Strictly controlled production of ethylene gas lies upstream of the signaling activities of this crucial regulator throughout the plant life cycle. Although the biosynthetic pathway is enzymatically simple, the regulatory circuits that modulate signal production are fine tuned to allow integration of responses to environmental and intrinsic cues. Recently identified posttranslational mechanisms that control ethylene production converge on one family of biosynthetic enzymes and overlay several independent reversible phosphorylation events and distinct mediators of ubiquitin-dependent protein degradation. Although the core pathway is conserved throughout seed plants, these posttranslational regulatory mechanisms may represent evolutionarily recent innovations. The evolutionary origins of the pathway and its regulators are not yet clear; outside the seed plants, numerous biochemical and phylogenetic questions remain to be addressed.

Ethylene gas is a crucial regulator of numerous aspects of plant development and physiology, including germination, seedling growth and morphology, fruit ripening, organ senescence, and stress and defense responses (Abeles et al., 1992). Biosynthetic capacity for ethylene production may be nearly ubiquitous throughout the plant body, but biosynthesis is generally maintained at low levels by regulatory circuitry that confers tight control while allowing rapid and dramatic increases under conditions such as wounding or fruit ripening (Vandenbussche et al., 2012). Ethylene biosynthesis levels change in response to endogenous developmental cues as well as exogenous signals including light, abiotic stress, and pathogens (for review, see De Paepe and Van der Straeten, 2005; Argueso et al., 2007; Lin et al., 2009; Rodrigues et al., 2014), and it is clear that both transcriptional and posttranslational mechanisms provide strict control of biosynthetic enzyme activity levels. Because of ethylene’s many roles during vegetative and reproductive development, stress and defense responses, and the postharvest phase, biosynthesis of this simple molecule is of considerable agricultural significance. During vegetative development (and during ripening of most fruits), ethylene perception typically down-regulates the biosynthetic pathway. However, ethylene metabolism is of particular interest in the biology of climacteric fruits, which achieve ripening through a coordinated burst of respiration and ethylene production (for review, see Alexander and Grierson, 2002; Klee and Giovannoni, 2011). Although the enzymology of climacteric ethylene production is unaltered, the regulation is changed profoundly, as the biosynthetic pathway becomes part of a positive feedback loop (autocatalytic). Here, we focus on recent advances in our understanding of the biosynthetic pathway, with particular emphasis on posttranslational modifications that control the stability of biosynthetic enzymes and on phylogenetic analysis of biosynthetic capacity in land plants.

HARDWARE OF THE BIOSYNTHETIC PATHWAY

The biosynthetic pathway for ethylene in flowering plants was defined over 30 years ago (for review, see Yang and Hoffman, 1984) and includes only two committed enzymatic steps (Fig. 1). The first, conversion of S-adenosyl-Met (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) and methylthioadenosine, is performed by the enzyme ACC synthase (ACS) in an elimination reaction that requires a pyridoxal phosphate (PLP) cofactor. In the second step, ACC is converted to ethylene, CO₂, and cyano by 1-aminocyclopropane-1-carboxylate oxidase (ACO). The toxicity of cyanide production is ameliorated at the cellular level by the detoxifying enzyme β-cyano-Ala synthase (Yip and Yang, 1988). Recent work has shown that the iron center in the ACO active site is also protected from cyanide poisoning; in the presence of oxygen, bicarbonate ion, and ascorbate cofactors, ACO breaks the ACC ring and releases the unstable cyanofomate ion, sequestering cyanide until the reaction products have diffused away (Murphy et al., 2014). ACC synthesis is generally rate limiting for ethylene production during vegetative growth, although ACO activity may be limiting under some conditions (e.g. in fruit and flowers; Yang and Hoffman, 1984). Both ACS and ACO are encoded by multigene families in seed plants.

This apparent simplicity overlies a dynamic interplay of metabolic branch pathways that modulate the availability precursors as well as the production of...
conjugated ACC derivatives (Fig. 1). First, reactions of the Yang cycle salvage the methyl-thio group of methylthioadenosine into Met, stabilizing Met pools for essential primary metabolic functions even in periods of rapid ethylene synthesis (for review, see Sauter et al., 2013). Similarly, SAM is not only the methyl group donor for many methylation reactions, but also the precursor for polyamine and nicotianamine synthesis, so its levels must be maintained by adequate SAM synthetase activity. Free ACC levels also are modulated by conjugation with malonyl-CoA (yielding 1-malonyl-ACC) and with glutathione (yielding γ-glutamyl-ACC), as well as conjugation to jasmonic acid (JA; yielding JA-ACC; for review, see Van de Poel and Van Der Straeten, 2014). This last conjugate suggests a mechanism for coregulating JA and ACC availability (Staswick and Tiryaki, 2004). The relative abundance of ACC conjugates varies significantly among species (Wang et al., 2007), and it is unclear whether ACC is recovered for ethylene synthesis from these derivatives, although exogenous JA-ACC apparently is cleaved to release ACC (Staswick and Tiryaki, 2004). Another route of possible hormone cross talk is suggested by the characterization of the REVERSAL OF SAV3 PHENOTYPE1 (VAS1) aminotransferase activity, a reaction that reduces pools of both auxin and ACC by converting the auxin biosynthetic intermediate indole-3-pyruvate to Trp using an amino acid (preferentially Met) as the amino donor (Zheng et al., 2013). Finally, ACC deaminase, an enzyme produced by numerous bacteria, can affect ACC pools and alter growth characteristics of plants that interact with these bacterial strains (for review, see Update by Gamalero and Glick [2015], in this issue).

Down-regulation of either ACO or ACS expression via gene silencing approaches can reduce ethylene production levels dramatically (for review, see Lin et al., 2009). Ethylene production can also be blocked or reduced by compounds that inhibit the enzymatic activities of ACO (e.g. cobalt or α-aminoisobutyric acid) or ACS (e.g. aminooxyacetic acid, which compete with the required PLP cofactor). New inhibitors that may show improved specificity for ACS were recently identified (Lin et al., 2010); these compounds do not compete with PLP, reducing side effects on other PLP-dependent enzymes. Nonetheless, severe effects on seedling root growth should be expected for all of these inhibitors of ethylene synthesis, because reduced ethylene synthesis appears to be a primary defect in PLP-deficient roots, leading to impairments in auxin synthesis and distribution and in apical meristem function (Boycheva et al., 2015). Intriguingly, genetic analysis revealed an essential embryonic requirement for ACS function (but not ethylene signaling) in Arabidopsis (Arabidopsis thaliana; Tsuchisaka et al., 2009), and additional work has suggested that ACC may act in an ethylene-independent mode to signal loss of cell wall integrity (Xu et al., 2008; Tsang et al., 2011).

Biosynthesis of ethylene frequently involves spatial or temporal separation of the two committed reaction steps. A recent study characterized the distribution of ethylene metabolic enzymes and intermediate metabolites in ripening tomato (Solanum lycopersicum) fruits and found the locular gel and central tissues to be sites where intermediates including free and conjugated ACC accumulate, whereas ACC synthesis is concentrated in central tissues and peak ethylene production maps to the fruit’s outer (pericarp) layer (Van de Poel et al., 2014). These data suggest that intermediates may be stored in tissue that has limited biosynthetic capacity. Recent work indicates that some cells actively export ACC (Pesquet and Tuominen, 2011), and that at least one amino acid transporter, the LYS HIS TRANSPORTER1 of Arabidopsis (Shin et al., 2015), promotes uptake of extracellular ACC. Additionally, the importance of long-range transport of ACC for optimal ethylene synthesis in specific tissues has been characterized in a number of contexts, particularly flooding and hypoxia (Bradford and Yang, 1980; for review, see Van de Poel and Van Der Straeten, 2014).

**KEEPING SYNTHESIS ON A SHORT LEASH**

Accumulation of both ACS and ACO mRNA is responsive to endogenous and exogenous cues. Several ACS genes are subject to negative feedback loops that reduce mRNA levels, whereas expression of several ACO or ACO-like genes is increased by ethylene treatment (for review, see Lin et al., 2009; Van de Poel

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**Figure 1.** Biosynthesis of ethylene and metabolism of ethylene precursors. The core pathway is shown in blue, with additional enzyme activities (italics) and reaction products indicated. Dashed lines indicate additional enzymatic steps.
and Van Der Straeten, 2014). Other hormones, circadian regulation, and light-signaling pathways show complex interactions with ACS and ACO mRNA accumulation, with auxin having a particularly strong positive effect on expression of ACS mRNAs (Tsuchisaka and Theologis, 2004b; Lin et al., 2009; Rodrigues et al., 2014). Furthermore, rapid effects mediated by posttranslational modification can be reinforced via coordinate transcriptional regulation (Li et al., 2012).

ACS activity levels are regulated by proteasome-mediated degradation and by reversible protein phosphorylation (Fig. 2). ACS isozymes belong to three different subclades or types with distinct regulatory features (Figs. 2 and 3; Chae and Kieber, 2005). The three isozyme types were originally defined by the presence or absence of regulatory motifs in the noncatalytic C-terminal domains (Fig. 2, A and B). Type 1 ACS isozymes carry target sites for mitogen-activated protein kinase (MAPK) phosphorylation; this MAPK motif lies immediately downstream from a putative CDPK phosphorylation site (Hernández Sebastià et al., 2004; Liu and Zhang, 2004; Kamiyoshihara et al., 2010). Type 2 isozymes carry only the putative CDPK target motif (Fig. 2, B and C), and type 3 isozymes have a short C-terminal extension that contains neither motif (Chae and Kieber, 2005). All three isozyme types are degraded during proteasome-dependent mechanisms (for review, see Lyzenga and Stone, 2012).

As discussed below, recent work has bolstered our understanding of ACS turnover. Regulatory circuits that govern ACS stability often affect multiple isozymes of a common type (e.g. Joo et al., 2008; Christians et al., 2009), suggesting that members of these subclades diversified within a common regulatory context. However, several newly identified regulatory factors target ACSs of different types (e.g. Lyzenga et al., 2012; Yoon and Kieber, 2013a), highlighting the layering and integration of mechanisms that control ACS turnover. Overall, the stability control mechanisms clearly link ethylene production to exogenous (e.g. light, wounding, pathogen infection, and abiotic stress) or endogenous signals (e.g. hormone levels, developmental transitions). Light affects ACS stability both positively and negatively; for instance, turnover of ACS5 is decreased (Yoon and Kieber, 2013a), whereas ACS7 turnover is increased (Xiong et al., 2014) by light. Exogenous cytokinin and brassinosteroid treatments increase ethylene production via posttranslational stabilization (Woeste et al., 1999; Hansen et al., 2009), and pathogen infection increases ACS abundance at both transcriptional and posttranslational levels (Han et al., 2010; Li et al., 2012). One challenging question for future analysis is how the homo- and heterodimerization of ACS isozymes affect the interplay of these turnover mechanisms.

Analysis of ACO gene regulation has been hampered by an incomplete definition of the gene family members. ACO belongs to a large family of dioxygenases with high sequence similarity (Zhang et al., 2004), and numerous ACO-like family members have been defined by their amino acid sequence similarity with a group of tomato gene products known to convert ACC to ethylene (for review, see Lin et al., 2009). However, only a subset of ACO-like gene products group closely with bona fide ACOs in phylogenetic analyses (see below), and only a few of these have been assayed for their enzymatic functions (Hamilton et al., 1991; Dong et al., 1992; Gómez-Lim et al., 1993; Bidonde et al., 1998). Interestingly, the ACO-like protein E8 may participate in a negative feedback loop that limits ethylene production in fruit, but this antagonistic relationship does not appear to involve direct interaction between ACO and E8 proteins (Van de Poel et al., 2014).

Phosphorylation and Proteasome-Mediated Degradation: Leitmotifs of ACS Regulation

Phosphorylation of the Arabidopsis type 1 isoforms ACS2 and ACS6 by stress-responsive MAPKs (MAPK3 [MPK3] and MPK6) results in increased ethylene synthesis through protein stabilization (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010). MAPK activity on ACS6 is counteracted by protein phosphatase2A (PP2A)-mediated dephosphorylation (Skottke et al., 2011); moreover, okadaic acid and calyculin (which inhibit both PP2A and PP1) have been shown to cause the accumulation of stabilized MAPK- and CDPK-phosphorylated SIACS2 in tomato fruit (Kamiyoshihara et al., 2010). Unphosphorylated type 1 isozymes are rapidly turned over via a 26S proteasome-dependent pathway, and the noncatalytic C-terminal region containing the CDPK and MAPK phosphorylation motifs is sufficient to confer instability on reporter protein fusions (Joo et al., 2008). In rice (Oryza sativa), salt stress activates an analogous MAPK cascade downstream of the Salt Intolerancel receptor-like kinase, promoting ethylene synthesis (Li et al., 2014), although target ACS isozymes were not identified in that work. CDPK-mediated phosphorylation of a tomato type 1 ACS (SIACS2) stabilizes the enzyme and leads to increased ACS activity in wounded tomato tissue (Tatsuki and Mori, 2001; Kamiyoshihara et al., 2010). However, the corresponding site in Arabidopsis type 1 isoforms (ACS1, ACS2, and ACS6) conforms poorly to the consensus site utilized by several CDPKs (Fig. 2C; Hernández Sebastià et al., 2004) and has not been shown to be phosphorylated in vivo or in vitro.

Type 2 ACS isozymes are recruited for ubiquitin-dependent proteolysis by ETHYLENE-OVER-PRODUCING1 (EOT1) and the ETHYLENE-OVER-PRODUCING1-LIKE (EOL1 and EOL2) proteins, which are subunits of Cul3a/3b-based E3 ubiquitin ligases (Chae et al., 2003; Wang et al., 2004; Gingerich et al., 2005; Yoshida et al., 2005; Christians et al., 2009). Ethylene overproduction in the Arabidopsis eot1 mutant is caused by decreased proteolytic turnover of type 2 ACS isozymes. ETO/EOL-mediated regulation requires recognition of a C-terminal TOE motif that is conserved in numerous species; in Arabidopsis, this motif is altered by the dominant acs5eto2 and acs9eto3 mutations (Fig. 2C). Each of these mutations stabilizes the ACSeto protein product.
Figure 2. Determinants of ACS stability regulate ethylene production. A, Model summarizing regulation of ubiquitin-dependent ACS turnover for representative Arabidopsis ACS isozymes. Sequences that regulate ACS stability (cyan) occur both N and C terminally, relative to conserved catalytic domains (green). Specific E3 ubiquitin ligases (orange ovals) acting on type 2 and type 3 isozymes, but not type 1 isozymes, have been identified. Kinase/phosphatase factors that positively regulate ACS stability are shown in blue, whereas negative regulators are shown in red. C-terminal motifs containing three Ser-Pro motifs phosphorylated by MAPKs (M) or a Target of ETO1 (TOE) interaction motif regulate turnover of type 1 and type 2 isozymes, respectively. The PP2C isoform AP2C1 negatively regulates MPK6 (Schweighofer et al., 2007). B, ACS isozyme types from Arabidopsis (ACS) and tomato (SlACS) share posttranslational stability determinants. Type 1 (light-blue), 2 (light-orange), and 3 (light-pink) ACS isoforms interact with regulatory factors that stabilize (S), destabilize (D), or have no effect on turnover of the proteins (U); some physical interaction assay results (+ and $\ ^{2}$) have not been for correlating effects on ACS stability. Predicted target sites for regulators are present (*) or absent (gray shading) but untested in several ACS isozymes. Protein half-lives have been measured for ACS6, SIACS2, ACS7, and ACS5 (Chae et al., 2003; Joo et al., 2008; Kamiyoshihara et al., 2010; Lyzenga et al., 2012; Xiong et al., 2014); for epitope-tagged reporters, the tag is indicated (myc, HA, or Flag). C, A complex overlay of motifs surrounds the TOE sequence in type 2 isozymes. Sequences from the corresponding region of several type 1 and type 2 isozymes are aligned, with the consensus motifs or target residue for calcium-dependent protein kinase (CDPK) phosphorylation (Hernández Sebastiá et al., 2004), ETO1 interaction (TOE; Yoshida et al., 2005, 2006), and casein kinase 1.8 (CK1.8) phosphorylation (Tan and Xue, 2014) indicated in red. Isoforms highlighted in orange are known to interact with ETO1, whereas gray highlighting indicates failure to interact (Yoshida et al., 2005; Christians et al., 2009). Nonconservative substitutions at conserved positions in the TOE motif are shown in lowercase in type 2 isozyme sequences. Residues highlighted in black have been shown to undergo regulatory phosphorylation (Tatsuki and Mori, 2001; Tan and Xue, 2014); residues highlighted in yellow are phosphorylatable residues at the position of CK1.8 phosphorylation in ACS5. Sequences matching the CDPK target motif are underlined. D, The N-terminal extensions of ACS7 and other type 3 isozymes show weak sequence conservation. N-terminal sequences of Arabidopsis ACS7 (AtACS7) and type 3 isoforms from several other species were aligned manually; the alignment shown ends at the first residue of the conserved Box1 motif (Yamagami et al., 2003), and the consensus sequence (ConS) shows invariant (underlined), strongly conserved (capital, uppercase), and weakly conserved (lowercase, lowercase) residues. The first 14 amino acids (bold face) in ACS7 constitute an N-terminal stability determinant (ACS7-N14; Xiong et al., 2014). Sequences in the N-terminal extension (pink shading) are not conserved, and similarity to the ACS7-N14 motif is found only in sequences from cruciferous species. Similar to ACS7, turnover of AtACS4 is regulated by XBAT32, but the N-terminal sequence of ACS4 (blue) lacks any similarity to ACS7-N14 and differs from the ACS7 consensus at several conserved positions (gray letters).
by preventing its interaction with ETO1 (Chae et al., 2003; Wang et al., 2004; Yoshida et al., 2006; Christians et al., 2009). Ethylene overproduction due to loss of ETO/EOL-mediated regulation is dramatic in etiolated seedlings but is also observed in light-grown seedlings and adult plants (Christians et al., 2009; Yoon and Kieber, 2013a).

Phosphorylation also regulates type 2 ACS turnover. A CK1.8 isoform was recently shown to phosphorylate a Ser residue in the TOE motif of ACS5 and thereby promote ETO1-mediated ACS degradation (Fig. 2, A–C; Tan and Xue, 2014), providing an example of a destabilizing phosphorylation signal. PP2A-mediated dephosphorylation stabilizes ACS5 (Skottke et al., 2011), but it has not yet been determined whether ACS5 is the direct target for phosphatase action, or whether this effect is ETO/EOL or CK1.8 dependent. The putative CDPK target motif of type 2 ACS proteins overlaps with the TOE sequence (Fig. 2C). This motif can be phosphorylated in vitro (Hernández Sebastià et al., 2004), but the role of phosphorylation at this site is uncertain. Substitution of either a phosphomimicking or a non-phosphorylatable residue for the putative target Ser does not affect the interaction of ACS5 with ETO/EOL proteins in a yeast two-hybrid assay (Christians et al., 2009).

Regulation of ACS7 levels is of particular interest because it is the sole representative of the type 3 class in Arabidopsis, and may be capable of forming active heterodimers with both type 1 (ACS6) and type 2 (ACS4, 8, and 9) isoforms in vivo (Tsuchisaka and Theologis, 2004a). Type 3 isoforms are encoded by small multisoform gene families in some other species (e.g. rice; Iwai et al., 2006), and Arabidopsis ACS7 appears to make a significant contribution to baseline ethylene production in light-grown seedlings (Tsuchisaka et al., 2009; Li et al., 2012) as well as in etiolated seedlings (Dong et al., 2011), indicating that regulation of type 3 function may be equally important to that of type 1 and 2 isoforms.

Analysis of ACS7 turnover raises the interesting and experimentally challenging question of whether ACS proteins may carry both N- and C-terminal destabilizing sequences. To allow specific detection of individual ACS isoforms, in vivo turnover experiments have usually relied on ACS alleles in which an epitope tag is fused at one end of the protein (most frequently the N terminus), potentially masking some determinants of protein stability. In the absence of isozyme-specific anti-ACS antibodies, these constructs have been invaluable tools; however, the weak phenotypes of acs single mutants have typically precluded rigorous complementation testing to confirm that the tagged alleles show native function. ACS7 lacks the C-terminal regulatory motifs recognized in type 1 and type 2 isoforms but is destabilized by the ubiquitin ligase XB3 ORTHOLOG2
IN ARABIDOPSIS (XBAT32; Fig. 2A; Lyzenga et al., 2012). Similar to the recognition of TOE motifs by ETO/EOL proteins, XBAT recognition of ACS7 can be reconstituted in a yeast two-hybrid assay and in vitro, and has been assayed using N-terminally tagged ACS7 constructs (Prasad et al., 2010; Lyzenga et al., 2012). Interestingly, though, turnover of a C-terminally epitope-tagged ACS7 requires a motif contained within the 14 N-terminal amino acids (ACS7-N14; Xiong et al., 2014). Neither the XBAT32 dependence of ACS7-N14-mediated turnover nor the ACS7 dependence of ethylene overproduction in xbat32 mutants has been directly tested, thus it is unclear whether these constitute a single regulatory mechanism or two different turnover systems for ACS7. The N-terminal sequence of type 3 isozymes is very poorly conserved (Xiong et al., 2014), and sequences resembling the ACS7-N14 motif are found only in type 3 isozymes from species within the Brassicaceae (Fig. 2D).

Newly Identified Posttranslational Mechanisms Regulate Multiple ACS Types

A network of regulatory inputs has been superimposed on the ACS subclades to provide very finely modulated control of ethylene synthesis. Although the type 1, 2, and 3 designations accurately represent ACS subclades (Fig. 3), several recently elucidated regulatory factors target ACSs of different types (Fig. 2B), suggesting rapid evolution of regulatory mechanisms. For instance, the putative target site for destabilizing phosphorylation by CK1.8 (T463) is present in type 2 isozymes ACS5 and ACS9 plus several different type 1 isozymes, but absent from type 2 isozymes ACS4 and ACS8 (Fig. 2B). Similarly, interaction with 14-3-3 proteins stabilizes at least one isoform of each type (ACS2, ACS5, and ACS7; Yoon and Kieber, 2013a, 2013b). An abundant family of small dimeric proteins that typically bind phospho-Ser, 14-3-3 proteins interact with a wide array of phosphoproteins; the effects of 14-3-3 binding are variable (Denison et al., 2011). The mechanism for 14-3-3 stabilization of ACS2 and ACS7 is not known. In the case of ACS5, 14-3-3 interaction antagonizes ETO/EOL-mediated turnover both by stabilizing the ACS protein and by destabilizing ETO/EOL proteins. Interaction of 14-3-3 with ACS is not dependent on the TOE sequence of ACS5 (Yoon and Kieber, 2013a). Because 14-3-3 binding is generally phosphorylation-dependent, the discovery of 14-3-3-mediated stabilization suggests that these isozymes may undergo stabilizing phosphorylation in the catalytic domain, the only domain that is common to all three types.

Interestingly, cell-free degradation assays show that XBAT32 not only regulates ACS7 (type 3) stability, but also promotes turnover of ACS4 (Prasad et al., 2010; Lyzenga et al., 2012), a type 2 isoform that is a predicted ETO1 target (Chae et al., 2003; Wang et al., 2004; Gingerich et al., 2005; Yoshida et al., 2005; Christians et al., 2009). In addition to its C-terminal TOE motif (Yoshida et al., 2006; Christians et al., 2009), ACS4 also carries a destabilizing N-terminal sequence (Schlögelhofer and Bachmair, 2002). This N-terminal stability determinant has not been mapped precisely; the 94-amino acid region shown to destabilize a reporter protein includes two of the conserved structural elements of the catalytic domain (Yamagami et al., 2003) but lacks any similarity to the ACS7-N14 motif (Fig. 2D). Thus, both ACS4 and ACS7 carry N-terminal stability determinants, and both are regulated by XBAT32; the signals recognized by XBAT32 remain to be identified.

EVOLUTION OF THE BIOSYNTHETIC PATHWAY

The three ACS isozyme types, with their characteristic C-terminal motifs, are represented in monocots as well as dicots (Fig. 3; Zhang et al., 2012), but these motifs are not conserved outside the flowering plants. Nonetheless, conifer ACS isozymes are clearly related to isoforms in the type 1, type 2, and type 3 subclades (Fig. 3; Ralph et al., 2007; Lyzenga et al., 2012; Zhang et al., 2012). This is consistent with the diversification of ACS isozymes into three types after the split of seed plants from other land plants (Fig. 3) and the innovation of posttranslational regulatory switches controlling ACS stability very early in flowering plant evolution. Conifers express ACO isoforms that group phylogenetically with the angiosperm ACOs known to synthesize ethylene as well as additional ACO-like isoforms, indicating that diversification of ACO isoforms occurred prior to the split of flowering plants and gymnosperms (Hudgins et al., 2006; Rudus et al., 2013). The conservation of ACS and ACO isoforms in conifers suggests that the flowering plant ethylene biosynthesis pathway is conserved throughout seed plants, although the posttranslational regulation of specific isoforms likely differs between gymnosperms and angiosperms, because the gymnosperm ACS isoforms exhibit none of the known regulatory sequence motifs.

Because of the conservation of ethylene responses, the evolutionary origin of the biosynthetic pathway is of considerable interest, and the ACS and ACO gene families are clearly present in seed plants. However, physiological studies of ethylene biosynthesis outside the seed plants do not provide a consistent mechanistic picture of a conserved biosynthesis pathway. In flowering plants and in all four gymnosperm groups (Osborne et al., 1996), feeding the biosynthetic intermediate ACC to whole plants or live tissue results in the production of ethylene, consistent with an ACO activity conserved throughout seed plants. Similarly, ACC treatment increases ethylene production in the charophyte Spirogyra pratensis, the chlorophytes Haematococcus pluvialis and Ulva intestinalis, and the red alga Pterocladiella capillacea, consistent with conservation throughout the plant kingdom (Maillard et al., 1993; Plettner et al., 2005; Garcia-Jimenez et al., 2013; Ju et al., 2015). However, treatment of liverworts, moss, and ferns with exogenous ACC does not yield increased ethylene production, despite detectable uptake and metabolism of labeled ACC (Osborne et al.,
These results are perplexing since they suggest that seed plants and red and green algae convert ACC to ethylene, whereas land plants other than seed plants use an alternate pathway. Additional work will be required to clarify the biosynthetic pathway in nonseed plants.

**ACS and ACS-Like Gene Families**

Aminotransferase activity likely represents the ancestral function of the ACS-like enzyme family. Phylogenetic analyses indicate a complex evolutionary history for the subclade of the PLP-dependent aminotransferase gene family that contains ACS, and suggest that the role of ACS in ethylene biosynthesis is an evolutionary novelty that arose specifically in plants. The ACS-like subfamily of amino transferases includes both true ACS isoymes and a group of related proteins that lack ACS enzymatic activity (Fig. 3). Members of the ACS-like protein family are conserved in vertebrates, and biochemical characterization of a mammalian isoform shows that it does not convert SAM to ACC at physiologically relevant rates, but does deaminate the ACS inhibitor 1-aminovinyl-Gly (Koch et al., 2001). Thus, animal ACS-like proteins may catalyze a similar but distinct deaminase or aminotransferase reaction (putative deaminase clade; Fig. 3). Similarly, the Arabidopsis ACS-like proteins ACS10 and ACS12 are Asp, Phe, and Tyr aminotransferases, not ACSs (Yamagami et al., 2003). ACS10 and ACS12 are members of an ACS-like clade specific to seed plants (putative aminotransferase clade; Fig. 3), whereas type 1, 2, and 3 ACSs form a separate clade shared throughout seed plants. These relationships suggest that true ACS enzymes and a separate group of ACS-like amino transferases diverged after the split of seed plants from mosses (Fig. 3; Ralph et al., 2007; Zhang et al., 2012). Furthermore, the ethylene-related clade is under positive selection (Zhang et al., 2012), consistent with diversification of an ancestral ACS-like aminotransferase clade and functional specialization of true ACS isoforms after the divergence of seed plants from other plants.

Under this model, the ACS-like sequences recently identified in green algae, red algae, glaucophytes, cyanobacteria (Ju et al., 2015), mosses (Rensing et al., 2008), and lycophytes (Banks, 2009) might catalyze amino transferase reactions rather than synthesize ACC. The recently identified putative ACS from S. pratensis (SpACS1 in Fig. 3; Ju et al., 2015) groups outside of the clade that contains both true ACS enzymes and ACS-like aminotransferases, leaving its function ambiguous. However, a simple amino acid sequence comparison (using BLAST) reveals that the S. pratensis ACS-like protein sequence (Ju et al., 2015) is more similar to that of ACS8 and other ACC-producing isoforms than it is to ACS-like amino acid aminotransferase proteins (e.g. ACS10 and ACS12). This suggests that ACS/ACS-like isoforms outside the seed plants may be dual-function ACC-producing aminotransferases, with subsequent subfunctionalization of ACC synthase and amino acid aminotransferase activities occurring in seed plants. Further characterization of nonseed plant ACS isoforms is needed to clearly define the enzymatic activities and metabolic roles of these proteins.

**ACOs Genes: Disambiguation Desired**

The ACO gene family is less well studied, and the available data on evolution of ACO function are not clear cut. ACO isozymes are members of a large Fe(II)-requiring dioxygenase/oxidase superfamily encompassing roughly 100 Arabidopsis proteins (Zhang et al., 2004), most of which do not exhibit known ACC oxidase activity. Only a few proteins (e.g. tomato ACO1–3 [Hamilton et al., 1991; Bidonde et al., 1998], MdACO1 from apple [Malus domestica; Dong et al., 1992], and Arabidopsis ACO4 [Gómez-Lim et al., 1993]) are known to exhibit ACC oxidase activity in vitro. Arabidopsis ACO2 and ACO3 and tomato ACO4 group with these active ACOs in phylogenetic analyses along with some conifer enzymes, whereas tomato ACO5 and Arabidopsis ACO1 and ACO5 cluster together in a sister clade that is separate from the group of enzymatically defined ACO isoforms (Hudgings et al., 2006; Ruduż et al., 2013). Furthermore, a set of Arabidopsis dioxygenase gene products with a more distant relationship to the known ACOs (Ruduż et al., 2013) have been designated as ACO6 to ACO13 on the basis of sequence similarity alone (Clouse and Carraro, 2014). Genes encoding isoforms of the dioxygenase superfamily have been identified in mosses, lycophytes, and green algae, but it is currently unknown whether these enzymes catalyze an ACO reaction (Clouse and Carraro, 2014). This uncertainty about which flowering plant dioxygenase genes encode bona fide ACOs has hindered analysis of ACO-like enzymes from other plants.

**PROSPECTS AND QUESTIONS IN ETHYLENE BIOSYNTHESIS**

The preceding summary clearly points to a number of questions for future research on the ethylene biosynthetic pathway and its regulation.

Are the ACC conjugates that have been identified in several systems simple sinks that reduce the ACC pools available for ethylene production, or are they nodes of hormonal cross talk and/or physiologically significant reservoirs for ACC that can be mobilized by conjugate cleavage?

What are the constituents of the circuits that provide feedback regulation on ACS and ACO mRNA levels?

How does ACS dimerization affect turnover? What are the mechanisms that regulate and integrate the protein kinase and phosphatase activities that modulate turnover?

How widely conserved are the posttranslational regulatory mechanisms that control ACS stability? Can the conclusions derived from analysis in Arabidopsis
and tomato be extrapolated to more distantly related, agriculturally important plants such as banana (Musa spp.), kiwi (Actinidia delicosa), and avocado (Persea americana)? Did ancestral ACS enzymes contain non-catalytic regulatory domains?

The evolutionary history of the biosynthetic pathway presents additional puzzles. Although components of the ethylene response pathway are distinctive and provide a clear phylogenetic signature, ACS and ACO are members of multiisoform gene families that catalyze distinct but chemically similar enzymatic reactions; gene family members are difficult to distinguish from each other on the basis of simple sequence similarity. Thus, the pathway for ethylene biosynthesis outside of the seed plants is somewhat unclear, because ACS-like enzymes in moss and algae may convert SAM to ACC, or they may perform a different but chemically similar reaction. The functions of ACO-like dioxygenases are even harder to predict in the absence of biochemical data. The phylogenetic position of true ACOs within the dioxygenase family will provide interesting insight into the evolution of this function and thus the configuration of the pathway in evolutionary history.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in Supplemental Table S1.

Supplemental Data
The following supplemental materials are available.
Supplemental Table S1. Names and accession numbers of protein sequences represented in Figure 3 phylogeny.

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