Chi-Kuang Wen *Editor*

Ethylene in Plants

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ISBN 978-94-017-9483-1 ISBN 978-94-017-9484-8 (eBook)
DOI 10.1007/978-94-017-9484-8

Library of Congress Control Number: 2014951662

Springer Dordrecht Heidelberg New York London

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

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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C.-K. Wen (ed.), *Ethylene in Plants*, DOI 10.1007/978-94-017-9484-8_1

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

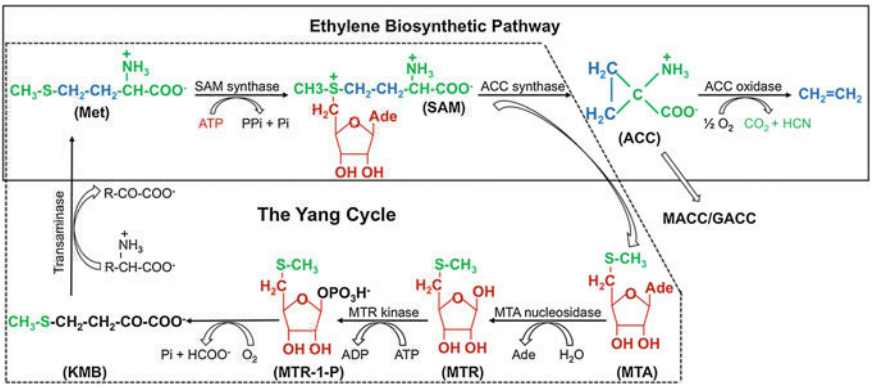

Fig. 1.1 Ethylene biosynthetic pathway and the Yang cycle. Ethylene is synthesized from Met via SAM and ACC. MTA, a byproduct when SAM is converted to ACC by ACS, is subsequently recycled to Met via a pathway known as the methionine cycle or the Yang cycle. ACC undergoes oxidative cleavage to form ethylene, 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

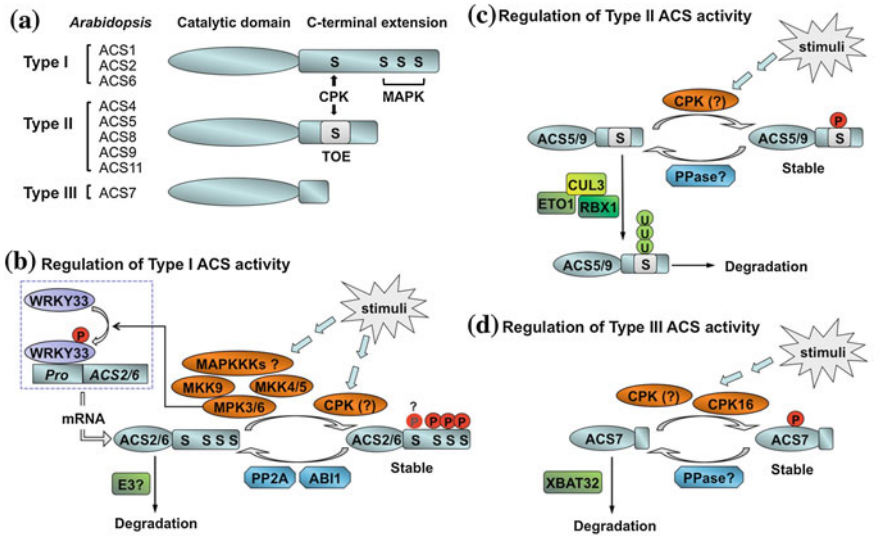

Fig. 1.2 Regulation of cellular ACS activity, a rate-limiting step in ethylene biosynthesis. **a** Classification of ACS members into three subgroups based on the presence and absence of MAPK and/or CPK phosphorylation sites in the C-termini of ACS. Only *Arabidopsis* ACS isoforms are shown. **b** Regulation 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.

Acknowledgments We apologize for not being able to cite all relevant publications due to page limitation. This work was supported by the National Nature Science Foundation of China (Grant No. 91317303) and the third stage of Zhejiang University 985 Project (No. 118000-193411801).