, or the moss *Physcomitrella patens* have been expressed in yeast or in the prokaryotic host *Escherichia coli*. The soluble protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) was expressed and isolated in functional form using insect cells (Huang et al. 2003). Further components of the pathway such as the positive regulator ETHYLENE INSENSITIVE2 (EIN2) and the *Arabidopsis* HISTIDINE-CONTAINING PHOSPHOTRANSFER protein AHP1 were purified from various *E. coli* strains (Scharein et al. 2008; Bisson et al. 2009). Likewise, the nuclear transcription factor ETHYLENE INSENSITIVE3 (EIN3) has been successfully expressed in *E. coli*, but was not purified to homogeneity yet (unpublished work by Kessenbrock et al.).

In contrast to studies on soluble proteins from the ethylene pathway such as CTR1, AHP1, and EIN3 or on the soluble extra-membranous domains of the receptors or EIN2, which can be directly released upon cell disruption and purified from the cell extract of the expressing host, studies on the transmembrane ethylene-binding domain of the receptors or on the intact integral membrane receptors require integration of these proteins into the membrane system of the host and subsequent isolation of these membranes or solubilization of the recombinant expressed proteins from these membranes. Accordingly, membrane fractions isolated from transgenic yeast cells expressing *Arabidopsis* ethylene receptors ETHYLENE RESPONSE1 (ETR1) and ETHYLENE RESPONSE SENSOR1 (ERS1) were used for biochemical characterization of ethylene receptor proteins (Schaller and Bleecker 1995; Rodríguez et al. 1999; Hall et al. 2000). Suitable detergents for mild, but efficient solubilization of ethylene receptor proteins from different species from membranes isolated from the expressing *E. coli* host cells have been identified in systematic solubilization studies (Classen and Groth 2012). In these studies, membrane pellets containing the overexpressed receptor proteins were treated with a set of 23 nonionic and zwitterionic agents that have been successfully applied for the solubilization of different human chemokinine receptors (Ren et al. 2009). Detergents were applied according to their critical micelle concentration (cmc). To improve extraction of the receptors from the purified membranes different temperatures and solubilization times were applied. Nonsolubilized material was removed by centrifugation and solubilized receptor proteins were collected from the supernatant. Analysis of the supernatant by western or dot blot identified Fos-Cholines and Dodecylmaltoside (DDM) as detergents for mild and efficient solubilization of the receptors. Chen et al. (2010) have successfully employed lysophosphatidylcholine (LPC), an ionic phospholipid containing a single fatty acid chain, or octylglucoside (OG), a nonionic detergent, for solubilization of the ethylene receptor family from *Arabidopsis* membranes. Voet van Vormizeele and Groth (2008) applied urea for solubilization of *Arabidopsis* ETR1 overexpressed in *E. coli*. Renaturation and functional refolding of the purified receptor was obtained by dilution in refolding buffer according to a protocol successfully applied before for the membrane proteins OmpC (Kumar and Krishnaswamy 2005) or the human voltage-dependent anion channels HVDAC1 and HVDACII (Engelhardt et al. 2007).

Recombinant membrane bound receptors and soluble proteins of the ethylene signaling pathway produced in bacterial, yeast, or insect cells are typically purified from their expression hosts by affinity chromatography making use of a characteristic sequence tag added to the N-terminus or C-terminus of the recombinant protein. Common affinity tags applied for the purification of receptors, CTR1, EIN2, EIN3, and AHPs are polyhistidine and glutathione S transferase (GST) (Hall et al. 2000; Huang et al. 2003; Voet van Vormizeele and Groth 2003; Xie et al. 2003; Zhang et al. 2004; Moussatche and Klee 2004; Scharein et al. 2008; Bisson et al. 2009; Bisson and Groth 2010). The polyhistidine-tag consists of 6–10 histidine residues. The histidine motif binds to several types of immobilized metal ions. The most common metal ion used for metal chelate affinity purification (IMAC) is nickel, although cobalt, copper, and zinc are also employed. Alternatively, immobilized anti-His antibodies can be used for purification of the fusion protein. The GST-tag encodes for a highly soluble enzyme of 26 kDa, which rapidly folds into a stable protein upon translation. Thereby, the GST tag frequently promotes folding and solubility of the attached recombinant protein. For purification, the GST-fusion protein is captured on immobilized glutathione. Thereafter, the captured protein is released from the glutathione affinity matrix under mild, nondenaturing conditions using reduced glutathione. To ensure mono-dispersity and homogeneity of the purified protein and to separate degraded or not completely translated proteins from the protein of interest, size exclusion chromatography (SEC) is sometimes applied as final step in the purification protocol.

Fundamental to any biochemical or biophysical characterization of individual elements of the ethylene pathway is that recombinant produced proteins or individual domains either in membrane fractions or in solubilized form reflect the physiological structure and function of these proteins *in planta*. Tools to evaluate proper function of the recombinant proteins involve studies on protein folding, catalytic activity, or specific interaction with upstream or downstream elements of the ethylene pathway. Methods to monitor these processes are presented in detail in the next sections.

12.2 Spectroscopic Characterization of Functional Folding and Protein Stability

Soluble expression and solubility in physiological buffers is an initial indication of proper folding for most soluble proteins. A more quantitative measure on protein folding and protein stability is provided by *CD spectroscopy*. Circular Dichroism (CD) measures the difference in the absorbance of right- and left-circularly polarized light by an optically active substance. The CD optical phenomenon is a function of the wavelength. Polypeptides and proteins are analyzed in the near and far UV at wavelengths of 180–260 nm. The distinct types of secondary structure present in proteins such α-helix, parallel and antiparallel β-sheet and turn have different discrete spectra and a spectrum of a complex polypeptide or protein can be approximated as linear combination of the CD spectra of each contributing

secondary structure type. Therefore, analysis of CD spectra of purified recombinant proteins can provide valuable information on the secondary structure content and on proper folding of the heterologously expressed protein.

Purified ethylene receptors from *Arabidopsis*, tomato, and the moss *Physcomitrella patens* were analyzed by CD spectroscopy to obtain information on their secondary structure and protein folding (Voet van Vormizeele and Groth 2008; Classen and Groth 2012). The receptor orthologs showed almost identical CD spectra characteristic of typical α-helical proteins. Secondary structure calculations by the software package *CDPro* (Sreerama and Woody 2000) suggest an α-helical content of 41–52 % and a β-sheet content of 9–14 %. These secondary structure contents agree well with the numbers predicted from the primary structure of the transmembrane domain of ETR1 (65 % α-helices), the crystal structure of the ETR1 receiver domain from *Arabidopsis* (45 % α-helices) solved by Müller-Dieckmann et al. (1999), and known structures of soluble bacterial histidine kinases which are characterized by a α-helical content of 35–40 % (Tanaka et al. 1998; Tomomori et al. 1999; Bilwes et al. 1999, 2001) and are thus indicative for a native protein structure of the recombinant receptors.

Soluble proteins and protein domains of the ethylene signaling pathway from *Arabidopsis* produced in *E. coli* were also analyzed by CD spectroscopy to probe folding and secondary structure composition of the purified recombinant proteins. Secondary structure calculations from CD spectra of the extra-membranous part of EIN2 with the *CDPro* software package revealed secondary contents of 39 % α-helix and 11–13 % β-sheet. Secondary contents of 46 % α-helix and 10 % β-sheet predicted from the EIN2 primary structure using the program PROF (Rost and Sander 1993; Rost et al. 1996) indicate that the recombinant protein adopts the functional native structure (Bisson et al. 2009).

Folding and protein stability of purified recombinant proteins can be also addressed by *fluorescence spectroscopy*. Tryptophan fluorescence is very sensitive to protein conformational changes and provides information about changes in the secondary and tertiary protein structure. Therefore, endogenous or engineered tryptophan residues in the purified recombinant proteins can be used to monitor protein stability and unfolding transitions (Eftink 1994). Our lab took advantage of this fact and analyzed fluorescence emission of endogenous tryptophan residues in ETR1 and various ETR1 mutants. The wild-type protein and mutant A102T hold seven endogenous tryptophan residues. Mutant receptor R118W and double mutant A102T-R118W contain an additional tryptophan. Fluorescence intensity of wild type and mutants was analyzed to probe for the structural stability of the purified recombinant protein as buried tryptophan residues, in general, show stronger fluorescence emission than solvent exposed residues (Lakowicz 2006). Purified ETR1 was titrated with increasing concentration of the denaturant guanidine hydrochloride (GdnHCl) and the fluorescence intensity at 342 nm was determined. Figure 12.1 shows the normalized intensity as a function of the denaturant concentration. All curves show a sigmoidal shape reflecting basically a two-state transition from the folded to the unfolded receptor. For both, wild type and mutants unfolding of the receptors starts at 1 M GdnHCl. But, wild-type ETR1 is more stable to chemical denaturation than the A102T or the A102T-R118W

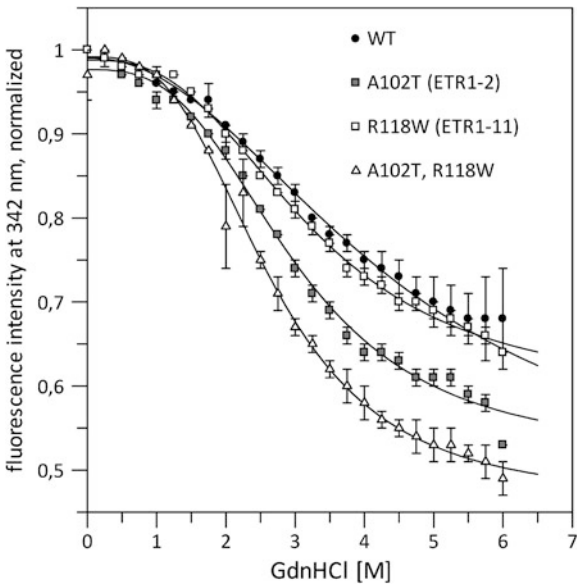

Fig. 12.1 Chemical stability of recombinant wild type ETR1, mutants A102T, R118W, and double mutant A102T-R118W. Unfolding of the receptor proteins was induced by titration with increasing GdnHCl concentration (0.25–6 M) and monitored by fluorescence spectroscopy. Changes in the fluorescence intensity at 342 nm were normalized by the initial intensity monitored in the absence of GdnHCl and plotted as a function of the denaturant concentration. Each data point represents two independent measurements. Standard deviations are indicated by *bars*

mutant as indicated by the smaller slope of the denaturation curve. As a quantitative parameter for denaturation and structural stability the inflection point of each curve was calculated which corresponds to the half-maximal concentration (DC_{50}) required for chemical denaturation of the protein. For wild-type ETR1 denaturation was half-maximal at 4.4 M GdnHCl. Single substitution mutants A102T and R118W show half-maximal denaturation at lower GdnHCl concentrations. The lower numbers compared to wild type are indicative that both mutations have a strong effect on the structural stability of the receptors and result in proteins that are less stable. The strongest effect of the GdnHCl treatment was observed for the A102T-R118W double mutant. Here, half-maximal denaturation was already achieved at a concentration of 2.6 M GdnHCl indicating that destabilization of the protein structure by the individual mutations is additive. In a similar approach intrinsic tryptophan residues in the EIN2 C-terminus have been used for characterization of protein stability and solvent accessibility to external quenchers. Analysis of the fluorescence emission spectra at different concentrations of the polar quencher acrylamide—a substance which leads to a decreased fluorescence of tryptophan residues (Eftink and Ghiron 1981)—indicate that 2–3 of the tryptophan residues in the EIN2 C-terminus are surface-exposed and solvent-accessible, while the remaining 6–7 residues are shielded from the aqueous environment.

For soluble proteins structural integrity of purified mutant proteins relative to the wild-type protein as well as the effect of ligand binding on protein stability can be also assessed by differential scanning fluorimetry (Pantoliano et al. 2001). In this assay, thermally induced unfolding of a protein is measured by an environmental sensitive fluorescent dye, such as SYPRO Orange. While the fluorescence of the dye is low in aqueous solution, the signal is increased upon binding of the dye to the hydrophobic core regions of the protein, which are exposed upon unfolding (Niesen et al. 2007). The temperature dependent unfolding process can be monitored in a Real Time PCR Instrument. The midpoint of the unfolding curve defines the melting temperature (T_m) of the protein (Matulis et al. 2005). Any change in the stability of a protein in its native state, or in site-specific or chemically modified forms is reflected by a change in the melting temperature (Bullock et al. 1997). Thermofluor analysis was applied to compare protein stability of wild type and a tryptophan-less mutant of EIN2 lacking the nine endogenous tryptophan residues in the C-terminal domain. Upon thermal unfolding the wild type shows a melting temperature of 53.4 °C. For the mutant, a T_m of 47.2 °C was obtained indicating that substitution of all in all nine residues affects protein stability only to a small extent (on average 0.7 °C per residue). Functionality of the tryptophan-free mutant has been demonstrated in previous complementation studies (Bisson and Groth 2010).

12.3 Characterization and Functional Analysis of the Ethylene-Binding Domain

Early work by Burg and Burg (1967) suggested that a transition metal cofactor plays a crucial role in ethylene binding. Based on the role of copper olefin complexes as catalytically active species or resting states in organic synthesis, Cu(I) was proposed as a possible cofactor of the receptor proteins (Sisler 1976, 1977). The proposed role of monovalent copper in ethylene binding in plants, was further backed up by the synthesis of Cu(I) complexes with imidazole-like ligands which formed rather stable Cu(I) adducts with ethylene and its agonists (Thompson et al. 1983; Thompson and Whitney 1984; Thompson and Swiatek 1985). Direct evidence for the requirement of copper for ethylene binding was provided by genetic and biochemical studies (Hirayama et al. 1999; Rodríguez et al. 1999). Rodriguez et al. (1999) analyzed the effects of various transition metals on the ethylene-binding activity of yeast membranes expressing ETR1 from *Arabidopsis*. Ethylene binding to the transgenic receptors was probed by *radiolabeled ethylene* ($[^{14}C]C_2H_4$). Only Cu(II) and Ag(I) significantly increased ethylene binding to the transgenic yeast membranes, whereas other divalent cations such as Fe(II), Co(II), Ni(II), or Zn(II) showed no effect on the ethylene-binding activity. Recent studies by Binder et al. (2007) identified Au(I) as another transition metal supporting ethylene binding to transgenic ETR1. In contrast to Ag(I), which serves as potent inhibitor of the ethylene signaling pathway, Au(I) like Cu(I) did not block ethylene action on plants. On the basis of sequence conservation in the ethylene-binding domains and mutagenesis studies

(Rodríguez et al. 1999), proposed a structural model for the ETR1 ethylene-binding domain. The model predicts a stoichiometry of one Cu(I) center per ETR1 dimer and coordination of the monovalent cation in the transmembrane, hydrophobic ethylene-binding domain by residues Cys65 and His69. Alternative models suggested that the binding side contains either one or two Cu(I) centers per dimer (Hirayama et al. 1999; Pirrung 1999; Klee 2002). Binding of copper to purified recombinant *Arabidopsis* ethylene receptors was recently demonstrated in our lab by *microscale thermophoresis* (MST) (for experimental setup and principle of thermophoresis see Sect. 12.5 this chapter). In contrast to previous studies on metal binding, this technique allows determination of the dissociation constant of the copper cofactor at the receptor (unpublished work by Kessenbrock and Groth).

In contrast to genetic screens on plant seedlings, where application of a gaseous molecule such as ethylene is straightforward, biochemical and biophysical studies on purified receptors addressing the effects of the plant hormone on the molecular level are more difficult and troublesome. Structural studies on the receptor, on conformational changes related to ethylene binding or on protein–protein interactions with other proteins of the ethylene pathway are complicated due to the gaseous character of the plant hormone. Various π-acceptor compounds, i.e., molecules which can accept electrons from metal centers, and structural analog of ethylene like carbon monoxide, cyanide, *n*-butyl isocyanide, phosphorous trifluoride, and tetrafluoroethylene have been shown to compete with ethylene for binding in tobacco leaf cells (Sisler 1979). However, aside from cyanide all of these compounds are gases, too. A recent study of our lab (Bisson and Groth 2012) demonstrated that the nongaseous compound cyanide is as a suitable substitute of the plant hormone for in vitro studies with purified proteins. Recombinant ethylene receptor ETR1 showed high level and selective copper-dependent binding of radiolabeled cyanide ([^{14}C]KCN). Immunoblotting and mass spectrometry (MS) of the purified protein confirmed that the binding observed in the radio-assay is solely attributed to ETR1. Moreover, the radioactive binding assays showed that replacement of the essential copper cofactor in the ethylene-binding site by silver still allows ligand binding, whereas replacement of Cys65 in the ethylene-binding domain by serine, corresponding to a mutant deficient in ethylene binding (Rodriguez et al. 1999), dramatically reduced binding of the radiolabeled ligand. Together with the strict copper-dependence of cyanide binding, our study disclosed that binding of cyanide in ETR1 takes places at the same site as ethylene binding. In order to determine the affinity of the binding site in the purified receptor for the nongaseous ethylene agonist, the concentration of radiolabeled cyanide was stepwise increased in the radio-assay. From these experiments, an apparent dissociation constant (K_d) of 280 μM was obtained which is notably higher than the nanomolar binding of ethylene obtained in ethylene-binding studies using transgenic yeast (Rodriguez et al. 1999). However, the difference in binding affinity of both molecules was attributed to the high solubility of ethylene in hydrophobic systems such as biological membranes in contrast with the charged cyanide which is well soluble in aqueous systems, but not in nonpolar hydrophobic environments. These physicochemical characteristics are likely to hamper the access of the cyanide molecule to the hydrophobic ethylene-binding pocket in the membrane domain of the receptor.

12.4 Characterization and Functional Analysis of In Vitro Kinase Activity

Based on sequence homology with bacterial sensor kinases the prototype of the *Arabidopsis* ethylene receptor family ETR1 was considered a putative histidine kinase. Gamble et al. (1998) demonstrated the proposed intrinsic kinase activity of ETR1 and thereby provided the first evidence on the existence of histidine kinases in higher plants. The kinase activity of ETR1 was monitored by a radio-assay using purified recombinant receptor and *radiolabeled ATP* as substrate. To this end, Gamble et al. cloned the gene of the *Arabidopsis* ETR1 lacking the transmembrane ethylene-binding domain fused to an N-terminal GST-tag into yeast. The construct representing the soluble C-terminal part of ETR1 ($ETR1^{164-738}$) was then heterologously expressed in yeast. Transgenic yeast cells were disrupted and centrifuged at 100,000 × *g* to separate the soluble cell fraction from cell debris. The supernatant was loaded on GST-agarose beads, and the recombinant kinase domain bound to the agarose beads was used for the in vitro assay. Most protein kinases require divalent metal cations (Me^{2+}) as a cofactor for ATP-binding and activity as they form a complex with the ATP substrate that shields the negative charge of one of the phosphoryl groups in the ATP molecule. Thereby, the nucleophilic attack on another phosphoryl group, mostly the γ-phosphate group, is facilitated. Therefore, the recombinant agarose-bound GST-$ETR1^{164-738}$ was incubated in the presence of 5 mM $MnCl_2$ and 0.5 mM radioactive labeled [γ^{32}P]ATP. As the terminal phosphoryl group of the ATP molecule is transferred on the protein in the phosphorylation reaction it is essential to use [γ^{32}P]ATP where the γ-phosphoryl group of the ATP is labeled. The radioisotope ^{32}P used for labeling of the substrate is a β emitter and is easily detected by autoradiography. Following this procedure, Gamble et al. (1998) confirmed the autokinase activity of isolated ETR1. After incubation of the agarose beads containing the recombinant GST-$ETR1^{164-738}$ with $MnCl_2$ and [γ^{32}P]ATP, the enzymatic reaction was stopped by adding SDS-PAGE loading buffer in order to unfold the recombinant protein by SDS. Subsequently, the samples were subjected to SDS-PAGE and protein samples were then electro-transferred from the gel onto a nylon membrane. The blotted nylon membrane was then subjected to autoradiography for quantification of incorporated radioactivity. When a mutant of the conserved histidine His353 in the kinase domain was applied in this assay no radiolabel was incorporated in the purified recombinant protein demonstrating that ETR1 is a histidine kinase, which autophosphorylates at residue His353. In their experiments, Gamble et al. also analyzed the cation dependence for autophosphorylation of ETR1. Autokinase activity was addressed in the presence of various divalent cations and the incorporated ^{32}P-radioactivity was monitored as a measure of the kinase activity. Gamble et al. found that Mn^{2+} is the preferred divalent cation for histidine kinase activity of ETR1. Low basal levels of kinase activity were obtained with Mg^{2+}, while no kinase activity at all was detected in presence of Ca^{2+}. In contrast to these results, many bacterial histidine kinases are known to prefer Mg^{2+}. However, several plant receptor-like kinases are also known

to prefer Mn^{2+}. Hence, it might be a general characteristic of plant protein kinases to favor Mn^{2+} over Mg^{2+} as divalent cation in the phosphorylation reaction.

Moussatche and Klee (2004) used a similar approach to analyze the kinase activity of all five members of the *Arabidopsis* ethylene receptor family. For their in vitro kinase assay, GST-fusions of all five receptor proteins lacking the transmembrane ethylene-binding domain were heterologously expressed in yeast and purified on a GST-column. In contrast to the work of Gamble et al. (1998), Moussatche and Klee (2004) eluted the recombinant proteins from the column using reduced glutathion prior the purified recombinant proteins were subjected to the kinase activity assay. For the kinase activity assay, the purified proteins were incubated with a divalent metal cation and [γ^{32}P]ATP, and subsequently subjected to a SDS-PAGE before samples were electroblotted to a PVDF membrane. Incorporated radioactivity was analyzed by autoradiography. By this protocol Moussatche and Klee were able to demonstrate autokinase activity for all five receptor proteins. The in vitro kinase assays described so far were all done with receptor proteins lacking the transmembrane domain. As ethylene binding was shown to be localized at the transmembrane domain of the receptor proteins (Rodríguez et al. 1999), kinase assays based on receptors lacking this domain fail to analyze the effect of the plant hormone on the kinase activity of the receptors. To address the effect of ethylene binding on the autokinase activity, Voet van Vormizeele and Groth (2008) developed a kinase assay with purified, detergent-solubilized *full-length ETR1*. Full-length ETR1 was fused to an N-terminal deca-histidine tag and the recombinant protein was heterologous expressed in *E. coli.* Bacteria cells expressing the recombinant receptor protein were disrupted, host membranes were solubilized and the protein was purified with an IMAC column (Voet van Vormizeele and Groth 2003). The purified, detergent-solubilized ETR1 receptor was subsequently subjected to the radioactive kinase assay. To this end, the recombinant protein was incubated with a buffer containing $MnCl_2$ and [γ^{32}P]ATP to provide the essential compounds for the kinase reaction. The assay was terminated by adding SDS-PAGE loading buffer to the samples followed by SDS-PAGE. The samples were electroblotted from the gel to a nitrocellulose membrane and incorporated radioactivity was analyzed by autoradiography. Autokinase activity was demonstrated in these studies for the purified full-length ETR1 receptor. In addition, by running the assay in the presence of ethylene, Voet van Vormizeele and Groth were able to show that autokinase activity of ETR1 depends on ethylene. Incubation of the purified receptors in the presence of the plant hormone results in a decreased incorporation of radioactivity supporting the idea that the receptor proteins are negatively regulated. Besides, Voet van Vormizeele and Groth analyzed the effect of the ethylene antagonist 1-methyl-cyclopropene (1-MCP), known to inhibit ethylene-induced ripening in plants (Hall et al. 2000) on the autokinase activity of the recombinant receptors. Their studies showed that 1-MCP competes with ethylene for the same binding site. Furthermore, the presence of 1-MCP led to a constitutive kinase activity of ETR1, supporting the idea that ethylene binding alters autokinase activity and thereby regulates signal transfer.

Another protein kinase involved in the ethylene signaling pathway, the negative regulator CTR1, has also been analyzed by an in vitro-based phosphorylation assay. Based on sequence homology CTR1 was predicted to be a Raf-like serine/threonine protein kinase and the putative head of a MAPK-cascade involved in ethylene signaling. Huang et al. (2003) analyzed the biochemical characteristics of CTR1. To this end, CTR1 protein was fused to a GST-tag and the recombinant protein was heterologous expressed in insect cells using the baculovirus transfection system. Recombinant GST–CTR1 protein was isolated from the insect cells by purification on a GST-column. Then, the purified protein was subjected to a radioactive-based kinase assay as described before. CTR1 was predicted to have trans-kinase activity. Thus, purified GST–CTR1 was incubated together with the myelin basic protein (MBP), a known substrate for Raf-1. However, in their kinase assays Huang et al. found that CTR1 contains both, an auto- and trans-kinase activity. Detection of incorporated radioactivity showed distinct signals for MBP, but also for CTR1. When repeating the CTR1 phosphorylation assay in the presence of AtMEK1, an *Arabidopsis* homologue of a MAP kinase kinase, Huang et al. observed only low phosphorylation levels for the putative AtMEK1 substrate at approximately 5 % of the MBP phosphorylation level. These results were taken as clue, that the *in planta* substrate of CTR1 phosphorylation is not a MAPKK. In a recent study, Ju et al. (2012) discovered that *in planta* in fact EIN2 is the phosphorylation target of CTR1 in the ethylene signaling pathway.

The in vitro kinase assays reported so far all rely on radiolabeled ATP as substrate. To overcome this limitation and to reduce the application of radiolabeled substances Kinoshita et al. (2006) developed a novel concept to detect phosphorylated proteins based on a dinuclear metal complex. The compound (1,3-bis[bis (pyridin-2-ylmethyl)amino]propan-2-olatodizinc(II)complex) has a very high affinity for anionic substrates, especially for phosphomonoester dianions at neutral conditions. The compound is called *Phos-tag* and allows the detection of phosphorylated proteins directly in a SDS-PAGE or western blot. When the Phos-tag compound is added to an SDS-PAGE, migration of phosphorylated proteins is substantially reduced due to complex formation between the phosphorylated amino acid in the protein and the Phos-tag molecule. By comparing the migration of phosphorylated and nonphosphorylated isoforms, the phosphorylated protein is easily identified. Kamiyoshihara et al. (2012) made use of the Phos-tag system to evaluate the phosphorylation state of the tomato ethylene receptor proteins LeETR4 and NEVER RIPE (NR). Kamiyoshihara et al. analyzed the microsomal proteins from tomato pericarp on SDS-PAGE, Phos-tag SDS-PAGE, and western blot using anti-LeETR4 antibody and anti-NR antibody. In immature fruits a high amount of phosphorylated LeETR4 was found, whereas the nonphosphorylated LeETR4 protein accumulated at different developmental stages of fruit ripening. Treatment with ethylene also led to an accumulation of the nonphosphorylated LeETR4 protein, whereas treatment with 1-MCP resulted in a higher amount of phosphorylated receptor protein. A similar phosphorylation pattern was observed for NR, another ethylene receptor protein in tomato. Taken together, the results of Kamiyoshihara et al. revealed that ethylene leads to an inhibition of the receptor's kinase activity in

tomato fruits. These results confirm previous results of Voet van Vormizeele and Groth (2008) who showed that ethylene in vitro reduced the autokinase activity of *Arabidopsis* ETR1, whereas 1-MCP had no effect on the phosphorylation state of the receptor.

In addition to phosphorylation studies on mutated receptors carrying non-phosphorylatable residues in the position supposed for phosphorylation such as the His353Ala mutant of ETR1, the identity of the phosphorylated residue can be disclosed by thin-layer chromatography. The fundamental principle of this analysis is to study the stability of the phosphorylated protein at different pH. Depending on the type of phosphorylated residue phosphate esters or amidates are formed showing different stability at acid and alkaline pH. As a start for *phosphoamino acid analysis* (PAA), the recombinant kinase is incubated with radiolabeled ATP and radioactivity incorporated into the protein is analyzed by autoradiography similar to assays addressing the activity of the purified receptor kinase. Then, radiolabeled protein bands are cut from the membrane and exposed to HCl or NaOH followed by a rescan of the autoradiography. The phosphoamidate bond between histidine and the phosphoryl group is unstable at acidic conditions, but stable at alkaline conditions. Thus, for phosphohistidines signals on the autoradiography can be only detected in the sample treated with NaOH, but not in the sample exposed to acidic conditions. In contrast, serine, threonine, or tyrosine residues form phosphoester bonds with the phosphoryl group, which are stable at acidic conditions, but are hydrolyzed at alkaline conditions. As a consequence, phosphoserine, -threonine, and -tyrosine show a radioactive signal after HCl treatment. Providing phosphorylation on serine, threonine, or tyrosine residues is detected by this protocol, the identity of the phosphorylated residue can be narrowed down with the appropriate phosphoserine, threonine, and tryrosine standards.

Xie et al. (2003) used PAA to study the kinase activity of ethylene receptor NTHK1 from tobacco. Surprisingly, phosphorylation on serine/threonine was detected for NTHK1, even though the receptor has a conserved histidine and thus was predicted to show histidine kinase activity. When analyzing NTHK2 from tobacco by a similar protocol Zhang et al. (2004) found that NTHK2 contains both, serine/threonine kinase activity and histidine kinase activity. The type of residue that was phosphorylated depended on the divalent cation used in the kinase assay. Moussatche and Klee (2004) analyzed kinase activity and identity of the phosphorylated residue of all five ethylene receptor proteins from *Arabidopsis*. The authors revealed serine/threonine phosphorylation for ETR2, ERS2, and EIN4 belonging to the receptor subfamily II. For ETR1 histidine kinase activity was confirmed as reported by Gamble et al. (1998). For ERS1 dual kinase specificity was observed depending on the divalent metal cation used. Still, the biological relevance of this dual kinase specificity, which fits to the phosphorylation specifity observed for NTHK2 by Zhang et al. (2004), is unclear. For CTR1 serine/threonine phosphorylation was confirmed by Huang et al. (2003).

Another technique to reveal the identity of the phosphorylated residues is *phosphoproteomics*. By this approach in addition to the type also the position of the phosphorylated amino acid in the protein becomes known. Additionally,

phosphoproteomics allow parallel analysis of several proteins or of an entire proteome. For a typical phosphoproteomic analysis, cultured cells are lysed and proteins are digested with a protease, usually trypsin, either directly in the polyacrylamide gel or in-solution. Subsequently, phosphopeptides are enriched. The most common technique used to enrich phosphopeptides is an IMAC based method, which takes advantage of the affinity of the negatively charged phosphoryl group to bind to positively charged metal ions, like gallium or titanium. Enriched phosphopeptides are then analyzed by MS, which allows detection of the mass and the charge of the peptides. Following this approach phosphorylated residues can be explicitly identified. MS is an analytical technique, which is routinely used for determining masses of particles and for deciphering the elemental composition of a sample or molecule. However, MS has the additional potential to describe transient, dynamic complexes, to map protein interfaces, and to resolve conformational dynamics in a protein or a protein–protein complex (for details see a review of Hernandez and Robinson 2001). The principle of MS is to generate charged molecules or molecule fragments of the laboratory sample, transfer them into the gaseous phase, and to measure their mass-to-charge ratios. For protein analysis, fragments for ionization are generated by proteolytic digestion and soft ionization techniques have to be applied. Among the most used soft ionization techniques are Electrospray (ESI) and Matrix Assisted Laser Desorption (MALDI). For a comprehensive introduction in phosphoproteomics and MS, please see Loroch et al. (2013), Gross (2011).

Chen et al. (2011) applied phosphoproteomics to analyze the response of dark-grown *Arabidopsis* seedlings treated with ethylene. These studies revealed that the positive regulator of ethylene signaling EIN2 is phosphorylated in the absence of the plant hormone. In contrast, ethylene leads to an accumulation of nonphosphorylated peptides of EIN2. The most significant sites of EIN2 phosphorylation identified are on Ser645 and Ser924. Both residues are highly conserved in EIN2 homologues from different species supporting a critical role of these residues in ethylene signaling. Studies of Ju et al. (2012) revealed that CTR1 is the kinase that phosphorylates EIN2 at these residues in absence of ethylene. In contrast, the presence of the plant hormone leads to an inactivation of the CTR1 kinase and thereby to dephosphorylation of EIN2.

12.5 Characterization and Functional Analysis of Protein–Protein Interactions

The *yeast two-hybrid assay* (Y2H), a widely used method for identifying and analyzing protein–protein interactions, was also used to identify and characterize protein–protein interactions of the ethylene signaling pathway. The technique was pioneered by Fields and Song (1989) and is based on the transcription factor GAL4, which regulates the expression of genes required for galactose utilization. GAL4 consists of an N-terminal DNA-binding domain (BD) and a C-terminal transcription

activating domain (AD). The Y2H assay makes use of this bipartite structure of the transcription factor by fusing BD and AD to potential protein–protein interaction partners and expressing the proteins of interest in a GAL4-deficient yeast strain. If AD and BD assemble due to complex formation of the fused proteins, functional GAL4 can activate gene expression for galactose utilization and the yeast strain can grow on selective galactose medium. Many protein–protein interactions of the ethylene pathway have been characterized by Y2H. For instance, Clark et al. (1998) used Y2H to identify interactions between the N-terminal regulatory domain of CTR1 and the kinase domain of receptors ETR1 and ERS1. Furthermore, using Y2H Qiao et al. (2009) found two F-box proteins, EIN2 TARGETING PROTEIN1 (ETP1) and ETP2, as direct interaction partner of the EIN2 C-terminus. Their work revealed that both F-box proteins are involved in proteasome-dependent degradation of EIN2 supporting the idea that EIN2 protein level triggers ethylene responses in living plants. Recently, Ju et al. (2012) applied Y2H to demonstrate a direct interaction between the EIN2 C-terminus and the CTR1 kinase domain. Further analysis showed that CTR1 phosphorylates EIN2.

Even though Y2H has been established as a reliable and versatile tool to identify protein–protein interactions, Y2H is limited on small, soluble proteins, which can enter the nucleus. Hence, the *split-ubiquitin system* (SUS) has been developed for overcoming this limitation and to enable identification of protein–protein complex formation between membrane proteins. SUS was developed by Johnsson and Varkshavsky (1994) and refined by Stagljar et al. (1998). The small regulatory protein Ubiquitin (ub) is recognized in vivo by ub-specific proteases (UBPs). Proteins tagged with ub are rapidly degraded by these UBPs. For the SUS assay, ub was split into two fragments, an N-terminal part (Nub) and a C-terminal part (Cub). However, Cub is additionally fused to a reporter (Cub-re) and Nub and Cub-re are fused to two different proteins, respectively. If the two fusion proteins form a complex, Nub and Cub-re get in close proximity resulting in the assembly of both domains to a functional ub. The reconstituted ub is subsequently recognized by UBPs, and the reporter, previously fused to Cub is cleaved. Then, the released reporter, usually a transcription factor, enters the nucleus and activates the reporter genes.

In 2008, Grefen and coauthors did make use of an advanced SUS, the *mating-based split-ubiquitin system* (mbSUS) to analyze heteromerization of the ethylene receptor proteins (Obrdlik et al. 2004; Grefen et al. 2009). For mbSUS, Nub and Cub fusion are expressed in different yeast strains and after mating of the protein-expressing yeast cells, protein–protein interaction of the fusion proteins is analyzed by cell growth on appropriate selective media. In their approach Grefen et al. (2008) individually fused each of the ethylene receptors in their full-length sequence to Cub-re (receptor-Cub-re) and each of the receptors lacking the transmembrane domain to Nub (ΔTM-receptor-Nub). Cells expressing the respective receptor-Cub-re and ΔTM-receptor-Nub fusion proteins were mated in every possible combination. Thereby, homo-and heteromerization of all members of the ethylene receptor family in all combinations was identified.

Another technique widely used to verify protein–protein interactions is *Co-immunoprecipitation* (Co-IP) (Ransone 1995). For a Co-IP, a cell lysate is incubated with an antibody-coupled resin. The antibody captures a particular protein of interest and purifies the primary target (bait) together with other proteins interacting with the bait molecule. Unspecific bound proteins are washed away and the interacting protein can be detected by a specific antibody directed against the secondary target (prey). Co-IP is a common tool to identify protein–protein interactions in soluble fractions. When membrane proteins are suspected to participate in the protein complex, solubilization of the cell lysate is required, which might mask weak and transient interactions as these complexes might disintegrate on this treatment. A variant of Co-IP is the *pull-down assay*. In a pull-down assay, the bait protein is recombinantly fused to an affinity tag. The affinity tag is used to bind the bait molecule to a resin. Then, the resin containing the immobilized bait protein is incubated with a cell lysate containing the prey protein. After washing of unspecific bound proteins, the protein complex can be eluted from the resin and analyzed via SDS-PAGE and western blot by antibodies directed against the prey protein. Alternatively, it is also possible to coexpress bait and prey proteins, either stable in *Arabidopsis* or heterologous in *E. coli* or yeast. After cell disruption, the complete cell lysate is loaded to the bait affinity resin, resulting in a copurification of bait and prey. In other words, the pull-down assay performed on a complete cell lysate is highly similar to Co-IP, it only differs by the method used to purify the bait–prey complexes. Co-IP and pull-down have frequently been used to identify protein–protein interaction in ethylene signaling pathway. As an example Gao et al. (2003) identified interaction partners of CTR1 by a fusion protein (CTR1-MT) carrying two affinity tags, a myc tag and TAP-tag. The myc-tag consists of an antibody epitope (EQKLISEEDL) derived from the human proto-oncogen $p62^{c\text{-}}$*myc*. The TAP-tag contains a calmodulin binding peptide and a protein A peptide. The CTR1-MT fusion protein was stable expressed in *Arabidopsis*. Membrane fractions of transgenic plants expressing CTR1-MT were solubilized and after centrifugation the supernatant was incubated with IgG beads in order to bind the protein A in the TAP-tag. Bound protein was analyzed by immunodetection using antibodies directed against CTR1 and ETR1, respectively. This analysis demonstrated that CTR1 copurifies with the ETR1 receptor. A similar approach was used to study the heteromeric interactions among ethylene receptor proteins (Gao et al. 2008). Receptors ERS1, ETR2, ERS2, and EIN4 were individually fused to a TAP tag and stable expressed in *Arabidopsis*. Transgenic plants expressing the individual receptor-TAP fusion protein were homogenized. Later, the membrane fraction was solubilized and the soluble supernatant was loaded to IgG beads. Analysis of bound proteins with anti-TAP and anti-ETR1 antibodies revealed that ETR1 was copurified with all other ethylene receptor proteins. Another example for the application of Co-IP for the analysis of protein–protein interactions in the ethylene pathway comes from Qiao et al. (2009). To support the interaction of the EIN2 C-terminus and the F-box proteins ETP1 and ETP2 identified in Y2H studies, EIN2 was fused to a GST-tag, expressed in *E. coli* and the EIN2-fusion protein produced was purified from the cell lysate. Furthermore, ETP1 and ETP2 were labeled by a HA

tag and the resulting fusion proteins were translated in vitro. Then, the EIN2-fusion protein was pulled-down on a GST-affinity resin together with the HA-fusion versions of ETP1 and ETP2, respectively. Immunodetection of resin-bound proteins with the anti-HA antibody directed against the ETP-fusion and the anti-GST antibody directed against the EIN2-fusion confirmed the interaction of EIN2 and both F-box proteins.

In recent years, *fluorescence-based imaging techniques* also called bioimaging techniques have increasingly gained in importance, as they simplify identification of the subcellular localization of the proteins of interest. Moreover, these techniques provide versatile tools to identify protein colocalization, homo-and heteromerization, and protein–protein interactions. Furthermore, depending on the microscope and the methodology applied, the dynamics of protein complex formation can be resolved. For cellular imaging of living cells and tissues confocal microscopy has become an invaluable tool. With this technique individual cellular compartments such as organelles or membranes can be visualized and cellular processes can be imaged with high spatial and temporal resolution. For visualization, fluorescent probes such as antibodies directed against distinct cellular structures or individual proteins as well as fluorescent fusion proteins that are expressed directly in the cell —constitutively or on demand—are used. The most commonly used fluorescent reporters are the green fluorescent protein (GFP) from *Aequorea victoria* and its derivatives, the red fluorescent protein (RFP) and the yellow fluorescent protein (YFP). By molecular cloning methods, the fluorescent reporter is fused, either N- or C-terminal to the protein of interest, and transferred into an expression system of choice. Various expression systems have been used in ethylene research, but the most common systems are the *Agrobacterium*-mediated stable expression in *Arabidopsis* plants (Clough and Bent 1998), the polyethylene glycol mediated transfection of protoplasts (Hayashimoto et al. 1990), and the transient expression in tobacco leaf cells (Voinnet et al. 2003). In 2008, stable expression in *Arabidopsis* and transfection of protoplast were used to identify the subcellular localization of RTE1 at the ER and Golgi membrane. Furthermore, these studies demonstrated the colocalization of RTE1 with ethylene receptor ETR1 (Dong et al. 2008). Similarly, the colocalization of all members of the ethylene receptor family at the ER membrane was identified in transiently expressing tobacco leaf cells (Grefen et al. 2008; Bisson et al. 2009; Bisson and Groth 2010). Also recent data on the ethylene-dependent subcellular trafficking of EIN2 and the phosphorylation of EIN2 by CTR1 were obtained in studies using *in planta* expression and analysis by confocal microscopy (Qiao et al. 2012; Wen et al. 2012; Ju et al. 2012).

Walter et al. (2004) established *bimolecular fluorescence complementation* (BiFC) as a suitable assay to study protein–protein interaction in stable transformed *Arabidopsis* cells and transiently transformed tobacco leaf cells. This technique is based on the complex formation of the N- and C-terminal fragment of YFP, which are only fluorescent when coming in close proximity (Hu et al. 2002). Therefore, BiFC is also called Split-YFP technique. Walter et al. fused the N- and the C-terminal fragment of YFP individually to putative interacting proteins. Providing the proteins form a complex and get into contact also the YFP fragments are

brought in close proximity forming a functional YFP. The fluorescence of the assembled functional YFP then is detected by a confocal laser scanning microscope. Dong et al. (2010) used this technique to successfully identify RTE1-ETR1 complex formation at the ER and Golgi membrane in transiently expressed tobacco leaf cells. Similarly, Ju et al. (2012) demonstrated interaction of EIN2 and CTR1 in tobacco leaf cells by BiFC.

Although the fusion proteins were properly expressed, interaction of the different members of the ethylene receptor family could not be demonstrated by BiFC. Consequently, Grefen et al. (2008) established a sophisticated assay to analyze protein–protein interactions of membrane proteins transiently expressed in tobacco epidermal leaf cells. The *membrane-recruitment assay* (MeRA) developed by these authors to analyze homo-and heteromerization of the different members of the ethylene receptor family at the ER membrane makes use of a RFP-labeled membrane-integrated anchor protein. A second soluble protein is labeled with GFP and coexpressed together with the RFP-anchor protein. Formation of a protein–protein interaction between both proteins leads to the recruitment of the soluble cytosolic GFP-tagged protein to the ER membrane. Following this protocol Grefen et al. (2008) deleted the transmembrane domain in the five *Arabidopsis* ethylene receptor and tagged the remaining cytosolic receptor domain with GFP (ΔTM-receptor:GFP). Later, each ΔTM-receptor:GFP fusion was coexpressed with another RFP-tagged full-length receptor protein. The experiments showed that the ethylene receptor proteins form homo- and heterodimers of all possible combination.

As another fluorescence-based imaging technique Bisson et al. (2009) applied the *FRET-based acceptor-bleaching method* (Karpova et al. 2003) to identify protein–protein interactions in the ethylene pathway in living cells. The acceptor-bleaching method relies on the fluorescence resonance energy transfer (FRET) between GFP and RFP. When both proteins are brought in close proximity, a *scenario* that happens upon complex formation, energy from the excited state of the GFP fluorophore can be transferred to RFP. Thereby, the GFP donor partially excites fluorescence of the RFP acceptor. Photobleaching by a strong laser pulse is used to turn off the acceptor fluorescence, resulting in an increased fluorescence intensity of the GFP donor. In their study, Bisson et al. (2009) fused GFP to ETR1 and mCherry, a RFP, to EIN2. Both fusion proteins were cotransformed in tobacco leaf cells and ETR1-EIN2 complex formation was identified by FRET. Later on, this technique was used to demonstrate complex formation of EIN2 with all five members of the *Arabidopsis* ethylene receptor family (Bisson and Groth 2010).

Notwithstanding, conventional and membrane based *yeast two-hybrid* systems as well as fluorescence-based approaches such as BiFC and FRET are easy to handle and provide a cellular environment for the analysis of protein–protein interactions, these techniques also have some pitfalls. The most common problem with these assays is the detection of false-positive interactions. By using an over-expressing system, no matter whether yeast or plants, the high local concentration of protein molecules frequently causes unspecific complex formations, which are then detected by the in vivo-based method although these complexes will not exist at normal cellular conditions. Therefore, it is essential to include negative controls

in these assays in order to verify the identified protein–protein interactions. Moreover, any protein–protein interaction identified by these techniques should be confirmed by independent methods, preferentially by quantitative in vitro-based methods, which are described in the next section.

Albeit the techniques for studying protein–protein interactions described in the previous sections are reliable and sophisticated, they give essentially qualitative insights on complex formation. In contrast, a comprehensive analysis of complex formation requires quantitative data on the protein–protein interaction. Quantitative analysis can discriminate intermediate states and resolve the effects of different physiological conditions or environments, on a protein–protein interaction, e.g., the effects of cofactor or ligand binding or protein phosphorylation.

At present, quantitative determination of protein–protein interactions in living systems is not feasible. Thus, quantitative data on complex formation are currently limited to in vitro studies on purified proteins. The dissociation constant (K_d) of a protein–protein interaction is a measure of the stability of the complex and describes the affinity between the two interacting proteins. When $c(A)$ and $c(B)$ are the concentrations of the interaction partners and $c(AB)$ the concentration of the protein complex, the K_d of the complex is defined as

$$K_d = \frac{c(A) \cdot c(B)}{c(AB)}$$

The lower the K_d value, the stronger the complex formation. In contrast, a high K_d value reflects a weak or unspecific interaction. The first protein–protein interaction quantified in the ethylene signaling pathway was the interaction of ETR1 and the AHP1 phosphotransfer protein. Scharein et al. (2008) applied *fluorescence polarization* (FP) to calculate the affinity of this interaction. FP of a labeled macromolecule depends on the rotational motion of the molecule or the complex of a molecule in solution and describes the competition between the molecular motion and the fluorescence lifetime of the molecule. Broadly speaking, the smaller a fluorescent molecule, the higher its mobility and vice versa. A high mobility is related to a low FP. A mathematical description of this phenomenon was established by Perrin (1926) providing a direct correlation of FP and molecular weight. Huff et al. (1994) pioneered FP to study protein–protein interactions. Scharein et al. (2008) fused GFP as a fluorophore at the C-terminus of AHP1 and expressed the recombinant AHP1-GFP fusion protein heterologous in *E. coli.* The expressed fusion protein and recombinant full-length receptor ETR1 were isolated and purified to homogeneity. Then, FP of AHP1-GFP was analyzed at increasing concentrations of ETR1. By plotting the change in FP of AHP1-GFP against the ETR1 concentration, an apparent K_d of the ETR1-AHP1 complex of 1.4 μM was obtained. Later on, Scharein and Groth (2011) used the FP-based assay to characterize ETR1-AHP1 complex formation with different phosphorylation mutants of ETR1 and AHP1. In their study they found, that the phosphorylation state of ETR1 and AHP1, affects the affinity of the ETR1-AHP1 complex.

Bisson et al. (2009) established a *fluorescence-quench based assay* to characterize and quantify ETR1-EIN2 complex formation. This technique makes use of the endogenous fluorescence of proteins that is based on their aromatic residues. At 295 nm wavelength excitation, protein fluorescence is essentially determined by tryptophan residues. As tryptophan fluorescence is very sensitive to conformational changes and to the microenvironment (see Sect. 12.2) complex formation with other proteins affects tryptophan fluorescence and typically results in a quenched fluorescence. The technique was applied earlier by Libich et al. (2003) to study the interaction between Ca^{2+}-calmodulin and MBP. A prerequisite of this method is that the interacting protein partner carries no endogenous tryptophans. Hence, Bisson et al. substituted all endogenous tryptophan residues in ETR1 and the carboxyl-terminal part of EIN2 for leucine and phenylalanine residues. Functionality of the tryptophan-mutant proteins was verified by demonstrating autokinase activity for ETR1 and by phenotypic rescue-studies of tryptophan-less EIN2 (Bisson and Groth 2010). Next, ETR1, the EIN2 C-terminus and their tryptophan-free mutants were heterologously expressed in *E. coli* and purified from the bacterial host. For steady-state fluorescence measurements, the protein containing tryptophan residues was excited at 295 nm and fluorescence emission was monitored at 345 nm. Then step-by-step titration of the tryptophan-free interaction partner was performed. The resulting fluorescence quench was used to calculate the fraction of bound protein according to Libich et al. (2003) and plotted against the concentration of the tryptophan-free interaction partner. A K_d of 400 nM was obtained from these data corresponding to a tight interaction of ETR1 and EIN2. Later on, Bisson and Groth (2010) used the tryptophan quenching method to analyze ETR1-EIN2 complex formation in the absence and presence of the ethylene analog cyanide and found that cyanide leads to a fourfold increase in the complex stability. Similarly, titration of ETR1 lacking the receiver domain, and thus representing receptor ERS1, with the tryptophan-free EIN2 in the presence and absence of cyanide was performed. The ETR1 mutant mimicking ERS1 had a similar affinity to EIN2 as full-length ETR1. These data imply that the kinase domain of the receptors forms the interaction site with EIN2. Finally, ETR1 phosphorylation mutants were analyzed by this method. No change in the affinity for EIN2 was found. In contrast, to wild-type ETR1 the interaction was insensitive to cyanide implying that the plant hormone might control ETR1-EIN2 complex formation due to the phosphorylation state of the receptor.

A recent technique for studying protein–protein interactions in a quantitative manner is *microscale thermophoresis* (MST). This technique is based on the motion of molecules in a temperature gradient. Thermophoresis depends on the size, the charge, and the solvation shell of a molecule. Typically at least one of these parameters is changed upon protein–protein interaction. Thus, highly sensitive quantification of the interaction is possible (Wienken et al. 2010; Jerabek-Willemsen et al. 2011). For quantification one of the binding partners is labeled by a suitable fluorescence dye, e.g., Alexa Fluor Dyes. Fluorescence of the labeled protein is monitored in a small glass capillary. Next, an infrared (IR) laser generates a temperature gradient leading to the diffusion of the labeled molecule. When the

temperature gradient is applied at different concentrations of a putative interaction partner, the affinity of the complex can be determined from the fluorescence changes of the labeled protein at the different concentration of the binding partner and a K_d value for the interaction can be calculated. To evaluate this new technique, Bisson and Groth analyzed the previously described ETR1-EIN2 interaction by MST. Purified ETR1 receptor protein was labeled with Alexa Fluor 488 and MST was performed at increasing concentrations of the EIN2 C-terminus. The K_d value for the ETR1-EIN2 interaction was calculated to 421 nM, which is highly similar to the K_d value obtained in tryptophan quenching studies. Due to its response to diverse parameter such as molecular size, charge, solvation entropy, and hydration, MST seems a promising tool for the analysis and quantification of protein–protein interactions in general and more specifically for further studies on the ethylene signaling pathway.

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Chapter 13
Research Tool: Ethylene Preparation: Treatment with Ethylene and Its Replacements

Mark Tucker and Chi-Kuang Wen

Abstract Ethylene gas is an important plant hormone that can be chemically prepared or biologically synthesized by microbes and plants. The gas can be commercially obtained in a pressurized gas cylinder or chemically prepared with necessary purifications. Laboratories that wish to perform experiments involving ethylene treatment need a convenient setup for ethylene preparation and delivery. When the use of a pressurized ethylene gas cylinder is not feasible, an ethylene response can be initiated in the plant or plant organs by treating the plant or organ with an aqueous solution of the natural plant precursor to ethylene, 1-aminocyclopropane-1-carboxylic (ACC), or 2,4-dichlorophenoxyacetic acid (ethephon), which decomposes slowly to make ethylene at a pH above 4.0. However, the release of ethylene for these applications is dynamic and not experimentally controllable, and, moreover, the replacement may produce unwanted side effects that can affect data interpretation. Therefore, a direct ethylene treatment is often favorable over the replacement. An alternative to a pressurized tank of ethylene is the chemical synthesis of ethylene by ethanol dehydration or ethephon decomposition, with a specific setup to collect the produced ethylene. This chapter discusses the advantages and disadvantages of direct ethylene treatment using ethylene gas or a replacement. Also discussed are the underlying chemical and biochemical reactions for ethylene production, and the setup for ethylene treatment in a closed system or a flow-through system. The goal is to provide readers with the necessary tools for ethylene treatment with easily accessed laboratory devices.

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13.1 Introduction

Ethylene is a gaseous plant hormone regulating many aspects of plant growth and development. For research purposes, there are advantages and disadvantages to working with a gaseous hormone. Because it is a gas, it is quickly and easily disseminated inside a closed chamber. Commercially, fruit (e.g., bananas, tomatoes, avocado) can be packed into a large chamber and cheaply and efficiently exposed to ethylene to induce ripening (Abeles et al. 1992). Biological experiments involving ethylene treatment require a pressurized ethylene gas cylinder as an ethylene source and necessary devices for handling and delivering the gas. Unlike other plant hormones that can be delivered and treated as a solution, the gas cylinder and necessary devices for ethylene treatment may not be readily available in laboratories that only occasionally perform the treatment. Nonetheless, unlike most other plant hormones, which have specific mechanisms for uptake, transport and metabolism, ethylene diffuses rapidly into the plant through stomata and is water and lipid soluble, which allows it to readily move across cell membranes with no specific transport mechanism (Abeles et al. 1992). Experimentally, because multicellular plants have interconnecting gas space to every cell and diffusion through gas is 10,000-fold faster in air than water, this means that, although the plant may synthesize more ethylene when exposed to ethylene, the concentration you present on the outside of the plant is the minimum concentration that a cell on the inside of the plant perceives at its surface. The concentration at the surface of a cell for other plant hormones (e.g., IAA, BA, ABA, JA) is not so easily known. However, there are experimental circumstances where a gaseous treatment is not feasible. In this case, a soluble replacement (e.g., ACC or ethephon) can be used to induce an ethylene response in the plant.

The ethylene biosynthesis pathway in higher plants begins with the amino acid methionine, which is converted to S-adenosyl-L-methionine (SAM). SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is the immediate ethylene biosynthesis precursor and is converted to ethylene and cyanide on oxidation by the ethylene-forming enzyme ACC oxidase (ACO) (Adams and Yang 1979; Peiser et al. 1984; Kende 1993; Yoon and Kieber 2013). Cyanide is a toxic byproduct of ethylene production with ACC oxidation and is removed immediately by the conversion to β-cyanoalanine and asparagines (Miller and Conn 1980). Of note, cynoformate is an intermediate product formed during the reaction to shuttle away the toxic cyanide from the active site of ACO, such that the iron-containing ACO is protected from the cyanide deactivation (Murphy et al. 2014). The formation of ACC is the rate-limiting step for ethylene production, and an elevated ACC level results in higher ethylene evolution. Therefore, ACC is

widely used as an ethylene replacement. However, ACC is consumed shortly after application (Zhang et al. 2010; Zhang and Wen 2010; Lavee and Martin 1981); replacing ethylene with ACC may not be ideal for quantitative experiments requiring a long response window.

Another replacement for ethylene is ethephon (also called Ethrel). Under alkaline conditions, ethephon (2,4-dichlorophenoxyacetic acid) decomposes to ethylene, phosphate, and chloride (Lavee and Martin 1981; Yang 1969; Biddle et al. 1976; Zhang and Wen 2010). Aqueous ethephon solutions are widely used as a replacement for ethylene treatment. The uptake and decomposition of ethephon *in planta* is unclear and not experimentally controllable, and the decomposition products phosphate and chloride that produce a low pH condition may have adverse effects on many aspects of plant growth and physiological processes (Reid et al. 1980; Goudey et al. 1987; Southwick et al. 1986; Zhang and Wen 2010). Thus, using ethephon as a replacement for ethylene treatment is not ideal for quantitative experiments requiring a long response window, and unwanted effects with the strong acid phosphate and chloride produced by ethephon decomposition must be evaluated.

For a plant laboratory that may not want to invest in a pressurized tank of ethylene and are concerned about applying ethephon directly on the plant, ethylene can be prepared by chemical decomposition of ethephon or complete ethanol dehydration. The ethylene released in these systems can then be used in a closed system to induce an ethylene response in the plant.

This chapter describes the use of ethylene replacements and possible unwanted effects associated with the replacements, the chemical preparation of ethylene with use of standard laboratory equipment, the delivery and transfer of the gas, the setup for an airtight chamber for ethylene treatment, and a flow-through system for experiments requiring a stable ethylene concentration environment. Researchers with different experimental needs may choose appropriate approaches for ethylene treatment.

13.2 Ethylene Treatment with the Use of Replacements

ACC and ethephon are the two most widely used replacements for experiments involving ethylene treatment. Both are solid, water soluble, and thus easily prepared. ACC is an intermediate product of ethylene biosynthesis and ethephon is not; the former can be oxidized to produce ethylene by ACC oxidase, whereas mechanisms for the absorption and decomposition for the latter *in planta* is unknown. Although convenient, both have limitations and drawbacks when used to replace ethylene. Ethylene is released within a short response window by the replacements, and the release is dynamic and uncontrollable; therefore, the treatment can be qualitative but not quantitative and thus the result is less reproducible. Nevertheless, the replacements may have other advantages. Several factors should be considered for proper experimental design with ethylene replacements.

13.2.1 ACC as an Ethylene Replacement

ACC, $C_4H_7NO_2$, with a molecular mass 101.1 g $mole^{-1}$, is a solid and is readily dissolved in water. The concentration and amount of ACC to be used is dependent upon the desired amount of ethylene production, which is not necessarily constant throughout the treatment window (Lavee and Martin 1981; Zhang and Wen 2010; Zhang et al. 2010). A sufficient supply of ACC is essential to ensure a prolonged ethylene exposure. Otherwise, the ethylene released by ACC oxidation will decrease over time because of ACC consumption. For experiments involving ethylene effects on inhibiting seedling hypocotyl growth, the seeds are germinated and grown on a relatively large volume of Murashige and Skoog (MS) medium in agar with a large amount of ACC at the necessary concentrations so that ethylene biosynthesis is sustained through a desired growth period. *ETHYLENE RESPONSE FACTOR1* (*ERF1*) is a primary target of the ethylene signal, and its induction is directed by the transcription factor ETHYLENE INSENSITIVE3 (Solano et al. 1998). The expression of *ERF1* is linked to the degree of the ethylene response and thus is a form of quantification for the ethylene response. *Arabidopsis* seedlings grown on MS-containing agar supplemented with ACC show nearly identical *ERF1* expression as those treated with a saturating concentration of ethylene, e.g., 10 μL L^{-1}. In contrast, *Arabidopsis* plants treated with a foliar spray of ACC do not show reproducible *ERF1* expression (Zhang and Wen 2010). For hypocotyl growth experiments, the seedlings in this short-term treatment only consume a small fraction of the supplemented ACC, and therefore, the conversion of ACC to ethylene is sufficient and sustained for the necessary time frame. When ACC was applied as a foliar spray, the amount of ACC absorbed by individual plants may have varied and consumed quickly. Thus, a foliar spray may be ideal for experiments requiring a short response window. Prolonged ethylene exposure would require periodic sprays of ACC over the entire experiment. Nevertheless, the amount of ACC sprayed on an individual plant may vary, and the ethylene produced also varies; thus, foliar sprays of ACC may be less reproducible.

Although ACC has been used to replace ethylene treatment for analysis of seedling growth inhibition, when non-maximal concentrations of ethylene and ACC are used, the effects on *Arabidopsis* growth inhibition differ slightly. Over a wide concentration range, the ethylene dose–response shows a concave curve, whereas the ACC dose–response curve is convex, with 50 % growth inhibition for 0.1–0.2 μL L^{-1} (ethylene) and 0.5 μM (ACC) (Zhang and Wen 2010). Aminoethoxyvinylglycine (AVG) is a potent ethylene biosynthesis inhibitor. Ethylene treatment with AVG supplementation does not affect *Arabidopsis* seedling growth inhibition over a wide ethylene concentration range. In contrast, with ACC treatment to replace ethylene, with AVG supplementation, seedling growth inhibition is in part alleviated over a wide range of ACC concentration. Thus, without AVG to prevent endogenous ethylene production, ACC treatment may trigger an increase in endogenous ethylene production, whereas treatment with ethylene does not.

Several factors should be considered when ACC is used to replace ethylene treatment. In higher plants, the amino acid methionine is adenylated to form SAM, an important methyl donor involved in many biological processes and the biosynthesis of polyamines and ACC (Pommerrenig et al. 2011). ACC is the immediate precursor for ethylene biosynthesis on oxidation by ACOs. Thus, replacing ethylene treatment with ACC requires sufficient oxygen to ensure the ethylene production reaction. ACC may not be efficiently converted to ethylene for experiments performed under hypoxia (or oxygen shortage). Thus, for an ACC treatment performed in small closed vials or containers, in which oxygen is used for respiration, oxygen availability may be insufficient to support prolonged growth, and the ACC oxidation reaction attenuated. No matter, hypoxia in itself can impose adverse effects on plant growth. For experiments carried out in a closed system, with ACC used to replace ethylene, sufficient oxygen is needed to support ACC oxidation and plant growth.

Of note, lower plants also produce ethylene, and the gas has biological effects on many aspects of growth and development in ferns. The unicellular spores of the fern *Onoclea sensibilis* can germinate in darkness and produce 2 cells by cell division, and the initial division is inhibited by ethylene as low as 0.1 μL L^{-1} (Fisher and Shropshire 1979; Edwards 1977). However, lower plants do not seem to use ACC as a precursor for ethylene biosynthesis. Although ACC is present in ferns, ACC treatment does not increase ethylene evolution. Treatment with aminoethoxyvinylglycine and α-aminoisobutyricacid, the inhibitors of the ethylene-forming enzymes ACS and ACO, respectively, does not inhibit ethylene production in the semi-aquatic ferns *Regnellidium diphyllum* Lindm. and *Marsilea quadrifolia* L. (Chernys and Kende 1996). When these plants were treated with radioactive [^{14}C]-ACC, the ACC was readily taken up and decarboxylated by the fern *R. diphyllum* and the liverwort *Riella helicophylla*, but the [^{14}C]-ethylene was not released (Osborne et al. 1996). Thus, ACC is not a replacement for ethylene for experiments involving lower plants.

In higher plants, ACC can be malonylated to form N-malonyl-ACC (MACC) by ACC N-malonyltransferase with malonyl-CoA used as the malonyl donor (Peiser and Fa Yang 1998; Finlayson et al. 1991; Martin et al. 1995; Martin and Saftner 1995). MACC is not normally converted back to ACC for ethylene biosynthesis. The conversion of ACC to MACC is probably a mechanism to consume excess ACC to prevent excess ethylene production (Hoffman et al. 1983). When an excess of ACC is present in the plant, a certain fraction of ACC may be converted to MACC, which would accelerate ACC consumption.

In addition to ethylene, cyanoformic acid is formed on the oxidation of ACC by ACO, and cyanoformic acid is spontaneously degraded into cyanide and carbon dioxide (Peiser et al. 1984; Murphy et al. 2014; Adams and Yang 1979; Kende 1993). Cyanide is toxic and can be metabolized to form β-cyanoalanine and asparagine. Recent studies suggest that cyanide formed by ACC oxidation may have a role in rice (*Oryza sativa*) resistance to the blast fungus *Magnaporthe oryzae*. Treatment with ethylene alone has little effect on rice resistance to the blast fungus, whereas potassium cyanide (KCN) and ACC each confers the resistance.

Salicylhydroxamic acid (SHAM) is an inhibitor of cyanide-resistant respiration (Seo et al. 2011). The mycelium growth of *M. oryza* on potato dextrose agar (PDA) supplemented with KCN and SHAM was substantially inhibited, whereas KNC and SHAM alone had a minor effect on the fungal growth. The synergistic effect indicates that the prevention of cyanide-sensitive and cyanide-insensitive respiration is fatal to the fungal growth. However, it should be noted that the amount of cyanide and ACC used in the experiment was relatively high (0.5–1 mM for KCN and 0.5 mM for ACC), that may not necessarily occur *in planta*. Of note, although ACC as an ethylene replacement has limited use, ACC is an ideal replacement for ethylene treatment in experiments when treatment with ethylene is technically difficult. For instance, ACC but not ethylene treatment can be used to observe the effects of ethylene at the subcellular level in live cells by microscopy.

13.2.2 Ethephon as an Ethylene Replacement

Ethephon, $C_2H_6ClO_3P$, with molecular mass 144.5 g mole^{-1}, is a solid and is readily soluble in water (123.9 g 0.1 L^{-1} at 23 °C). The amount of ethephon needed for ethylene replacement can be determined experimentally. Ethephon is a dibasic acid (pKa1 = 2.24 and pKa2 = 6.97 at 25 °C), and commercially available ethephon solutions are acidic (pH about 2.3). Ethephon is in the form of monoanion at low pH and dianion at high pH. About 10 and 90 % of ethephon is in the dianion form at pH 6 and 8, respectively. Above pH 9, ethephon is 100 % in the dianion form, which is the form that undergoes decomposition into ethylene (Reid et al. 1980; Biddle et al. 1976; Yang 1969):

$$ClCH_2CH_2PO_3^{2-} + H_2O \rightarrow Cl^{-1} + C_2H_4 + H_2PO_4^-$$

The foliar application of aqueous ethephon solution has been widely used in agriculture. Lodging results in yield loss in tall cereals, such as barley and corn, and can be reduced with ethephon treatment to promote yield increases (Dahnous et al. 1982; Norberg et al. 1988). Ethephon treatment also increases the tiller number, millable canes, and yield of sugar cane (Li and Solomon 2003).

Foliar sprays or ethephon in hydroponic culture has been used to replace ethylene treatment in biological experiments. The release of ethylene from ethephon is dependent on both the pH and temperature of the aqueous environment. Thus, the buffering capacity of the solution the ethephon is dissolved in can have profound effects on the rate of ethylene released. Moreover, ethephon itself can contribute to buffering capacity when the treatment solution has weak or little buffer capacity. In weakly buffered solutions, the release of ethylene will decrease as the pH of the solution decreases as a result of ethephon decomposition. Also important to the efficacy of ethephon treatment is the uptake and movement of ethephon in the plant. Thus, complications with buffering capacity and movement of ethephon in the plant

require that appropriate treatment conditions be empirically determined. This limits its utility for research but has been very useful for well-characterized commercial applications.

Acids produced by ethephon may have other effects that are coupled with or not induced by ethylene and also promote ethylene biosynthesis to induce ethylene responses (Goudey et al. 1987; Reid et al. 1980; Zhang and Wen 2010). Of note, the amount of ethylene produced by ethephon decomposition is undetermined and may be dynamic over a response window; the combined effects of ethylene and the produced acids complicate the treatment, and the interpretation of the experimental data is difficult.

13.3 Chemical Preparations of Ethylene

Ethylene is widely used in the chemical industry and can be mass produced in the petrochemical industry by steam-cracking hydrocarbons; the procedure is complicated, energy intensive, and requires facilities that are not affordable for most laboratories to synthesize the gas. Ethylene and ethanol are interchangeable by hydration/dehydration reaction and ethanol dehydration with catalysts such as sulfuric acid, and aluminum oxide produces ethylene. The production of ethylene with industrial approaches may not be ideal for laboratories without the facilities to perform the reaction and purification. Nevertheless, ethylene can be produced by ethephon decomposition under mild conditions. Plant laboratories can easily set up ethylene production with the use of ethephon.

13.3.1 Ethanol Dehydration for Ethylene Production

Cracking ethanol to give ethylene and water can be used to produce ethylene by removal of the hydroxyl (–OH) group and hydrogen atom from the second carbon in the chain. With the use of acid as a catalyst for ethanol dehydration, the hydroxyl group is protonated by an acid and leaves as a water molecule; the methyl group of ethanol is then deprotonated by the conjugated base of the catalyst, and the hydrocarbon rearranges into ethylene. The reaction is zero-order and endothermic occurring at elevated temperature (180–500 °C) that shifts the equilibrium toward ethylene production. Of note, reactions to form diethyl ether or acetaldehyde are favored outside the temperature range, and byproducts are generated (Zhang and Yu 2013). Ethanol can also be oxidized by concentrated sulfuric acid to carbon dioxide and the acid reduced to sulfur dioxide at the same time. The ethanol dehydration by concentrated sulfuric acid is dangerous in a regular laboratory, and the produced ethylene contains byproducts that need to be removed before biological treatment to avoid unwanted effects.

Various solid acid catalysts such as zeaolites and silica-alumina have been used for ethanol dehydration to produce ethylene in industry (Takahara et al. 2005). γ-alumina (Al_2O_3) is an alternative catalyst for industrial ethanol dehydration; the ethylene yield is relatively low (80 %) and contains byproducts, and the reaction temperature is high (450 °C). With various modifications, γ-alumina-catalyzed ethanol conversion has been improved with a higher ethylene selectivity (Fan et al. 2012). Nevertheless, the reaction needs high temperature, and the produced ethylene must be purified to remove byproducts. In addition to γ-alumina, several nanoscale catalysts have been developed for ethanol dehydration, with relatively high conversion rate and ethylene selectivity at lower temperature (as low as 220 °C).

Although the production of a small amount of ethylene for a biological treatment does not have to consider conversion efficiency and cost-effectiveness, the ethylene produced by ethanol dehydration must be purified to prevent unwanted effects exerted by the byproducts. With a reaction taking place at high temperature, with various byproducts, ethanol dehydration can be dangerous and not a favored approach for laboratories that do not have the facilities for the reaction and purification.

13.3.2 Ethylene Production by Ethephon Decomposition

Ethephon has been widely used as a replacement for ethylene treatment. The chemical reaction that takes place for ethephon to produce ethylene is described above (Sect. 13.2.2). Providing a constant reaction condition that favors the decomposition of ethephon facilitates the production of the ethylene gas that can be used directly for an ethylene treatment without purification. Ethephon decomposition is favored under alkaline conditions. At pH > 9.0, the chemical is nearly completely in the dianion form, which decomposes readily into ethylene (Biddle et al. 1976; Yang 1969). Of note, ethephon decomposition produces acid compounds, phosphate and chloride, that will reduce the pH of the solution and slow the decomposition reaction (Sect. 13.2.2). Thus, to ensure constant decomposition, a strong buffer capacity is needed to maintain the reaction at a high pH. Because the products of the decomposition are ethylene and acids and the latter remain in the solution, the ethylene gas produced can be easily collected for use without the need for purification.

Chemical conversion of ethephon to ethylene for subsequent treatment of plants in a closed system has been tested (Zhang and Wen 2010). A kinetic analysis for the ethylene production with ethephon decomposition in the solution containing disodium phosphate (5 mM) as the reaction buffer showed that ethylene production was tightly correlated with the amount of ethephon, with $R^2 > 0.99$. Ethylene from a commercial gas tank and the ethylene produced by ethephon decomposition gave identical ethylene dose–response curves, when measuring hypocotyl length of etiolated *Arabidopsis* seedlings. Also, the purity of the ethylene produced from ethephon was examined by gas chromatography (GC), and no other hydrocarbon

species were detected. Thus, the ethylene produced from ethephon can be used directly for biological experiments. Of note, production of ethylene by ethephon decomposition is a cost-effective alternative to purchasing a compressed tank of ethylene. The decomposition of 8.3 μmole ethephon in a 2-L container produces an ethylene concentration of 102.83 ± 6.15 μL L^{-1} and 0.83 μmole ethephon an ethylene concentration of 9.47 ± 0.17 μL L^{-1}. Both ethylene concentrations are sufficient to saturate ethylene responses for most biological experiments.

For laboratories without the equipment to deliver the ethylene, the ethephon decomposition can occur in an airtight, closed chamber, along with the biological material to be treated, to produce ethylene without further gas handling. For example, in an experiment to determine the ethylene dose–response of etiolated *Arabidopsis* seedlings, ethylene introduced from a pressurized commercial tank and ethylene produced from ethephon decomposition produced nearly identical results (Zhang and Wen 2010; Zhang et al. 2010). For ethylene responses that require a short response window, the pH of the buffered solution needs to be higher than 9.0 to rapidly release ethylene and mixed with the ethephon while inside the closed chamber. This can be accomplished by injecting the buffer through a septum into a container of ethephon (see below). When the exact concentration of ethylene must be known, ethylene in the chamber can be quantified as described in Chap. 14.

13.4 Handling of the Ethylene Gas

Unlike many other plant growth substances that can be prepared in aqueous solutions, ethylene is a gas and its handling requires special equipment. Two main approaches for ethylene gas treatments are described: (1) a closed system where ethylene of a known concentration (e.g., 1,000 μL L^{-1}) is injected into a chamber of known volume (e.g., 1.0 L), and (2) a regulated concentration of ethylene in air is passed continuously through the chamber. Of note, in the dark, plants and plant organs consume oxygen in respiration and emit CO_2, water vapor, and other volatiles, and in the light the amount of oxygen produced and CO_2 consumed can vary. In other words, in a closed system, the concentration of gases can change, while in an open flow-through system, the gas concentrations are kept more constant.

13.4.1 Ethylene Treatment in a Closed System

For a closed system, the ethylene gas must be transferred from a source to an airtight chamber in which the plant material is placed. The gas concentration for ethylene is usually presented as μL L^{-1}, and, for most experiments, ethylene responses can be saturated within a concentration range of 1–10 μL L^{-1}. The gas chamber can be an airtight container of any type; a canning jar (Mason jars, with a band and lid) is ideal for small plant materials (such as seedlings, detached leaves

on *Petri* dishes, or small fruits) or a desiccator or custom-made acrylic chamber of for larger plant materials (such as plants in pots).

For most gas chambers, a rubber stopper can be sealed in the top or side of the chamber to create a gas inlet/outlet to facilitate the injection of the ethylene gas and the sampling of the chamber gas for ethylene and other volatile measurement. To create the gas inlet/outlet, a hole is drilled in the container, and a rubber stopper is pushed into or placed over the hole and sealed with silicone sealants. The rubber stopper can be of any kind; a re-sealable stopper is preferred because it can allow for repetitive injection and sampling of the gas, with a syringe and a needle, with little impact on airtightness. A variety of sleeve and vaccine stoppers can be used. A medical syringe needle with a bevel tip is not recommended for multiple sampling, because it can destroy the airtightness of the rubber stopper or the septa pinhole. We prefer to use the inlet septa for GC (part number: 5183-4761 from Agilent Technologies or its replacements) as the stopper and a GC manual syringe because the re-sealable pinhole at the septa can be repeatedly used with the GC syringe, of which the cone tip needle does not destroy the septa and will maintain airtightness.

The ethylene source can be a pressurized gas tank or a flask containing the ethylene gas at a known concentration. For most treatments, the amount of ethylene to be administered is very small. For instance, only 100 μL ethylene is needed for a 10 μL L^{-1} ethylene treatment in a 10 L chamber. Typically, the pure ethylene stock is diluted by injecting a known concentration of ethylene into a closed flask that you have measured the volume of by weighing the empty flask and then filing it to capacity with water and weighing again (water is 1.0 g mL^{-1}). It is essential that the flask be completely dry before making the gas dilution because ethylene is partially soluble in water. Often, if pure ethylene is used as a starting material, a series of dilutions will be required to sufficiently reduce the ethylene concentration.

The pressure in a pressurized tank is generally quite high, and taking the gas directly from the tank can be highly dangerous and technically difficult. A reducing valve (or pressure regulator) connected to the gas tank reduces the gas pressure to facilitate the safe use of the gas. A double-stage regulator is preferred because it has two valves. A rubber or silica tubing connected to the pressure regulator is used to collect pure ethylene from the tank. To do this, a clamp seals the end of the tubing and low-pressure ethylene is released from the tank, then the clamp is released to flush the air away. The flushing is repeated several times to ensure that the air in the tubing is flushed away and replaced by ethylene. Alternatively, the end of the tube can be sealed with a sleeve-type stopper with a small-gauge needle (#26) inserted in the stopper to continuously evacuate the gas for a few minutes before withdrawing pure ethylene from the tube. A medical syringe (preferably an insulin syringe) with a bevel tip needle can be used to withdraw the ethylene gas from the tubing.

Of note, an accurate concentration is difficult to achieve when a fairly small amount of gas is transferred for dilution, because a very small variation that occurs on the stock measuring will result in a large variation in the final concentration. For example, measuring 100 μL with a standard 1.0 mL insulin syringe is not particularly accurate. A larger volume is more accurately measured than a smaller one. For an ethylene dose–response assay, it is particularly useful to dilute the ethylene

stock by serial dilution. To improve accuracy of the dilutions, we use a constant volume of gas (e.g., 1.0 mL) when transferring the gas from the stock to each descending dilution. As long as the dilution flasks are completely sealed, the diluted ethylene stocks can be prepared in advance.

As mentioned previously, an alternative to a pressurized tank is preparation of ethylene by decomposing a necessary amount of ethephon with an alkaline solution in an airtight container. The airtight container could be a container separate from the treatment container, wherein ethylene is withdrawn and injected into the treatment container or the actual treatment container in which the alkaline solution is injected into a vial containing the appropriate amount of ethephon. If necessary, the concentration of ethylene can be confirmed by gas chromatography. We showed that the commercial ethylene and chemically prepared ethylene have identical biological effect on the growth of *Arabidopsis* seedling and induction of the ethylene-inducible *ERF1* gene (Zhang and Wen 2010; Zhang et al. 2010).

The setup of ethylene treatment in a closed system is simple and cost-effective and can be versatile with the use of nearly any airtight container with a gas septa inserted or sealed onto the chamber. Here we describe two types of containers that have been tested for ethylene treatment in a closed system (Fig. 13.1).

For treating small plant materials, such as *Arabidopsis* seedlings and detached leaves on a *Petri* dish, any airtight container with a diameter greater than that of a *Petri* dish will suffice. The volume of the *Petri* dish (including the growth medium) can be estimated. Because the amount of ethylene that partitions into aqueous solutions (media) is small compared to the amount in the gas space, the volume the *Petri* with media can be deducted from container volume. For instance, if the net gas volume of the container is 0.5 L and the ethylene stock is 1,000 μL L^{-1}, 0.5 mL of the ethylene stock is injected to achieve a final ethylene concentration of 1 μL L^{-1}. To ensure airtightness, we usually seal the "mating surfaces" on the lid and the container with grease (such as Vaseline) to prevent gas leakage. The gas inside the container can be sampled periodically and quantified by gas chromatography to determine changes in the amount of ethylene in the container. For ethylene treatment of larger plant materials, such as *Arabidopsis* rosettes or rice (*Oryza sativa*) seedlings, we use a custom-made acrylic container. The "mating surface" of the chamber is sealed with a flat rubber gasket to ensure airtightness when lids are closed. Spring toggle latches (spring-loaded latch) on each side of the chamber secure the lid. A hole is drilled on the lid and a piece of gas septa is attached to the hole and sealed by silicone sealant. The container volume can be calculated by the chamber size, and the volume for the plant material (i.e., the pot volume) can be estimated and deducted from the chamber volume. An appropriate amount of ethylene stock is injected and can be quantified as described above. We do not recommend the use of laboratory glassware (such as a test tube or an Erlenmeyer flask) coupled with a matching rubber stopper for ethylene treatment. The stopper may slip over time without being noticed, and the slipping may result in gas leakage.

Precautions should be noted for ethylene treatment in a closed system. Plants emit water and the humidity in a closed chamber/container may increase throughout

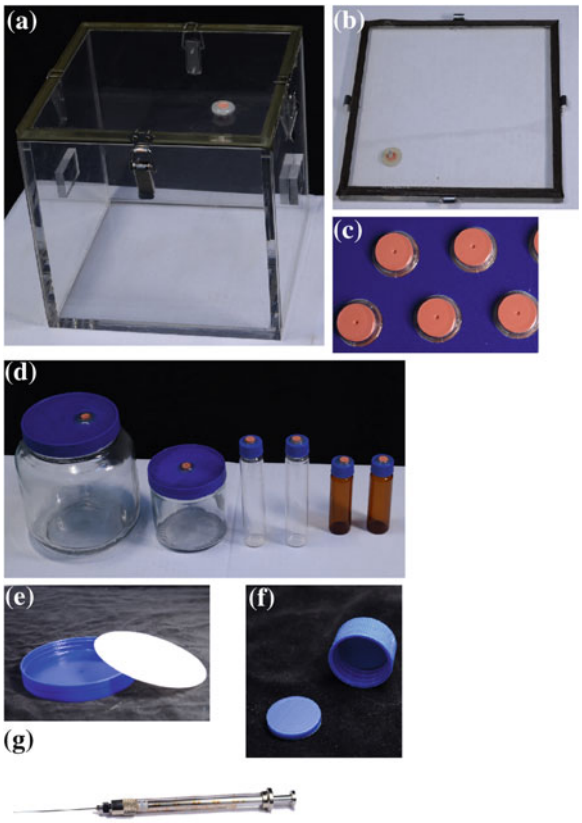

Fig. 13.1 Setup for ethylene treatment in an airtight closed system. **a** An airtight acrylic container for ethylene treatment in a closed system. Spring toggle latches on each side of the chamber fasten the lid. **b** On the lid, a hole is sealed with a gas chromatography (GC) inlet septa (*red*) that has a re-sealable pinhole for repeated gas delivery and sampling and a flat rubber gasket (*dark*) on the mating surface to ensure airtightness. **c** The GC septa inlet has a re-sealable pinhole. **d** An example of a container of other types that can be used for ethylene treatment. **e**, **f** A gasket or pad on the matching surface inside the lid of a container to ensure airtightness. **g** A GC syringe with a cone tip needle to deliver the gas

the entire treatment when the water content in the soil is high. As a result, water will condense and accumulate on the leaves to produce unwanted effects to the plant material. To prevent this situation from happening, absorbing paper is placed underneath the pot to absorb excess water, and the soil water content is kept at a low level sufficient for normal plant growth at the beginning of the treatment. Given that plants produce the ethylene gas, the ethylene concentration will also increase over time in a closed chamber. To minimize ethylene concentration fluctuation, a container or chamber of a large volume is preferred so that the emitted ethylene has less impact on the final ethylene concentration.

13.4.2 Flow-Through System for Ethylene Treatment

In a closed system, the concentration of ethylene may change slightly over time. Typically, the ethylene concentration will increase because the plant synthesizes ethylene. A marked decline in ethylene usually indicates a small leak in the system. However, if the duration of the experiment is fairly long, several hours to days, the change in CO_2, oxygen, and other volatiles can have marked effects on plant growth and the plants response to ethylene. When a more constant level of ethylene, oxygen, and CO_2 concentration is required, a flow-through system can greatly reduce variation in the composition of the gas environment. A fairly simple and inexpensive setup for doing this is in Fig. 13.2.

The central component in a flow-through system is a chamber that can be sealed except for a gas inlet and outlet. A vacuum desiccator can be used for this purpose. Some chambers come with two ports and others will need a port added. It is worth mentioning that, even though most chambers come with good quality latches, in some chambers it is difficult to achieve a 100 % seal on the door when no vacuum is being applied. However, in a flow-through system, a small leak less than 10 % the outlet flow can be tolerated because these leaks will have a negligible effect on the concentration of ethylene and other gases inside the chamber. If gas sampling is required to confirm the ethylene concentration inside the chamber, a silicone hose can be connected to the outlet and a gas sample withdrawn. The gas sample must be withdrawn slowly so as not to pull outside air into the open end of the tube and into the syringe.

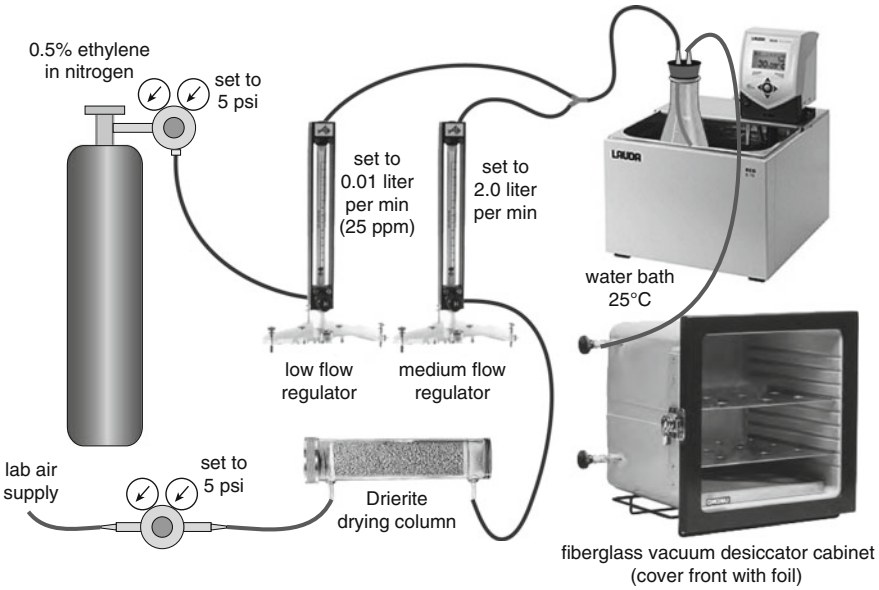

Fig. 13.2 Setup for flow-through ethylene treatment of plant material

Light and diurnal circadian rhythms affect most ethylene responses and need to be addressed. We use an opaque fiberglass chamber with a clear Plexiglas door, which has an approximate volume of 33 L. The clear door must be covered with aluminum foil or black paper to maintain darkness. We collect plant tissue at the same time each day (e.g., morning), and place the tissue in the dark chamber for ethylene treatment. However, for a light–dark cycle, a clear Plexiglas vacuum desiccator chamber can be obtained and kept in a lighted incubator.

Also important to any biological system is maintenance of temperature. The chamber can be kept inside a constant temperature room or incubator, but the gas mixture may be passed through a sealed flask held in a temperature-regulated circulating water bath to bring all the gases to the desired temperature before entering the sealed chamber (Fig. 13.1). If the chamber is kept in a room where the temperature is relatively close to the desired temperature, passing the gas mixture through the flask is sufficient to maintain a constant temperature in the chamber. We use a 2 L Erlenmeyer flask containing approximately 100 mL distilled water. The flask is sealed with a rubber stopper. Small holes are bored through the stopper and plastic inlet, and outlet tubes are pushed through the holes to make an airtight fit. To improve temperature equilibration and humidify the gas, Tygon tubing is connected to the inlet on the inside of the flask so that the tube extends down to the surface of the water in the bottom of the flask. The gas exits the flask through an outlet that extends only a short distance into the flask.

Preparing the gas mixture requires an air supply and a source of a high concentration of ethylene. We use the building air supply for our air source, but an inexpensive pump that can deliver 2 L min^{-1} with an outlet pressure of 5 psi or greater will suffice. The pressure of our lab air supply varies. We use a single-stage gas regulator to maintain a constant pressure of 5 psi at the outlet. We pass lab air through an approximately 0.5 L acrylic Drierite gas drying column (DRIERITE cat no. 26800), which removes moisture but also acts as a rough filter. Condensation must be prevented in the flow regulators that follow the Drierite column. Condensation will clog the regulators and change the flow rate. Most lab air has a low level of ethylene in it. Generally this can be ignored. However, if necessary for a control treatment, the air supply can be scrubbed of ethylene and other hydrocarbons by adding a second gas column packed with Purafil Select Media (a potassium permanganate product).

For our ethylene source, we use a 2,000 psi 44 L tank of 0.5 % ethylene in nitrogen (specialty gas mixture from Airgas). A different concentration can be used, but this works well with our flow regulators, flow rate, and final concentration. A tank of this size and concentration will last for quite a long time. A two-stage nitrogen gas regulator is required to reduce the outlet pressure to 5 psi. These gas mixtures are already dry and do not usually require a drying column.

The flow rate of the air and ethylene gases must be regulated and mixed to obtain an ethylene concentration in air of between 5 and 25 μL L^{-1}. In most plant systems, 0.1 μL L^{-1} ethylene in air produces a half-maximal ethylene response (Abeles et al. 1992). A concentration of 10 μL L^{-1} is commonly used as a saturating concentration to produce a maximal ethylene response (Abeles et al. 1992; Lincoln and

Fischer 1988); however, to insure a more rapid response for shorter time intervals, we use 25 μL L^{-1} ethylene in air with a final flow rate of 2.0 L min^{-1}. To obtain this concentration and flow rate, we use two rotameters (gas flow meters). A variety of flow meters can be used for this task. We use a flow meter that uses a 150 mm flow tube. These devices are fitted with different-sized tubes and valves to achieve the required flow rates. One of the flow meters should accurately (±5 %) measure an airflow between 0.5 and 5.0 L min^{-1} (LPM). The other flow meter should measure an air (nitrogen) flow between 0.002 and 0.050 LPM. This will dilute the 0.5 % ethylene in nitrogen (5,000 μL L^{-1}) with air to achieve an ethylene concentration between 5 and 25 μL L^{-1}. In our setup, for the air supply, we use a flow tube FM4331 with a standard metering valve 0202-4113 (L) from Specialty Gas Equipment (ASGE). The low-flow meter for the ethylene gas is fitted with a flow tube FM4334 with a standard metering valve 0202-4114 (M). After metering, the two flows are then combined before entering the Erlenmeyer flask in the water bath and then the chamber. We have successfully used this system for many years (Kalaitzis et al. 1995; Tucker et al. 1988; Tucker and Yang 2012).

13.5 Concluding Remarks

As a plant hormone important to many aspects of plant growth and development, by itself and in combination with other hormones and biotic and abiotic cues, ethylene or its replacements are widely used for biological experiments to address the aforementioned phenomena and underlying mechanisms. For simplicity and short duration experiment, a closed system works well for ethylene treatments; for longer exposures to ethylene, a flow-through system is superior to a closed system. When pressurized gas cylinder is not conveniently available, replacements for ethylene can be used instead. Small amounts of ethylene can be generated efficiently and inexpensively by chemical conversion of ethephon to ethylene in an alkaline solution. In a closed system, the ethylene gas produced from ethephon works as well as ethylene from a pressurized gas cylinder. Alternatively, ACC and ethephon can be applied directly to the plant in media or a spray. However, precautions need to be considered for the use of the ethylene replacement: (1) aqueous ethephon solutions are of low pH and the ethephon decomposition to produce ethylene produces acid species, which may lead to unwanted effects that complicate data interpretation; (2) the conversion of ACC to ethylene is an oxidation reaction and a sufficient oxygen supply will ensure the reaction to proceed; (3) ACC can be alternatively converted to MACC, which cannot be converted to ethylene; (4) results from ACC application by foliar spray may be less reproducible because of early ACC consumption and unequal spraying; (5) treatment with ACC or ethephon to replace ethylene treatment is not ideal for reactions that require a long response window; and (6) lower plants produce ethylene via unidentified pathway(s) that do not use ACC as a precursor, so ACC is not an ideal replacement for ethylene treatment for lower plants.

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Chapter 14
Research Tools: Ethylene Detection

Simona M. Cristescu, Ernst Woltering, Christian Hermans, Frans J.M. Harren and Sacco te Lintel Hekkert

Abstract Over the last decades, ethylene detection in plant physiological studies is mainly done through enclosing of the plant material under study for a period of time in a closed flask or container and analyzing the accumulated ethylene in the headspace using a gas chromatography equipped with a flame ionization detector. In most of the studies, this will give a reasonable measure of the in vivo ethylene production. However, especially the changing composition of the atmosphere may influence plant behavior and subsequently its ethylene production. Also, if such measurements are mostly done on excised plant parts, the induced wounding also may affect the total ethylene production. Therefore, there is clearly a need for more sensitive equipment to measure ethylene of whole plant or plant parts (*in planta*) in a flow-through situation. One direction is to further optimize standard GC equipment. This can be done, e.g., by using a more sensitive photoionization detector or through improved sampling and preconcentration devices. Another route is provided by other techniques including the laser-based detection or mass spectrometry that are inherently more sensitive and fast for ethylene measurement. This chapter

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discusses several of the available techniques for ethylene detection as well as the gas sampling approaches. Guidelines for proper selection and use of the described methods are provided together with examples of applications of monitoring ethylene production from various biological samples.

Keywords Gas sampling · Gas chromatography · Mass spectrometry · Electrochemical sensing · Optical sensor · Laser-based detector · Real-time detection

14.1 Practical Approaches for Headspace Sampling and Analysis

With the exception of fruit storage under controlled conditions, most of the measurements involving ethylene monitoring take place in laboratory environment under controlled temperature and light intensity. Headspace from plant parts/organs or from the whole plant is usually collected for analysis. Ethylene is sampled either from detached plant/plant parts or when possible from attached plant parts in order to avoid any wounding effect. In case that systemic induced response is of interest, in situ enclosing plant material is the optimum choice. Ethylene sampling can be performed from static or dynamic headspace, depending on the concentration levels and the available method for analysis.

14.1.1 Sample Enclosure

The chamber where the whole plant or the plant part is enclosed can be a cuvette, container, vial, or jar usually made of glass, a material that has been proven to be inert to ethylene. Moreover, light can easily reach the plant tissue and photosynthesis can take place. Transparent plastic bags (e.g., polyester) are not so common for ethylene measurements, since they cannot be sealed properly, but are recommended as more convenient than glass containers during the collection of other volatile organic compounds (VOCs). When no light is required as for example during seed germination or fruit storage, other material may be also considered including Teflon or metal. The volume of headspace in the glass cuvettes should be as small as possible, but sufficient to enable optimum conditions for plant growth. The glass cuvettes can be custom designed for specific application and have various geometries and volumes ranging from few milliliters up to few litters (Figs. 14.1 and 14.2). Glass petri dishes (PD) can be ingeniously used as cuvettes especially for germinating seeds or seedlings, which do not need to be transferred onto a sampling cuvette, or single plant attached leaf (Fig. 14.1c). Another example using petri dishes (glass or plastic) is a custom-made, sandwich-type cuvette; the PD

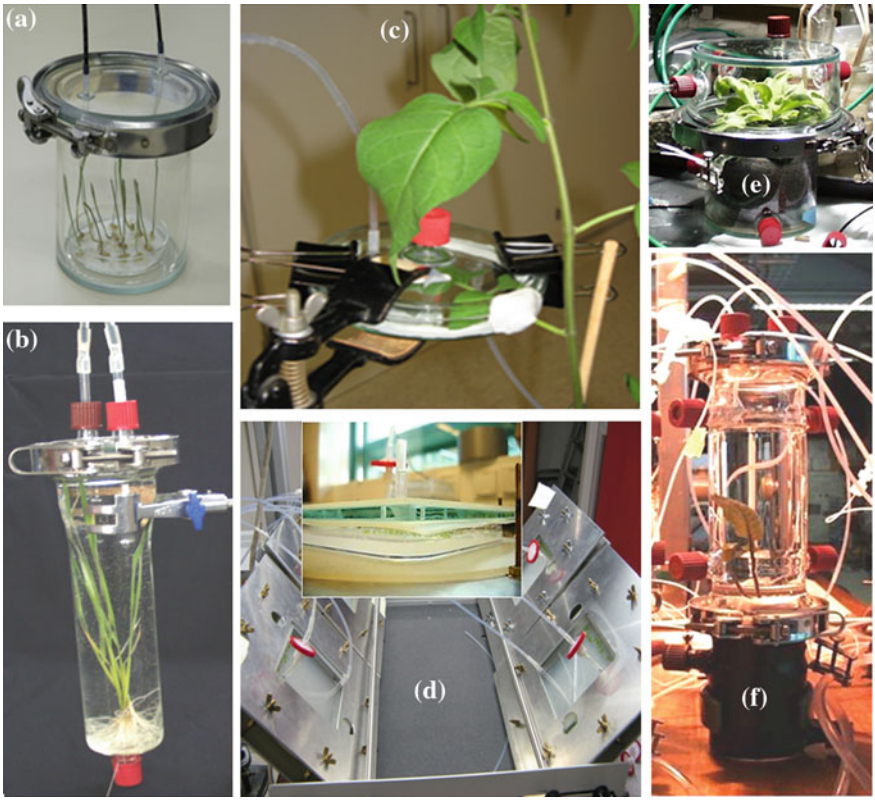

Fig. 14.1 Presentation of sampling cuvettes: **a** glass cuvette with different volume with two ports for the carrier air (e.g., 1–5 l) (suitable for fruit, potato tubers, seedlings, or plants on pots), **b** glass vessel with three ports (suitable for submerged plants, the *upper part* is not completely filled with water to allow air circulation, **c**, **d** sandwich-type cuvette using petri dishes (suitable for in vitro cultures, attached leaf, germinating seeds, seedlings) **e**, **f** two compartment cuvettes where above ground is separated from below *ground part* (suitable for independent ethylene monitoring from root and shoot from the same plant, e.g., in systemically-induced ethylene studies)

containing the biological material is closed with a glass cover having an inlet and outlet for gas flow, and assembled between two metal pieces (Fig. 14.1d). Commercially available glass vials with volumes from 10 ml up to 150 ml sealed with aluminum caps with septum or rubber padding are very suitable for repeated gas sampling or dynamic sampling from small samples, such as seeds, seedlings, hydroponically grown plants, microcuttings, or tissue cultures (Fig. 14.2).

Nevertheless all the sampling vessels need to be tested for tightness using either an ethylene standard (applicable for static sampling) or a flow controller (for dynamic sampling). The stability of ethylene concentration in such vials has been reported for static headspace over 48 h (Fiserova et al. 2008).

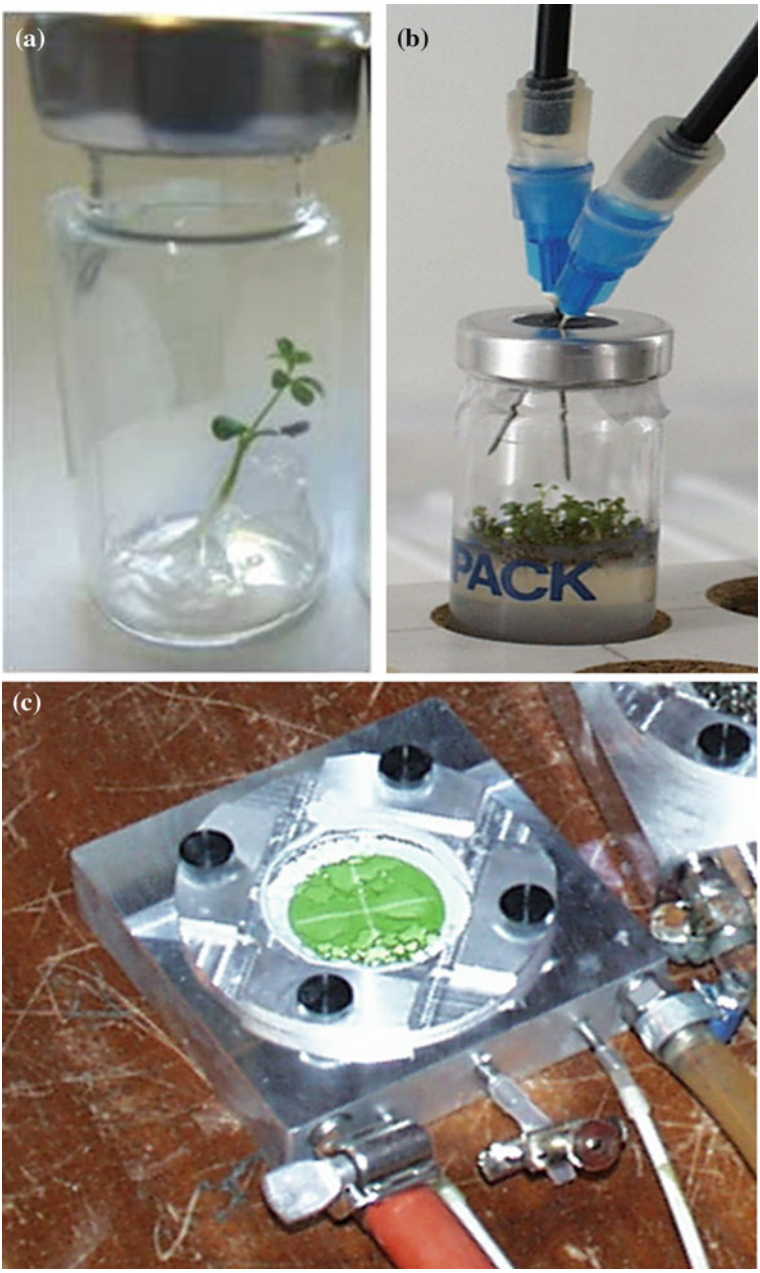

Fig. 14.2 Cuvettes for small biomass samples. **a**, **b** Glass vials with cap with septum/rubber padding suitable for tissue cultures, isolated seedling, seeds germination, stem microcuttings, algae, etc. **c** Microincubator for small (liquid) samples for microbiology

As a general rule, ethylene emission from a reference cuvette that can be empty, containing liquid or solid growth medium, soil, filter paper, etc. should also be measured in the same experimental conditions as the plant material and subtracted it from the ethylene released in the plant containing headspace in order to overcome undesired additional effects. For example, a nylon membrane filter placed onto the surface of a medium as support for seedlings growth releases ethylene (Moniuszko et al. 2011); this additional emission will not only contribute to the ethylene production measured in the headspace of the sampling cuvette, but may also influence the growth of the seedlings.

The effects of enclosing on the plant itself are often ignored. A critical assessment of the merits and limitations of glass cuvette versus plastic bags approaches has been performed (Stewart-Jones and Poppy 2006). An enclosed plant material is constantly exposed to changing concentrations of CO_2, O_2, and C_2H_4 due to plant's metabolism. Carbon dioxide is an essential cofactor for ACC oxidase (Dong et al. 1992) and variation of its concentration in the air inside the cuvette can induce stress in plants and influence the ethylene biosynthesis. For cut or damaged plant material, the wound effect may add further complications due to increase in respiration rate and ethylene production. Moreover, wounding causes local production of ethylene and other VOCs that can induce rapid systemically changes in volatile emissions from unwounded parts (Schmelz et al. 2001). Therefore, using intact plant tissues is preferred.

14.1.2 Static Headspace Sampling

For static headspace, the plant material is placed inside a cuvette and no air is circulated inside. Ethylene can be sampled with a gas syringe and injected directly into the GC column. This is the case when the accumulated gas reaches concentration levels within the detection limit of the gas chromatograph. Thus, the method is limited by the sensitivity of the measuring instrument.

Direct sampling is quick and apparently time saving. However it might not be possible to perform the GS analysis immediately after sampling. Therefore, to ensure a sufficient number of replicates the gas samples need to be stored in syringes having the needle inserted into a rubber cork or a flask to avoid leakage. Even so ethylene preservation is significantly altered after 24 h (Fiserova et al. 2008).

Alternatively, low ethylene emissions can be trapped onto an adsorbent over a certain period of time typically hours (e.g., Tenax or solid phase microextraction—SPME) and thereafter released by thermal desorption into a GC column. This method is time consuming and requires further instrumentation such as thermal desorption unit in combination with a GC or GC-MS (for SPME). Commercial headspace samplers (including the SPME option) allow nowadays automated sampling.

Overall static headspace sampling presents several disadvantages. Maintained over periods of few hours up to 1–2 days it will favorize increase of humidity and

temperature (less if cold lamps are used) and induce changing of CO_2 and O_2 that may affect the plant physiology and bias the ethylene outcomes. Moreover, higher humidity promotes pathogen growth and subsequently influencing ethylene biosynthesis (Lund et al. 1998). Temperature is also influencing the ethylene biosynthesis which has a maximum within the range of 25–35 °C. Additionally, accumulated levels of ethylene can induce autocatalytic ethylene production (e.g., in ripening climacteric fruit) and stimulate several processes in plant such as seed germination, adventitious root formation, respiration, and phenylpropanoid metabolism, abscission, and senescence, etc. (Saltveit 1999). Since there is no ventilation inside the sampling cuvette, also other VOCs can accumulate in the static headspace and have reciprocal effect.

14.1.3 Dynamic Headspace Sampling

Dynamic headspace sampling is currently mostly used technique in plant volatile analysis. A carrier gas, usually air at constant flow rate is passed through closed cuvette containing the plant material or purged out of the cuvette. In this case the cuvette is equipment with minimal two ports for gas inlet and outlet, respectively. Extra ports may be considered for example for addition of chemicals during the experiment without disturbing the headspace composition. This method has clear advantages over the static headspace sampling namely, eliminating the effect of humidity, temperature and volatile accumulation. Figure 14.3 shows the comparison between the ethylene released by *Botrytis cinerea* grown in vitro on different methionine concentrations measured with two techniques, namely online by laser-based detector (Cristescu et al. 2002) and after 7 days accumulation with GC

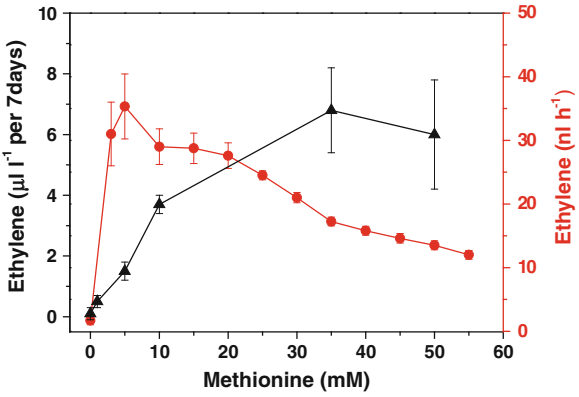

Fig. 14.3 Online versus accumulation. Ethylene production from *Botrytis cinerea* grown in vitro medium supplied with different methionine concentrations was measured online by laser-based technique (circle) (Cristescu et al. 2002) and compared with the ethylene accumulated for 7 days and measured with GC (triangle) (Qadir et al. 1997)

(Qadir et al. 1997). Although the growing conditions were similar the accumulation induced other effects on ethylene production. Depending on the available detection method, ethylene released by plants can be easily transported directly into the measuring device (see Sect. 14.2) or trapped into an adsorbent material and processed afterwards by GC. A detailed description of several dynamic headspace sampling systems is given elsewhere (Tholl et al. 2006). Hydrocarbon-free air obtained, for example, after passing through a catalyzer is usually used as carrier air. Commercial cylinders with compressed air are also of option if they contain 300–400 ppmv (parts per million by volume) CO_2. Caution should be taken in using the proper flow rate to flush the cuvette. This is determined by the volume of the cuvette, the ethylene production rate, and optimal gas exchange between the plant tissue and the headspace air. A large volume cuvette and/or high air flow rate may dilute the ethylene concentration released in the headspace. A small cuvette and/or small flow rate will improve the ethylene signal, but it may affect the plant physiology (i.e., insufficient CO_2 over day and high release of CO_2 during the night).

Automated dynamic headspace sampling system is commercially available in combination with online thermal desorption units for monitoring the time-dependent changes. However, this approach is mainly used for VOCs measurements, rather than for single gas detection, due to its high costs. Alternatives include electrochemical or optical sensors that will be described in Sect. 14.2.

The time at which the headspace sample is collected is important when considering discrete sampling (usually for GC analysis) instead of online or real-time measurements. Ethylene emission is highly dependent on the light conditions and most plants show diurnal and circadian rhythms.

14.2 Professional Equipment and Methods

14.2.1 Gas Chromatography

Due to its simplicity and relatively low cost, a standard gas chromatograph (GC) is most often used in physiological studies to measure ethylene. In its simplest form, a GC consists of an injector port, a 1–2 m 1/8″ copper or stainless steel tube (column) packed with a suitable matrix (such as activated aluminum oxide or Porapak) held at a fixed temperature (e.g., 60–100 °C) and a detector. The column is flushed with a carrier gas that may be nitrogen or helium at constant flow rate. Following injection of a limited amount of gaseous sample, the compounds in the sample are separated from each other due to differential interaction with the matrix and will arrive at the detector at different times after injection (retention time). Using a calibration gas with known amount of ethylene, ethylene in the sample can be identified based on retention time and quantified based on peak area as measured with suitable software.

The first GCs that were used to measure ethylene in plant physiological studies were equipped with a thermal conductivity detector (Burg and Stolwijk 1959). The relatively low sensitivity of such detectors for ethylene allowed to measure ethylene down to approximately 10 ppmv. Later, flame ionization detectors (FID) became standard and these allow to measure ethylene down to about 10 ppb (parts per billion), depending on the stability and configuration of the apparatus.

Generally, the configuration of a GC with FID is such that often a mixture of nitrogen (or helium) and oxygen (e.g., 80 % N_2 and 20 % O_2) is used as a carrier gas and that H_2 is added at the detector to fuel the flame. Alternatively, both H_2 and O_2 may be added at the detector and N_2 or He may be the carrier gas. A potential of a few hundred volts is applied across the burner tip and a collector electrode located above the flame and the resulting current is monitored. Ions formed during combustion of organic materials that enter the flame affect the current; the current is proportional to the number of carbon atoms.

Over the years researchers have experimented with the settings and configuration of the standard GC to optimize the ethylene measurements. The longer the column the better the separation (resolution) of the compounds in the sample, but the peaks will be broader and the detection limit will get worse; increasing the column diameter generally broadens the peaks, but allows injecting higher volumes of sample which may eventually improve the detection limit. Using a capillary column improves the separation of the compounds and yields sharper peaks but does not allow injecting bigger sample volumes. In general, most standard GC's are suitable to measure ethylene with high sensitivity when settings are optimized. A serious problem when measuring ethylene from biological samples is the existence of many other volatile compounds that may interact with the matrix and will show a response at the detector. Some of these compounds may move very slowly through the column. When no temperature programming is possible, slow compounds from earlier samples may interfere with ethylene from later samples. An important improvement to a standard GC set-up is the use of a back flush system. In this case, the column is split into two parts (e.g., 1/3:2/3). As ethylene generally moves relatively fast through the matrix, the direction of the carrier gas flow through the first 1/3 part of the column is reversed as soon as ethylene has arrived in the second part of the column. All compounds that are slower than ethylene will now be flushed back and will not reach the detector (Fig. 14.4). At the moment ethylene reaches the detector, the flow in the pre-column can be reversed again. Often the back flush option is combined with a sampling loop. When the pre-column is flushed back, a new sample can be injected in the sample loop. The "pressure peaks" due to sample injection and the switching of, e.g.. the backflush valve need special attention.

Modern GCs are equipped with high-quality flow controllers, advanced electronics and may contain (multiple) sample loop(s). Together with the use of ultrapure gases this improves stability to the system and allows performing measurements of ethylene very fast in succession; generally injections can be done (automatically) each minute. A potential interfering compound produced by plant material is ethanol. In some configurations, retention times of ethylene and ethanol

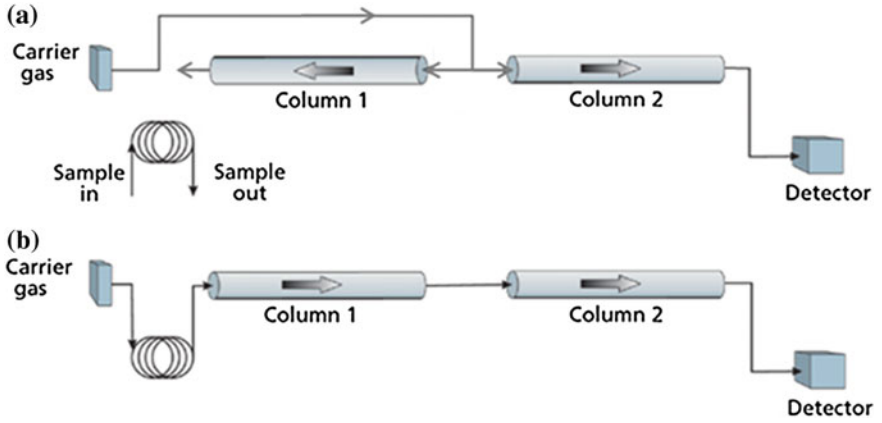

Fig. 14.4 Operation of standard GC equipped with sample-loop and back flush system. **a** Samples are injected into the sample loop when the GC is in back flush mode. **b** When GC is put in injection mode, the sample is brought onto the pre-column (*Column 1*). As soon as the ethylene is in the analytical column (*Column 2*), the flow over the pre-column is reversed **a**. The switching actions may be performed through multichannel rotary valves or through a combination of single valves

may be close to each other and this should be checked by injection of a low concentration of vaporized ethanol.

Most researchers use the GC to measure ethylene from the headspace of vials containing plant material following a couple of h of incubation. The obvious disadvantage of incubating the materials in a closed vial (changes in oxygen, carbon dioxide, humidity, and other volatiles) can be overcome when samples are incubated under a continuous flow. The ethylene concentrations are than often too low for detection by GC. To further improve the sensitivity of the standard GC as to allow the use of a continuous flow system several options are available. In one configuration, combining a sample loop and a relatively long and wide column (2.5 m long and ¼ inch diameter) allows to inject sample volumes up to 10 ml. Using this set-up concentrations down to 1–2 ppbv could be measured in a reproducible way (Woltering and Sterling 1986). Another option to improve sensitivity is the use of a Photo Ionization Detector (PID) instead of a FID. A PID uses high-energy photons, typically in the ultraviolet range, to ionize the molecules. The positively charged ions create a current that is monitored. As molecules need to be excited by the specific wavelengths, the PID is more selective than the FID. For ethylene detection, a lamp with an ionization energy of near 10.5 eV is required. Such an approach improves the sensitivity several times and allows to reliable measure ethylene even under 1 ppbv (Bassi and Spencer 1985). Several options are available to pre-concentrate ethylene before analysis with a GC. A first application was described by Degreef and Deproft (1978). Using a flow-through system, the airstream from the plant cuvettes was first led through a column filled with KOH/$CaCl_2$ to remove water vapor and CO_2, whereafter the air was directed through a

small pre-column (packed with Porapak-S) cooled at −95 °C. Ethylene trapped on the column was released by immersing the column in boiling water and subsequently directed to a second pre-column cooled at −95 °C to further concentrate the ethylene. Following heating of the second column the ethylene was introduced into the GC. This system was successfully used to measure ethylene production of plants in a continuous flow system. Nowadays several commercial "purge and trap" options are available to combine volatile trapping with standard GC.

Another option to avoid the undesired effects of the stagnant atmosphere during incubation of plant material in a closed vial for measurement of ethylene production includes a system where CO_2 and O_2 were held constant during the incubation period. In this closed system, CO_2 produced by the plant material was removed by pumping the air through a KOH solution. The resulting drop in pressure activates a valve through which pure O_2 is injected till the pressure is back to atmospheric pressure. During longer incubation periods (up to 24 h) the ethylene accumulation was regularly measured and production rate over time was calculated. In this way long incubation times are allowed and even extremely low productions (down to 0.01 nl g^{-1} h^{-1}) can be measured accurately.

14.2.2 Proton-Transfer Reaction Time-of-Flight Mass Spectrometry

Proton-transfer reaction mass spectrometry (PTR-MS) emerged during the last decade as an on-line technique that allows sensitive and real-time detection of plant volatile organic compounds (VOCs) (Hansel et al. 1995; de Gouw et al. 2003; Boamfa et al. 2004; Danner et al. 2012). Several compounds including aldehydes, ketones, alcohols, oxygenated compounds that have proton affinity higher than of water (165 kcal mol^{-1}) are easily ionized via a proton-transfer reaction with H_3O^+. The ions produced are further mass filtered with a quadrupole mass spectrometer based on their mass to charge ratio (m/z) and quantified by a secondary electron multiplier.

Ethylene cannot be ionized in this way since its proton affinity is lower than of water (162.6 kcal mol^{-1}). However it can efficiently react with O_2^+ and get ionized via a charge transfer mechanism and detected at m/z 28.031. For its identification a high mass resolution is necessary. Therefore the PTR-MS was coupled with other detector than a quadrupole mass spectrometer, namely time-of-flight mass spectrometer (PTR-TOF-MS) (Cappellin et al. 2013), as produced for example by Ionicon Analytik GmbH (Fig. 14.5).

Interestingly, O_2^+ as well as NO^+, is freely emitted during the generation of the primary ions (H_3O^+) via the discharge; i.e., the ion at m/z 30 is mostly representing NO^+, and the ion at m/z 32 represents O_2^+ with similar origin. Both NO^+ and O_2^+ are preset in a few percentages and as parasitic ions they can ionize VOCs without transferring a proton. The ionization efficiency is further improved in the newly developed instruments that have switchable reagent ion capability (Jordan et al. 2009).

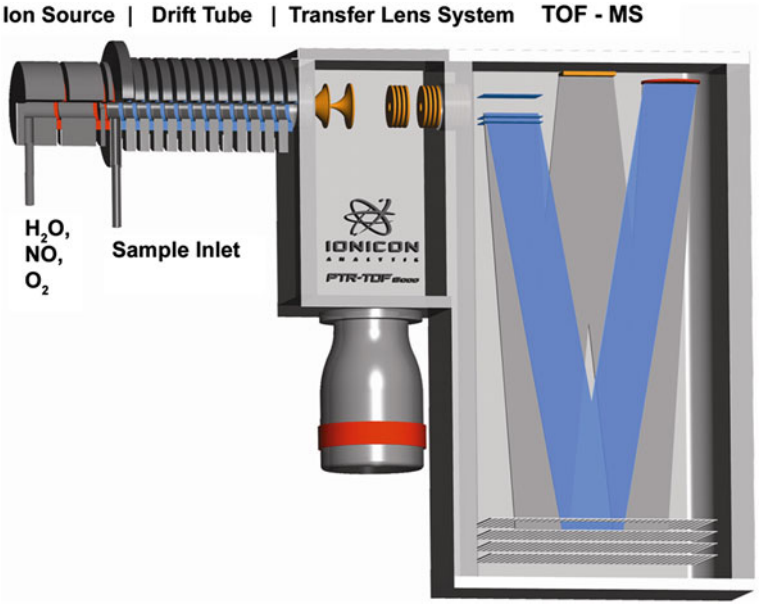

Fig. 14.5 Proton-transfer reaction time-of-flight mass spectrometer (PTR-TOF-MS). An external ion source generates the reagent ions such as H_3O^+, $NO^{+\cdot}$ O_2^+ (for ethylene detection O_2^+ are the most efficient ones). Ethylene is inserted via the sample inlet in the drift tube where get ionized by proton-transfer reactions. The protonated molecules are guided via the transfer lens system and analyzed into the time-of-flight mass spectrometer (TOF-MS) (Courtesy of Ionicon Analytik GmbH)

The major drawback of PTR-MS remains the identification of compounds, which is known to be difficult, because the detected m/z value can be a parent molecule, fragments of parent molecules, water clusters, etc. This makes the identification of compounds mostly tentative. PTR-TOF-MS overcomes this disadvantage, but it also provides a far more complex spectrometric data. In addition, the fragmentation patterns are necessary to be known for data analysis and interpretation.

The PTR-TOF-MS instrument is able to sensitively detect other VOCs than ethylene and in some application the use of one instrument, although expensive, it would be desired. Demonstrations of such system have been recently reported for measuring the production of ethylene and other aroma compounds in apple and correlate them with Quantitative Trait Locus QTL mapping approach (Costa et al. 2014).

14.2.3 Electrochemical Sensors

Electrochemical sensors are based on the chemical binding of ethylene on an active sensing material which in the presence on an electric circuit will induce a change of

current, resistance or capacitance, hence the name of amperometric, chemoresistive, and capacitive, respectively.

Specifically, by applying a voltage applied between a sensing electrode (anode) and a reference electrode submerged into an electrocatalytic solution, ethylene is oxidized at the anode surface and the resulted electrons are transferred to the electrode, resulting in a current proportional to the ethylene concentration. Efforts were dedicated to improve the quality of material for electrodes and electrolytes as well as the sensor design (Cristescu et al. 2013). The most recent developments involve nanoparticle technologies and the use of gold in fabrication of the anode or even of both electrodes (Fig. 14.6). Esser et al. (2012) measured ethylene concentration in the range of 0.5–50 ppmv using a mixture of copper complex with carbon nanotubes (i.e., sheets of carbon atoms rolled into cylinders that act as "superhighways" for an electron flow) as sensing material placed between two gold electrodes. As the electrolyte may be consumed or dries out, instead of the acid electrolyte another attempt has been proposed using ionic-liquid layer (Zevenbergen et al. 2011). The response time is only a few seconds, and the detection limit (defined as three times the noise level, 3σ) is about 760 ppbv. A nanoporous layer of gold plated onto

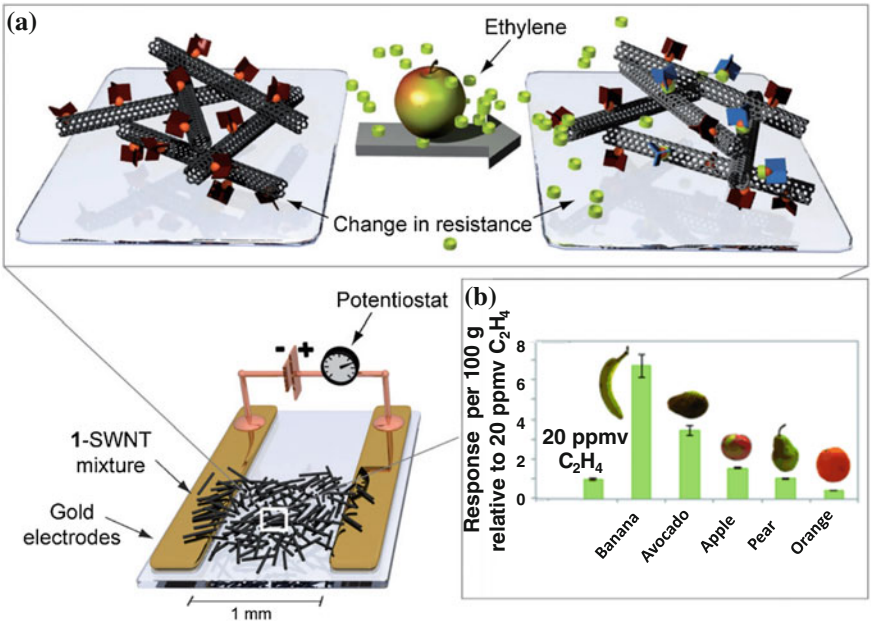

Fig. 14.6 Chemoresistive carbon nanotube-based sensor for ethylene detection at sub-ppmv level (ref). **a** A mixture of Single-Walled Carbon NanoTubes (1-SWNTs) and copper complex is placed between gold electrodes, forming the sensing element. The copper complexes bind to ethylene and induces a change in the resistance of the nanotubes according to the gas concentration. **b** Responses of the sensor to 100 g of different fruit relative to 20 ppmv ethylene (Adapted from [Esser et al. 2012])

a Nafion anode is used in combination with sulphuric acid as electrolyte (Shekarriz and Allen 2008). The sensor (e.g., model ETH-1010 manufactured by Fluid Analytics Inc. and licensed to Absorger, CID, and EMC) is portable, can be equipped with a rechargeable battery for up to 8 h of operation and claims a detection limit of about 10 ppbv in controlled and constant conditions within 2 min response time.

Without doubts, these sensors offer several advantages such as: good repeatability and accuracy and relative fast response time to ethylene (below 1 min) combined with a recovery time of minutes. Portability and low cost are the main rationales for using these sensors in fruit storage where ethylene emission is in the range of hundreds of ppbv and ppmv levels. Several attempts have been made for monitoring ethylene in greenhouses; however, no trustable results so far indicate them suitable for this field mainly because of insufficient sensitivity and selectivity. The drawbacks remain the poor selectivity, sensitivity to temperature and humidity, oxygen requirement, and limited shelf life especially when exposed to higher ethylene concentrations. The paradox is that these sensors are performing the best at high ethylene levels (by overruling the interfering gases), with the price of losing in sensitivity because of the anode exhaustion.

14.2.4 Optical Gas Sensors

Ethylene, like many other gases can show distinct absorption patterns in the mid-infrared (MIR) region, the so-called the fingerprint region (2–20 μm). By knowing the absorption strength of ethylene at a specific infrared frequency that is well documented in available database, the molecular concentration can be quantified (Brewer et al. 1982).

Optical sensors have been developed based on this principle, using light sources like broadband (called nondispersive infrared sensors) or lasers. The light is sent through an absorption cell where ethylene released by biological sample is transported. As result of this interaction, ethylene absorbs the light energy, which can be detected in several ways.

One approach is the detection of the absorbed energy with a microphone. This energy is internally converted into kinetic energy by exchange processes (e.g., by collision with other molecules), generating a local heating of the gas sample. By switching on and off the light with a certain frequency, rapid heating/cooling can occur, giving rise to a periodical pressure change, i.e., sound wave. The amplitude of this sound wave is proportional to the absorbed energy, i.e., the concentration of the ethylene in the absorption cell and can be detected very accurately with sensitive microphones (Kreuzer 1977). Thus, absorbed light is detected as sound, hence the name of the technique: photoacoustic (Fig. 14.7).

Since the nondispersive infrared (NDIR) sensors are using broadband sources, the absorption spectrum contains the molecular fingerprint of ethylene as well as of other gases present in the emission range of the light. Therefore, using NDIR optical filters are included for improving ethylene selectivity and attenuation of

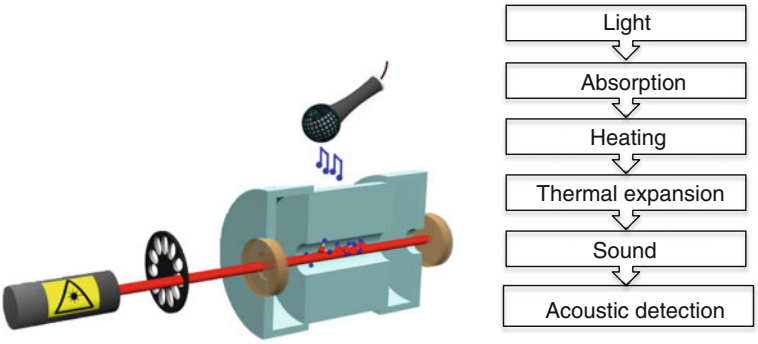

Fig. 14.7 Photoacoustic ethylene detection. The absorbed energy from the light causes local heating and through thermal expansion a pressure wave (sound) is generated and detected with sensitive microphones (Adapted from [Cristescu et al. 2013])

undesired absorbents; a mathematical model based on nonlinear compensations is considered for data analysis. The detection limits may vary from μl l^{-1} to few hundreds of nl l^{-1} level (LumaSense Techn. and Gasera Ltd.).

Laser-based sensors take the advantage of using laser to selectively emit in the range where ethylene presents absorption features. Several types of laser in combination with adequate detection techniques have been proposed for ethylene detection such as near-infrared diode laser (Pan et al. 2012), CO_2 laser (Cristescu et al. 2008; Fink et al. 1996; Nagele and Sigrist 2000), and quantum cascade laser (Manne et al. 2010; Weidmann et al. 2004). The laser-based sensors offer the best sensitivity achieved so far in the range of ppbv and even sub-ppbv within seconds/minutes time scale. Additionally, they have good selectivity and allow real-time monitoring.

To ensure selectivity, single gas detection is common characteristic for laser-based sensors. Multispecies is possible by using more than one laser or broadband emitting laser; the price will scale proportionally. Although the laser-based sensors are more expensive than the electrochemical ones, they offer incomparable features (i.e., sensitivity and real-time measurements) which no other existing sensors can do. In the next session, several applications are presented to illustrate their abilities.

At the Life Science Trace Gas Facility (Nijmegen, the Netherlands), the potential of laser-based ethylene detection has been exploited for more than 25 years in various biological applications. The laboratory set-up best sensitivity was ethylene monitoring in a continuous flow system down to 10 pptv (1 pptv = parts per trillion per volume = $1{:}10^{-12}$) pl l^{-1} over 90 s (Harren et al. 1990). The system was used to monitor various dynamic processes in plants and microorganisms such as real-time measurements from a single plant or plant organ without accumulation periods (Harren and Cristescu 2013). Since a decade, the development of these detectors has moved toward commercial instruments (ETD-300, Sensor Sense BV) (Fig. 14.8). Presently, this is the only commercially available laser-based ethylene detector. A detailed description of the ETD-300 instrument is given elsewhere

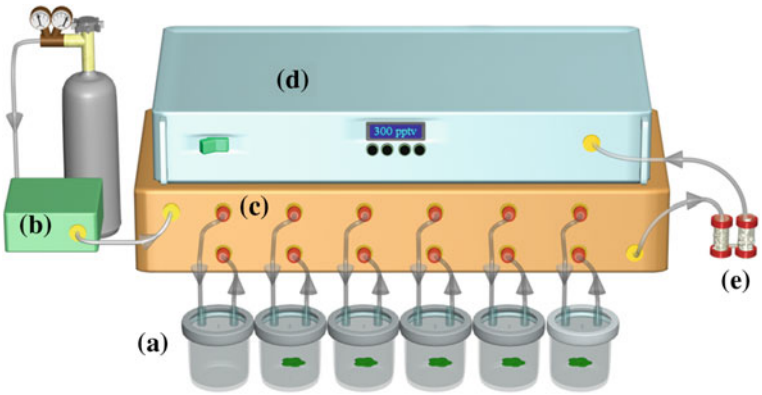

Fig. 14.8 Online ethylene monitoring using laser-based photoacoustic detector. The cuvettes (**a**) are flushed with hydrocarbons-free air obtained from compressed air passed through a catalyzer (**b**). The air flow-through system (**c**) consisting of automatic control of flow is placed in line with sampling cuvettes and allows alternately connection of each cuvette to the ethylene detector (**d**). Water and carbon dioxide are chemically eliminated (**e**) before entering the detector. The detector sensitivity is about 300 pptv in 5 s (Cristescu et al. 2008)

(Cristescu et al. 2008; De Gouw et al. 2009). Briefly, the ETD-300 uses a CO_2 laser emitting light in the 10 μm region where ethylene presents its strongest absorption. The ETD-300 is able of measuring ethylene online in the 300 pptv (pptv = parts per trillion volume, $1{:}10^{12}$) range within a 5 s time scale, which is the best achieved sensitivity outside laboratory settings. In addition to its high sensitivity and selectivity, the ETD-300 offers wide dynamic range, user friendly real-time data analysis, operational simplicity, relative portability, easy calibration, no need for sample preparation.

14.3 Sensitive, Real-Time Ethylene Monitoring

Due to their high sensitivity and fast response time, the laser-based detectors allow real-time continuous monitoring of dynamics of ethylene production from plants over periods of days or longer. For this, a flow-through system consisting of automatic control of gas flow and switching between the cuvettes is placed in line with sampling cuvettes like in Fig. 14.8. In the next sections, examples for the recording of ethylene production from various biological samples as provided by the ETD-300 ethylene detector in each of its mode of operation, namely continuous flow, stop-and-flow and sampling mode, respectively, are shown.

Since the last decade, the ETD-300 laser-based photoacoustic detector has been applied to monitor on-line ethylene released in various plant processes, such as signaling (Ellison et al. 2011; McDaniel and Binder 2012), plant development (Nitsch et al. 2012; Gallego-Bartolome et al. 2011; Piechulla et al. 2009; Millenaar

et al. 2009), abiotic stress (Clarke et al. 2009; Forni et al. 2012), plant–pathogen interaction (Lloyd et al. 2011; Cristescu et al. 2006; Mur et al. 2012), plant–insect interaction (Schroder et al. 2007), postharvest (Salman et al. 2009; Pranamornkith et al. 2012), programmed cell death (Yordanova et al. 2010), and mineral nutrition (Hermans et al. 2010, 2011; Benlloch-Gonzalez et al. 2010; Lequeux et al. 2010).

14.3.1 Continuous Flow

To conduct continuous flow experiments, the plant material is placed into sampling cuvettes and continuously flushed with air at a constant flow rate of maximum 5 l h^{-1}. Ethylene emitted in the headspace is transported to the ETD-300 detector alternately, allowing a succession of typically 10 up to 30 min for each cuvette. Thus, the dynamics of the ethylene release can be investigated in details over certain time period (from hours to weeks or longer). As an example, ethylene production of three *Nicotiana suaveolens* flowers was continuously monitored during flower development over 7 days (Piechulla et al. 2009).

The single flower remained attached to the plants and was enclosed into special designed glass cuvette (25 ml volume), and continuously flushed with air at constant flow of 2 l h^{-1} (Fig. 14.9). Each cuvette was measured for 30 min. From the obtained emission rates, readings of an empty cuvette are subtracted to adjust for externally

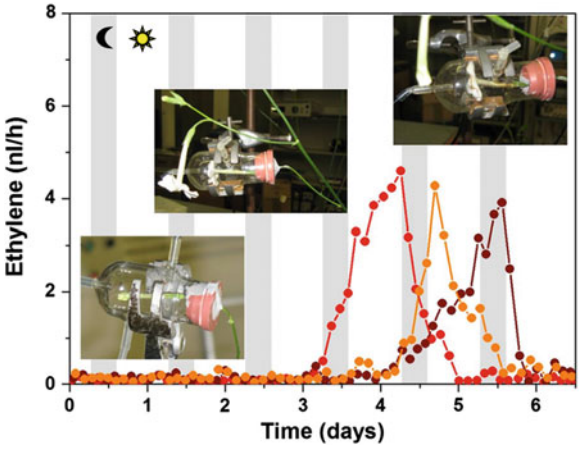

Fig. 14.9 Ethylene emission during flower development of three individual flowers of *Nicotiana suaveolens*. Single flower remained attached to the plant and was enclosed into custom designed glass cuvette sealed around the stem with a harmless flexile clay that were continuously flushed with air at constant flow of 2 l h^{-1} for 7 days. Ethylene production was measured in real-time with a laser-based photoacoustic detector (ETD-300) (Piechulla et al. 2009) following flower-close stage (very low ethylene emission), through anthesis (ethylene starts to rise) till senescence (ethylene reaches a maximum and decreases)

induced variations. For a good overview, the average value of ethylene production from the last 20 min out of the 30 min of sampling is displayed, indicating the ethylene emission rate every 2 h. The ethylene production was related to the emission rate by multiplying the measured value with the flow rate, and expressed in nl h^{-1}.

The white petals of *N. suaveolens* flowers emit methylated volatiles preferentially nocturnal and their synthesis is catalyzed by O-methyltransferases, which use S-adenosyl-L-methionine (SAM) as methyl donor. The enzymes involved in the regeneration of SAM (SAM synthase and methionine synthase) present rhythmic mRNA profiles during flower development with maximum levels during the day and reduced levels during the night (Piechulla et al. 2009). Due to the fluctuations of SAM, it was expected that ethylene would present a rhythmic emission. However, this was not the case and a burst of ethylene emission was monitored by the end of the life span of the *N. suaveleons* flowers (Fig. 14.9). The ethylene peak correlates with increased ACC oxidase mRNA and self-fertilization; this explains why the maxima occur at different moments for all individual flowers.

14.3.1.1 Stop and Flow

In a stop-and-flow mode, ethylene is allowed to accumulate for a short period of time, typically 1 or 2 h, while the headspace conditions remain the same for not altering the plant physiology. The accumulation time is practically determined by the sub-ppbv level of ethylene emission. Usually this is the case when plant samples with small biomass (e.g., germinating seeds or seedlings at early developmental stages, etc.) are analyzed. However, very small plant material such as root nodules of legumes in vitro can produce ethylene in higher concentration than potato plants (nonclimacteric). For example, this mode is indicated for recording ethylene emanation in response to exposure to abiotic factors or biotic agents upon in vitro culture. The example illustrates the close interrelation between ethylene and mineral nutrition in plants, which is largely documented (Cristescu et al. 2013; Iqbal et al. 2013). Biomass allocation between root and shoot, as well as root architecture is highly variable and varies according to the nutritional environment. For instance, low nitrogen availability increases the root to shoot biomass ratio and promotes lateral root outgrowth in search of the limiting N resource (De Pessemier et al. 2013). The involvement of ethylene is reported in the sensing and the acquisition of nitrate species (Leblanc et al. 2008; Tian et al. 2009). More precisely, plants growing at low N supply emanate more ethylene than satiated controls (Khan et al. 2008; Iqbal et al. 2011). Nonetheless, transfer to high N concentrations also result in an ethylene burst and that transient rise is considered as a potential inhibitory factor for lateral root growth inhibition (Tian et al. 2009). Besides those observations, our comprehension of the crosstalk between ethylene and N availability is still incomplete.

Ethylene released by 2 weeks old *Arabidopsis thaliana* seedlings, grown in vitro vertically on agar media (on sandwich-type cuvettes as described in Fig. 14.1d) is shown to illustrate the procedure (Fig. 14.10). The depicted experiment consists of successively measuring two cuvettes with wild type Columbia (Col-0) *Arabidopsis*

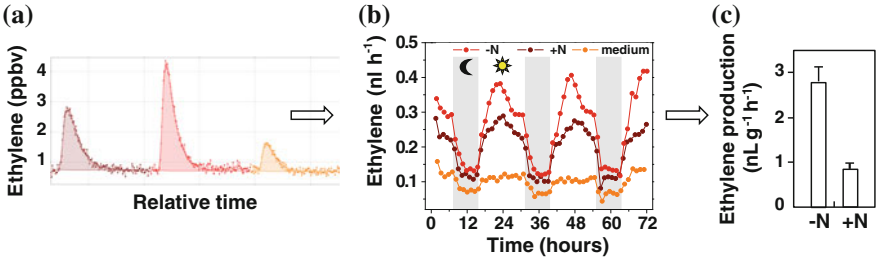

Fig. 14.10 Ethylene emission from 2 weeks old *Arabidopsis thaliana* seedlings, grown in vitro vertically on agar media measured in stop and flow mode. **a** Ethylene was accumulated for 1 h for each of the three cuvette containing seedlings on low nitrate –N, on high nitrate +N, and media, respectively, and then flushed out with an air flow of 3 l h^{-1} for 15 min. **b** The area of the ethylene peaks is then calculated and divided by the accumulation time, resulting a production rate (in nl h^{-1}). **c** An average over 24 h including day/night period is then performed and the results are normalized with the fresh weight: ethylene production rate per fresh biomass and per hour. n = 3 measured Petri plates containing 20 seedlings ±SE

seedlings grown on moderate nitrate substrate (as controls) and on low nitrate substrate (−N), respectively. The third cuvette contains agar medium without seedlings. Ethylene is accumulated for 1 h for each cuvette and then flushed out with an air flow of 3 l h^{-1} for 15 min (Fig. 14.10a). The area of the ethylene peaks is then calculated and divided by the accumulation time; the result is expressed as a production rate (in nl h^{-1}) (Fig. 14.10b). The experiment was performed over 3 days and revealed the day/night rhythm in ethylene emission. Depending on the research purpose, a period of several hours (here, 24 h including the day/night period is considered) should be carefully chosen for averaging the ethylene production. The final result is ethylene production rate per fresh biomass and per hour (for n = 3 Petri plates containing 20 seedlings ±SE) (Fig. 14.10c). The first indication of the experimental setting accuracy is when ethylene measured from the medium shows lower values than any of the cuvettes containing the biological samples on that medium. The illustration shows higher ethylene production upon nitrate starvation (Fig. 14.10c), which is accordance with previous published reports (Khan et al. 2008; Iqbal et al. 2011).

Both the continuous flow and the stop and flow mode allow real-time visualization of the ethylene dynamics and permit the researcher to correct/intervene while the experiment is running (e.g., identifying leaks, checking the ethylene level of the empty cuvette/media).

14.3.2 Sampling Mode

The sampling approach is very useful for applications that require multiple samples measurement, typically hundreds. Ethylene emission needs to be quantified once for each biological sample, rather than monitored over time. There are two ways to collect the ethylene released in the headspace. One way is to extract with a syringe

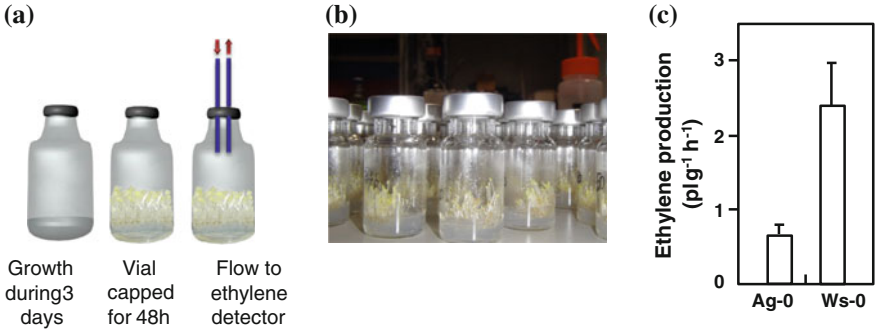

Fig. 14.11 Ethylene production of etiolated Arabidopsis seedlings grown in vitro in small caped vials. Experimental procedure. **a** Seeds of Argentat-0 (Ag-0) and Wassilewskija-0 (Ws-0) accessions were stratified for 48 h at 4 °C and germinated in vitro in small vials (10 ml) for 3 days in the dark. Agar media (0.8 %) contained 1× MS strength with 10 mM NO_3^- as the sole source of nitrogen. Vials were capped for 48 h prior to directly connecting to the ethylene detector ETD-300. **b** Capped vials with etiolated seedlings plants 5 days after germination. **c** Ethylene release from ~300 seedlings per fresh biomass and per hour. $n = 3$ measured vials ±SE

1–2 ml from the headspace of encapsulated plant samples and transfer it by injection into an empty vial (e.g., 10–20 ml volume) (Fig. 14.11a). The advantages are that large amount of samples can be collected within a short time period and that vials can be stored prior to measuring with the ETD-300 (Fig. 14.11b). The detector needs to be calibrated with known ethylene concentrations under similar gas transfer procedure. Another way is to measure the headspace by directly connecting the sampling cuvettes to the detector and flushing them with constant air flow for several minutes. The result of such measurement is a peak of ethylene; the area under the curve is further calculated and divided by the accumulation time.

This sample mode approach offers, for example, the opportunity to conduct genome-wide association (GWA) or linkage disequilibrium-based mapping (Atwell et al. 2010) for identifying alleles or loci responsible for the variation of ethylene production in natural populations of Arabidopsis. Indeed, that species has a broad geographical distribution and consequently is subject to varying environments which makes it a useful model for studying adaptation and selection. Over the last decade, the first successful wave of GWA studies has identified functional genetic variants associated with the natural variation of a given quantitative trait (Myles et al. 2009; Korte and Farlow 2013). Typically, those studies involve the phenotyping of a large diversity panel of accessions for which genotype data are available. Nonetheless, the main bottleneck to many GWA studies is often the collection of high-quality phenotypes (Myles et al. 2009). Our last application meets that requirement. For example, ethylene production of etiolated seedlings, which are grown in the dark, can be measured simultaneously and reliably in a large collection of plants (Fig. 14.11c). In a pilot experiment with a set of more than 100 accessions, Argentat-0 (Ag-0) and Wassilewskija-0 (Ws-0) were, respectively, identified as the accessions producing the lowest and highest ethylene amounts. The extension of

such screening procedure could identify genetic polymorphisms that are associated to functional alleles namely in the ethylene biosynthetic pathway.

14.4 Conclusions and Outlook

Next to gas chromatography, several types of sensors for monitoring ethylene production in plants were presented. The choice is dictated by the specific application. Gas chromatography is useful if more gases are needed to be measured. When no high sensitivity is required, but portability is highly desired, the electrochemical sensors can be used, preferably under constant measuring conditions. The laser-based detectors are most suitable for high sensitivity and real-time monitoring. The commercially available technologies offer solutions for various fields of applications. As the practice has demonstrated, setting up a proper design of a plant ethylene experiment is crucial. Several external factors, such as the air composition in the headspace around the plant, possible mechanical damage, temperature and light conditions, cuvettes design, etc. should be considered, as they can bias the ethylene measurement outcomes.

Yet, there is still space for improvements of the existing methods as well as for the development of new ideas in the future.

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