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Something old, something new: conservation of the ethylene precursor ACC as a signaling molecule

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Author Comments:	

Cover Letter

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Dr. Keiko Sugimoto
Guest Editor, *Current Opinion in Plant Biology*
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Dear Dr. Sugimoto,

Thank you for providing us with the reviewers' comments. Their comments were valuable and very helpful in improving our manuscript. We have provided a point by point response to each of the Reviewer's suggestions in the file "Response to Reviewers".

In addition to the above revisions, we have updated our article by including a citation to an upcoming paper [Ref 37]: Althiab-Almasaud R, Sallanon H, Chang C, Chervin C: **1-aminocyclopropane-1-carboxylic acid (ACC) stimulates tomato pollen tube growth independently of ethylene receptors**. *Physiol Plant*, accepted. We have added a paragraph describing this work on lines 235-241 and added a figure from this paper in Figure 4 (4e). We have also included a rating (•) and annotation for this paper. We made corresponding changes to the main text (e.g., replaced "*A. thaliana*" with "angiosperms "or with "*A. thaliana* and *S. lycopersicum*").

We have also added a diagram to Figure 3a in order to show the gemmaling cauloid lobe and column regions (to go along with two of the Reviewers' requested revisions to Figure 3b).

Thank you for giving us the opportunity to contribute to *Current Opinion in Plant Biology*, and thank you very much for handling our manuscript.

Sincerely,

Caren Chang

Something old, something new: conservation of the ethylene precursor ACC as a signaling molecule

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Ethylene, precursor, ACC, biosynthesis, signaling, hormone, evolution, liverwort, Marchantia, Arabidopsis

Abstract

In seed plants, 1-amino-cyclopropane-1-carboxylic acid (ACC) is the well-known precursor of the plant hormone ethylene. In non-seed plants, the current view is that ACC is produced but is inefficiently converted to ethylene. Distinct responses to ACC that are uncoupled from ethylene biosynthesis have been discovered in diverse aspects of growth and development in liverworts and angiosperms, indicating that ACC itself can function as a signal. Evolutionarily, ACC may have served as a signal prior to acquiring its role as the ethylene precursor in seed plants. These findings pave the way for unraveling a potentially conserved ACC signaling pathway in plants and have ramifications for the use of ACC as a substitute for ethylene treatment in seed-plants.

Introduction

The plant hormone ethylene (C_2H_4) regulates well-known aspects of growth and development, such as fruit ripening, senescence, abscission, and responses to stress, and controls diverse, lesser-known processes in many plants ranging from algae to angiosperms [1]. The ethylene signaling pathway, which was mainly elucidated in *Arabidopsis thaliana*, was likely functional at least 450 million years ago, as evidenced by conservation of the pathway in a Charophycean alga [2]. Ethylene signaling may have evolved prior to the ability to synthesize ethylene, given that the ethylene receptor likely originated in cyanobacteria [3] and because environmental ethylene is produced by microorganisms as well as by chemical breakdown of organic matter.

The ethylene biosynthesis pathway in angiosperms was uncovered in the late 1970s. A nonproteinogenic amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC), is the immediate precursor of ethylene [4]. In the first committed step of ethylene biosynthesis, the aminotransferase enzyme ACC-synthase (ACS) makes ACC from the universal methyl donor S-adenosyl-L-methionine (SAM); ACC is then converted to ethylene gas by ACC-oxidase (ACO), a dioxygenase that requires oxygen (Figure 1 [4]). ACS synthesis is regulated transcriptionally and post-translationally in response to a wide range of external and internal stimuli [4, 5]. The efficient uptake and conversion of ACC to ethylene by seed plants has enabled plant biologists to use ACC treatment as a substitute for ethylene gas to induce ethylene responses. However, as described in the next section, even though non-seed plants produce ethylene, non-seed plants that take up exogenous ACC produce little or no additional ethylene. Moreover, *ACO* homologs are absent in the available genome sequences of non-seed plants (Figure 2 [6]). In contrast, *ACS* homologs are widely conserved in land plants (Figure 2 [6]), raising the possibility that ACC synthesis evolutionarily preceded

the efficient conversion of ACC to ethylene in angiosperms and gymnosperms. If indeed this was the case, what was the original role of ACC biosynthesis in non-seed plants?

In this article, we review the evidence that ACC serves as a plant signaling molecule independent of its well-known role in ethylene biosynthesis. We also speculate on mechanisms of ACC signaling. By definition, a plant signal operates at low concentrations, evokes local or distant physiological responses, and can be transported. ACC is known to be transported, and the evidence discussed below shows that ACC responses can be distinct from those induced by ethylene and occur even when ethylene signaling is blocked. In evolution, ACC may have served as a signal prior to acquiring its role as the ethylene precursor, yet ACC has unique signaling roles even in angiosperms.

Emerging views of ACC function in non-seed plants

Do non-seed plants produce ACC?

An essential question with respect to ACC signaling is whether a plant is capable of producing ACC. Consistent with the presence of *ACS* homologs in the genomes of non-seed plants, both ACS activity and ACC have been detected in the ferns *Regnellidium diphyllum* and *Marsilea quadrifolia* [7, 8]. ACC was also measured in the liverwort *Riella helicophylla* [8] and the moss *Funaria hygrometrica* [9]. More recently, ACC was measured in the liverwort *Marchantia polymorpha*, and single and double knockout mutants were generated for the two *M. polymorpha* Mp*ACS* homologs [10••, 11••]. Li et al. [10••] observed dramatically less ACC in the Mp*acs1*Mp*acs2* double knockout mutants compared to the wild type, and conversely, detected ACC produced by yeast heterologously expressing each Mp*ACS* gene. In contrast, using pooled lines of Mp*acs1*Mp*acs2* knockout mutants in a different *M. polymorpha* genetic background, Katayose et al. [11••] observed no change in endogenous ACC levels (but raising an interesting question concerning the source of the endogenous ACC). Moreover, both ACS homologs in the moss *Physcomitrella patens* exhibited C_β-S lyase activity instead of ACS activity [12], bringing into question whether *P. patens* synthesizes ACC at all, and whether ACS homologs of other plant species are C_β-S lyases. C_β-S lyases catalyze the cleavage of carbon–sulfur bonds of L-cystine and L-cysteine and are involved in amino acid metabolism. Further studies are required to obtain a clearer view of ACC synthesis and the functions of ACS homologs in non-seed plants.

ACC is only weakly converted to ethylene in non-seed plants

Consistent with the absence of *ACO* homologs in non-seed plant genomes, a number of reports have

indicated that ACC is not the main ethylene precursor in non-seed plants. Studies in the 1990s concluded that there must be an alternative ethylene biosynthesis pathway for mosses, liverworts, and ferns [7, 8, 13]. In key experiments, treatment with radiolabeled [^{14}C]-ACC in the liverworts *R. helicophylla* and *M. polymorpha* [8] and in the ferns *R. diphyllum* and *M. quadrifolia* (ferns) [7, 8], resulted in very little [^{14}C]-ethylene (e.g., 0.005%). In other studies (e.g., in a red alga [14], Chlorophycean algae [15-17], a Charophycean alga [2], and ferns [18]), treatment with non-radiolabeled ACC (ranging between 200 μM–10 mM) yielded only a fraction of the ethylene levels known to be produced by angiosperms treated with similar ACC concentrations. Assuming there was uptake of the ACC in these experiments, such doses were likely above the physiological range. In more recent studies, *M. polymorpha* took up ACC from the growth medium [10••] as shown previously for liverworts [8], but only the highest doses of ACC tested (500 μM and 1 mM) gave a detectable increase in ethylene [10••, 11••, respectively]. In Mp*acs1*Mp*acs2* double mutants, ethylene production was 60% that of the wild type in one study (suggesting ACC could have at least a partial role in ethylene biosynthesis)[10••], but remained unchanged in another study [11••]. It is worth mentioning that exogenous ACC taken up by the plant can potentially be converted to ethylene by non-enzymatic reactions or by unknown enzymes, or conceivably trigger ethylene biosynthesis from a different precursor. Overall, the current opinion is that ACC is at best a weak ethylene precursor in non-seed plants.

The alternative pathways of ethylene production in non-seed plants have yet to be identified. Certain bacteria and fungi produce ethylene from 2-oxoglutarate using the Ethylene Forming Enzyme (EFE) [19] or by a non-enzymatic conversion of α-keto ɣ-methylthiobutyric acid [20]. Still other bacteria convert carbon monoxide or sulfur-compounds to ethylene using vanadium nitrogenase or methylthio-alkane reductase enzymes, respectively [21-23]. However there is currently no evidence indicating that these particular mechanisms exist in plants.

Evolutionarily, aquatic species that subsequently gave rise to land plants would not have benefitted from the synthesis of high ethylene levels. This is because ethylene is removed only by diffusion and diffuses 1000 times slower in water than in air, thus precluding the rapid removal of cellular ethylene in aquatic organisms. We speculate that ethylene may have even served as a sensor of land versus water, or dry versus wet times, and that perhaps ACO activity was acquired as the earth's environment became markedly drier in the Permian period following the wet Carboniferous period when the earliest seed plants appeared. Interestingly, *ACO* genes have been lost in *Potamogeton pectinatus* [24], and both the ethylene

biosynthesis and signaling pathways have been lost in *Zostera muelleri* [25] and *Zostera marina* [26]. These are all angiosperms that have (re)adapted to a fully submerged lifestyle, in contrast to angiosperms that grow on the surface of water, such as *Spirodela polyrhiza*, which has retained the ethylene hormone pathways [27].

Evidence for ethylene-independent ACC signaling in liverworts

The above evidence that non-seed plants can have detectable ACC, but do not necessarily rely on ACC for ethylene production, is compatible with ACC having unique signaling functions independent from its role as the ethylene precursor. In 1989, contradictory effects were reported for ethylene and ACC treatments in the liverwort *R. helicophylla*; ethylene induced cell elongation in the column region of gemmalings (Figure 3a [28]), whereas ACC (5-200 µM) inhibited cell division and reduced column length (Figure 3b [29]). Distinct ethylene and ACC responses were recently reported in another liverwort *M. polymorpha* [10••, 11••]. Ethylene treatment promoted thallus growth (Figure 3c [10••, 11••]), which was also shown by analyzing knockout mutants of positive (Mp*ein3*) and negative (Mp*ctr1*) regulators in the ethylene signaling pathway 10••]. The phenotypes of these mutants additionally indicated that ethylene promotes both cell division and cell enlargement in the gemma epidermis [10••]. In contrast to ethylene, ACC treatment (100 µM) suppressed thallus growth of the wild type [10••, 11••]. Notably, this response was observed even in ethylene-insensitive Mp*ein3* mutants using lower doses (e.g., 10 µM ACC) (Figure 3d [10••]). In Mp*ein3* gemmae, ACC treatment (100 µM) resulted in significantly fewer newly-generated epidermal cells with no difference in cell size, suggesting that ACC inhibits cell division but not cell expansion [10••]. By studying gene expression, Katayose et al. [11••] found that the ethylene-induced transcription factor gene, Mp*ERF1*, is not induced by ACC treatment (100 µM), and conversely identified two genes (Mp*GST2* and Mp*HEL*) induced by ACC treatment but not ethylene (Figure 3e).

Single knockout mutants of each of the Mp*ACS* homologs produced less ACC and were larger in thallus size compared to the wild type. Notably, the single (Mp*acs2*) and double (Mp*acs1*Mp*asc2*) mutant gemmae displayed a higher frequency of abnormal apical notches (meristems), which was not seen in ethylene-insensitive mutants (Figure 3f [10••]). This provides evidence for endogenous ACC having a signaling role in liverworts, and together with the other findings in non-seed plants, suggests the possibility that, evolutionarily, ACC was a signal before serving as the ethylene biosynthesis precursor.

ACC signaling roles in angiosperms

ACC has various roles as an ethylene-independent signal even in *A. thaliana*, an angiosperm in which ACC is unquestionably the ethylene precursor. The untangling of responses that are due to ACC versus ethylene in *A. thaliana* has relied on an array of genetic tools (such as well-characterized ethylene signaling mutants) and pharmacological tools (including chemical inhibitors of ethylene biosynthesis and ethylene perception) [30], with the caveat that chemical inhibitors can have unknown actions and side effects. In this section, all of the studies in which exogenous ACC was applied used concentrations that were likely to be in the physiological range.

ACC was first implicated as an ethylene-independent signal in *A. thaliana* in the regulation of root cell wall biosynthesis via the FEI pathway [31]. FEI1 and FEI2 are leucine-rich repeat receptor-like kinases, and the *fei1fei2* double mutant has shorter and swollen roots in the presence of high concentrations of sucrose or salt resulting from defective cellulose synthesis. The anisotropic expansion defect in the roots was rescued by treatment with either the ACS inhibitor 2-aminooxyacetic acid (AOA) or the ACC structural analog α-aminoisobutyric acid (AIB), which has been typically used as an ACO inhibitor [31]. However, the defect was not reverted by blocking ethylene perception with 1-Methylcyclopropene (1-MCP) or silver ions, nor by using mutations that confer ethylene insensitivity (Figure 4a [31]). The authors therefore hypothesized that ACC could be a signal that regulates root cell expansion. To explain the rescue of the *fei1fei2* defect by AIB, the authors proposed that AIB acted as a competitive inhibitor of an unknown ACC receptor.

Additional evidence for ACC having a role in cell expansion involved the cellulose synthase inhibitor isoxaben, which causes root swelling and shortening. Root elongation was restored by the ACS inhibitors AOA, aminoethoxyvinylglycine (AVG) and 2-anilino-7-(4-methoxyphenyl)-7,8-dihydro-5(*6H*)-quinazolinone (7303) or the ACO inhibitor AIB, but not by blocking ethylene perception (with silver ions or norbornadiene) [32]. Similar to isoxaben, ACC treatment induced a short-term inhibition of root elongation of the ethylene-insensitive mutant *ein3eil1*, indicating that ACC, not ethylene, is involved in the root response to cell wall damage stress [32]. Both auxin signaling and superoxide production function downstream of ACC to inhibit root elongation [32].

In a major advance in 2009, genetic evidence for ACC having ethylene-independent signaling roles was obtained through the creation of an *A. thaliana acs* octuple mutant in which all eight functional *ACS* genes were disrupted [33]. (Due to incomplete silencing of two of the *ACS* genes in this *acs* octuple mutant, a

true knockout mutant of ACC synthesis has yet to be obtained.) As expected, the *acs* octuple mutant displayed phenotypes shared by ethylene-insensitive mutants (an initial growth delay and delayed senescence) but also had phenotypes that appeared unrelated to having reduced ethylene levels (smaller cotyledons, a shorter primary root with a proliferation of root hairs, downward curling leaf tips, reduced branching, early flowering, shorter siliques, and fewer seeds) [33].

More recently, some of these potential ACC-specific phenotypes have been investigated in more detail. In the light, ACC treatment inhibited growth of the rosette (50-100 μM) and primary root (10-50 μM), as observed when ethylene signaling was blocked by 1-MCP treatment of the wild type or by the *ein2-1* ethylene-insensitive mutation (Figure 4b [34•]). In the dark, ACC treatment (1 μM) reduced hypocotyl elongation and primary root growth in wild-type and *ein2-1* seedlings in the presence of AIB, which presumably inhibited ethylene biosynthesis as an antagonist of ACO; however, AIB acting as an agonist or antagonist of a hypothetical ACC receptor has not been ruled out [34•].

ACC also modulates the symmetric division of guard mother cells (GMCs) into two guard cells (GCs) during stomatal development. Treatment of epidermal cells with AVG led to the formation of single guard cells (SGCs), whereas no response was observed when ACO activity was blocked (using AIB or Co^{2+}), nor in the wild type treated with 1-MCP nor in ethylene-insensitive mutants (Figure 4c [35•]). A similar SGC defect was observed in the *acs* octuple mutant, and treatment with ACC (10 μM) partially rescued the defect, whereas ethylene had no effect [35•]. ACC regulated GMC division via a cell cycle dependent pathway [35•].

Whereas Tsuchisaka et al. [33] proposed that embryonic lethality was an underlying cause of the lower number of seeds in the *acs* octuple mutant, a recent study determined that reduced seed set was the result of reduced pollen tube attraction by the octuple mutant ovules (Figure 4d [36••]). Mou et al. [36••] found that ACC in the sporophytic tissue of the ovules plays an ethylene-independent role in pollen tube attraction. Compared to wild-type ovules, a higher proportion of *acs* octuple mutant ovules showed greater retention of the LURE1.2 peptide in the synergid cells instead of trafficking to the filiform apparatus or micropyle where LURE1.2 (along with other LURE1 peptides) is known to serve as a pollen tube attractant [36••]. ACC treatment (1 μM) of *acs* octuple ovules promoted LURE1.2-eGFP secretion and restored pollen tube attraction, whereas ethylene did not [36••].

ACC might play a role in pollen tube growth as well. In *Solanum lycopersicum*, *in vitro* pollen tube growth was promoted by applying low concentrations of ACC (0.1-100 μM) when ethylene receptor signaling was inhibited [37•]. Unlike the ACC-specific responses discussed above, promotion of tomato pollen tube

growth can be induced by both ethylene and ACC. Notably, treating pollen grains and pollen tubes with ACC stimulated the expression of an ethylene-responsive reporter fusion (*EIN3 Binding Site (EBS):GUS*) either in the ethylene-insensitive Never-ripe mutant (e.g., Figure 4e) or in presence of 1-MCP, suggesting that ACC might activate ethylene response signaling at a point downstream of the ethylene receptor [37•].

Speculation on ACC signaling mechanisms

A candidate ACC receptor

The ethylene-independent functions of ACC above suggest that plants have ACC-specific signaling pathways. Mou et al. [36••] raised the possibility that ACC could signal via the gating of glutamate receptor-like (GLR) ionotropic channels. GLRs are homologs of mammalian ionotropic glutamate receptors (iGluR), which are ligand-gated ion channels; when activated by glutamate and other amino acids, they allow cations to flow across the cell membrane [38]. ACC, a non-proteinogenic amino acid (not naturally present in animal tissues), was found to be a partial agonist of mammalian iGluRs [e.g., 38-40]. In plants, GLRs can be stimulated by various amino acids [41], but the physiological ligands remain unknown, and the roles of GLRs are only starting to come to light. Mou et al. [36••] demonstrated that ACC elicits GLR-dependent Ca^{2+}-containing ion currents in *A. thaliana* root protoplasts (using 250-500 μM ACC) and triggers transient cytosolic Ca^{2+} elevation in ovules (using 500 μM ACC). Further, in a mammalian cell expression system, ACC treatment (500 μM) stimulated *P. patens Pp*GLR1-dependent elevation of cytosolic Ca^{2+} fluxes to a greater extent than any of the twenty proteinogenic amino acids. (The high concentrations of ACC used as a ligand in these studies are typical of electrophysiological studies and imaging in heterologous systems.) The LURE1.2 secretion defect in *acs* octuple ovules was rescued by increasing cellular Ca^{2+} levels with a Ca^{2+} ionophore, thus leading Mou et al. [36••] to propose that ACC might serve as a GLR-gating ligand that induces Ca^{2+} elevation in the ovule, which then promotes LURE1.2 secretion [36••]. Whether ACC signaling operates through GLRs and Ca^{2+} spikes that evoke downstream responses requires further investigation. As implicated by other studies, downstream signaling components of ACC signaling appear to involve auxin signaling [31], superoxide production [31], and/or the cell cycle [35•].

ACC as an internal or external sensor?

We speculate that ACC, which is typically present at low concentrations, may conceivably reflect cellular metabolic homeostasis, similar to how the non-proteinogenic amino acid GABA is considered to be a proxy

for the plant cell nitrogen or amino acid content [42], or how threhalose-6-phosphate, a low-abundant sugar-phosphate, mirrors sucrose levels in plants and controls the energy status [43]. Although this has not been investigated, ACC might have a nitrogen or metabolic sensing function, perhaps linked with changes in Ca^{2+}-fluxes caused by GLR channels. *A. thaliana* has 20 GLRs with distinct sub-cellular membrane localizations, raising the question of whether GLR-mediated Ca^{2+}-fluxes could be linked to ACC-specific responses in different organs.

ACC, or one of its derivatives [4], could potentially serve in a role outside of the cell. This idea is corroborated by the identification of ɣ-glutamyl-ACC (GACC), a low abundance ACC conjugate [44] that is made in the apoplast by ɣ-glutamyl transpeptidase in *A. thaliana* [45]. Secreted ACC could serve as a cellular messenger to alter the extracellular environment to control, for example, cell wall integrity and/or flexibility, perhaps involving plasma membrane-localized GLRs. Other extracellular Ca^{2+}-channels in plants operate as environmental sensors, such as the Na^{+}-sensor MOCA1 [46] and the osmotic sensor OSCA1 [47]. Apoplastic ACC sensing might play a role in plant-microbe interactions, as certain beneficial bacteria are known to consume ACC as a nitrogen source using ACC deaminase [48]; so perhaps apoplastic ACC can modulate the symbiotic interaction with plant growth-promoting bacteria.

Dual ACC functions

How might seed plants use ACC for both ethylene production and independent signaling? The function of ACC could be dependent on ACO expression/activity for ethylene production and the availability of ACC signaling components. A switch between ACC signaling and ethylene production could conceivably involve feedback mechanisms based on ACC and/or ethylene levels. ACC levels are controlled through ACC homeostasis, which is mainly regulated through synthesis (by ACS), conversion to ethylene, conjugation to malonyl, γ-glutamyl, or jasmonyl ACC [4], transport (e.g. via LYSINE-HISTIDINE TRANSPORTER1 (LHT1) and LHT2; [49, 50•]) and storage (e.g. in the vacuole [51]). There might also be feedback mechanisms involving an interplay of ACC and ethylene based on their downstream responses.

Conclusions and future directions

It is increasingly clear that ACC can function as a signaling molecule independent of its role as the ethylene precursor. ACC is active at low concentrations (e.g., 1 μM), can induce local or distant responses (e.g., Figure 4b), and can be transported via LHT1 and LHT2. Current data suggest that ACC may have been a signaling molecule prior to acquiring its predominant role in ethylene biosynthesis. This scenario has some

similarities with the biosynthesis of the plant hormone jasmonate (JA) in that bryophytes lack the enzymes that convert the precursor dinor-12-oxo-phytodienoic acid (dn-OPDA) to JA-Ile, and dn-OPDA itself serves as a signaling molecule. However, unlike dn-OPDA, which is the ligand of a co-evolved form of the JA receptor COI1 [52], there is no evidence that ACC signals through the ethylene receptors, given that ACC and ethylene responses are generally distinct, and ethylene-insensitive mutants still respond to ACC.

Given the role of ACC as a signal, caution is required when using ACC as a substitute for ethylene treatment. In maize, for example, ACC-induced resistance to the fungal pathogen *Fusarium graminearum* was attributed to ethylene but could have been due to ACC [53]. In the red alga *Pyropia yezoensis*, ACC-induced (50 μM) sexual reproduction was attributed to ethylene [14], while subsequent findings suggest that the response was likely due to ACC [14, 54]. This highlights the importance of verifying the effects of exogenous ACC using ethylene gas. Some other limitations of treating plants with ACC, particularly when investigating ACC signaling responses, include not knowing the fate of the ACC taken up by the plant and distinguishing between ACC signaling versus a stress/toxicity response at particular doses of ACC.

There are a number of future directions for the emerging topic of ACC signaling. The likely identification of additional ACC responses in the plant lineage will provide further impetus for a new focal point on the elucidation of ACC signaling pathways. This will include how ACC signaling is regulated, particularly in seed plants that rely on ACC for ethylene production, and whether there is interplay with ethylene signaling and other plant hormones. In non-seed plants, there are basic questions concerning whether endogenous ACC is synthesized and transported, how ACC is converted to ethylene, and what the primary mechanisms of non-ACC-based ethylene production are. An interesting broader question involves the evolutionary relationships between ACC biosynthesis, ethylene biosynthesis, ACC signaling, and ethylene signaling.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest

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    In this study, ethylene and ACC responses in the liverwort *Marchantia polymorpha* are described and found to be distinct, based on ethylene and ACC treatments, as well as on analyses of CRISPR/Cas9-generated mutants in predicted ethylene signaling and ACS genes. Genetic analyses of Mp*acs1*Mp*acs2* double mutants confirmed that ACC is not primarily used for ethylene biosynthesis, but has its own ethylene-independent roles in aspects of growth and development, such as the size and number of gemmae cups and the development of apical notches (meristems).

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In this study, genetic and chemical analyses reveal several distinct responses between ACC and ethylene in both light- and dark-grown seedlings of *Arabidopsis thaliana*. Independently of ethylene, ACC influences several vegetative developmental processes, such as inhibiting rosette growth and hypocotyl elongation.

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This study uncovers an ethylene-independent role of ACC in *Arabidopsis thaliana* guard cell development. Using both chemical and genetic analyses, the authors find that ACC is required for the symmetrical division of the single guard mother cell into a fully functional two-celled guard cell.

36. •• Mou W, Kao YT, Michard E, Simon AA, Li D, Wudick MM, Lizzio MA, Feijo JA, Chang C: **Ethylene-independent signaling by the ethylene precursor ACC in Arabidopsis ovular pollen tube attraction**. *Nat Commun* 2020, **11**:4082.

This paper investigates the underlying basis for why the Arabidopsis *acs* octuple mutant has a reduced seed set, revealing that ACC in the sporophytic tissue of the ovules plays a role in pollen tube attraction by promoting the secretion of the peptide LURE1.2, a pollen tube chemoattractant, needed for successful fertilization. Furthermore, the paper showed that ACC can activate glutamate receptor-like (GLR) channels, triggering elevation of cytosolic Ca^{2+} levels that are involved in secretion of LURE2.1 peptide. ACC, a nonproteinogenic amino acid, induces the highest PpGLR1-dependent cytosolic Ca^{2+} elevation levels (in a heterologous expression system) compared to all twenty amino acids, raising the possibility that ACC is a physiological ligand for GLRs.

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This study reveals that treatment with either ethylene or ACC stimulates tomato pollen tube growth, yet ACC does so even when ethylene receptor signaling is inhibited. Moreover, ACC treatment stimulates expression of an ethylene-responsive *EBS:GUS* reporter in pollen grains and pollen tubes when the ethylene receptors are inhibited by 1-MCP or by the ethylene-insensitive Never-ripe mutation, indicating the possible activation of ethylene signaling by ACC downstream of the ethylene receptors.

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Little is known regarding ACC transport in plants. The authors follow-up on the prior discovery that the lysine histidine transporter, LHT1, can transport ACC, by now showing that overexpression of the LHT1 paralog, LHT2, rescues the *lht1* mutant, restoring uptake of ACC and rescuing an early senescence phenotype. In contrast, overexpression of *LHT3* and *AMINO ACID PERMEASE 5* (AAP5) do not confer rescue. Transport assays in *Xenopus laevis* oocytes confirmed that LHT1 and LHT2 transport ACC, but also transport overlapping sets of other amino acids.

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Figure Legends

Figure 1. Ethylene biosynthesis in seed plants and the dual roles of ACC.

Ethylene biosynthesis is a two-step pathway from SAM, which is synthesized from methionine. In the first step, SAM is cleaved and converted to ACC by the enzyme ACS. In the second step, ACC is converted to ethylene by the enzyme ACO. ACC can also induce responses that are distinct from those induced by ethylene via an unidentified ACC signaling pathway (dotted line). This pathway is in contrast to the known, conserved ethylene signaling pathway.

Figure 2. Evolution of ACC and ethylene biosynthesis in relation to the phylogeny of the plant lineage.

ACS homologs are present in genomes of essentially all sequenced land plants (including non-seed plants), whereas *ACO* homologs have been found only in the genomes of spermatophytes (gymnosperms and angiosperms), which arose about 370 MY ago. Thus, the synthesis of ACC by ACS is potentially more ancient than the efficient conversion of ACC to ethylene in seed plants. The solid red line representing ACC synthesis indicates the presence of supporting evidence, whereas the dotted red line indicates the lack of evidence. In terms of ethylene signaling, the ethylene receptor gene may have been acquired ~2 BY ago from an endosymbiotic cyanobacteria that became the plastid. The complete ethylene signaling pathway as characterized in angiosperms was likely assembled during the evolution of Charophycean algae, prior to the origin of land plants ~450 MY ago.

Figure 3. Distinct ethylene and ACC responses in liverworts.

(a) Ethylene (C_2H_4) treatment (10 ppm for 72 hrs) enhances column elongation in *R. helicophylla* gemmalings. Scale bars = 50 μm. Diagram on the left shows the gemmaling cauloid lobe (c.l.) and the column (c.) regions. Photographs reproduced from Stange and Osborne [28] with permission. **(b)** ACC treatment inhibits the elongation growth of column cells in *R. helicophylla* gemmalings. The Y-axis indicates the lengths of both the cauloid lobe and the column. Modified from Stange and Osborne [29] with permission. **(c)** Ethylene treatment (100 ppm) increases thallus size and stimulates the formation of gemma cups (indicated by red arrows) in *M. polymorpha*. Reproduced from Li et al. [10••] with permission. Scale bars = 1 cm. **(d)** ACC treatment (20 μM) inhibits thallus growth of the ethylene-insensitive mutant

548 Mp*ein3* in *M. polymorpha*. Reproduced from Li et al. [10••] with permission. **(e)** Treatment with ACC (100
549 µM for 1 d) versus ethylene (50 ppm for 1 d) induces differential expression of three *M. polymorpha* genes
550 (*GST2*, *HEL* and *ERF*). Adapted from Katayose et al. [11••] with permission. **(f)** A gemma of the *M.*
551 *polymorpha* Mp*acs1*Mp*asc2* double mutant shows abnormal apical notches (meristems) in terms of their
552 number, shape and position (indicated by yellow arrows) compared to wild-type notches (indicated by
553 white asterisks) . Scale bars = 100 µm Reproduced from Li et al. [10••] with permission.

554

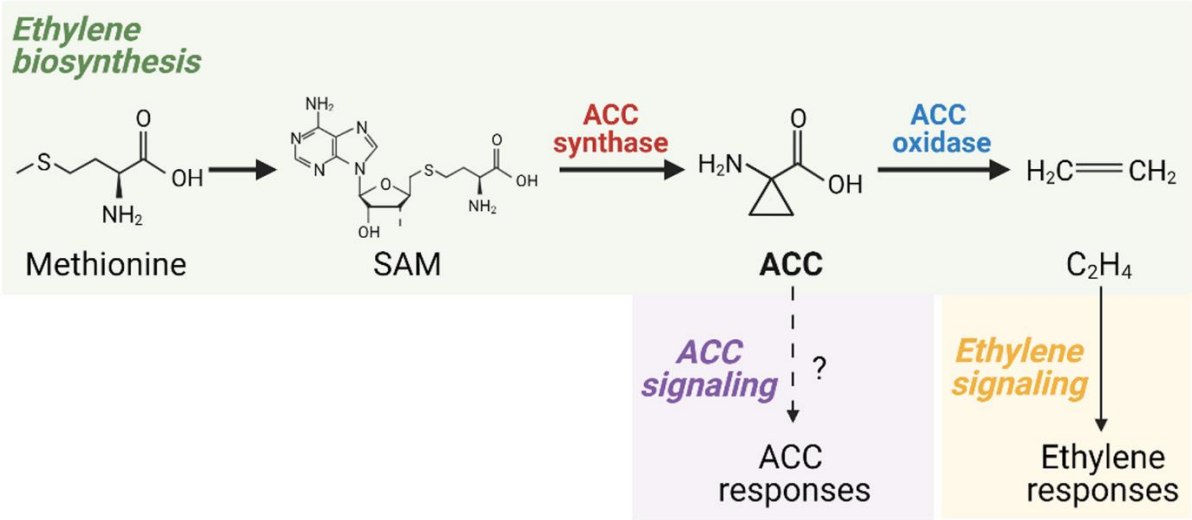
555 **Figure 4.** Ethylene-independent responses of ACC in angiosperms.

556 **(a)** ACC regulates anisotropic expansion of *A. thaliana* root cells via the FEI pathway. The swollen root tip
557 of the *fei1fei2* mutant is rescued by inhibiting ACC biosynthesis (using AOA treatment; 0.375 mM), but is
558 not reverted in an ethylene-insensitive mutant (*ein2-50*). Scale bar = 1 mm. Reproduced from Xu et al. [31]
559 with permission. **(b)** ACC inhibits the rosette growth of light-grown *A. thaliana* seedlings when ethylene
560 signaling is either chemically blocked by 1-MCP (250 ppm) treatment or genetically blocked by the *ein2-1*
561 mutation. Reproduced from Van Der Straeten et al. [34•] under the Creative Commons CC BY license. **(c)**
562 ACC signaling is essential for the symmetric division of *A. thaliana* guard mother cells to develop normal
563 stomata. Reduced ACC synthesis by AVG treatment (25 µM) leads to the formation of single guard cells
564 (SGC, indicated by red arrows), which are not observed when treated with either AIB (25 µM) or 1-MCP.
565 Similar SGCs are observed in the *acs* octuple (oct) mutant, but not in the constitutive ethylene-response
566 mutant *ctr1* nor in the ethylene-insensitive mutant *ein3eil1*. Scale bar = 20 µm. Modified from Yin et al.
567 [35•] under the Creative Commons CC BY license. **(d)** ACC plays a role in *A. thaliana* ovular pollen tube
568 attraction. Left side: The *acs* octuple (oct) mutant has reduced seed set compared to the wild type. Scale
569 bar = 5 mm. Right side: The basis for the reduced seed number is that fewer ovules in the *acs* oct pistil are
570 capable of attracting pollen tubes compared to the wild-type ovules. Pistils were hand pollinated with
571 wild-type pollen expressing β-glucuronidase (GUS) from a pollen-specific promoter (*proLAT52:GUS*) then
572 stained for GUS activity. Each blue dot of GUS staining within the ovule indicates fertilization by the pollen.
573 Scale bar = 0.5 mm. Adapted from Mou et al. [36••] under the Creative Commons CC BY license. **(e)** ACC
574 treatment stimulates expression of an ethylene-responsive *EBS:GUS* reporter in pollen grains, germinating
575 pollen, and pollen tubes of the Never-ripe (ethylene-insensitive) mutant of tomato. The Control consisted
576 of no treatment. Scale bar = 30 µm. Reproduced from Althiab-Almasaud et al. [37•] with permission.

577

578 **Figure 1**

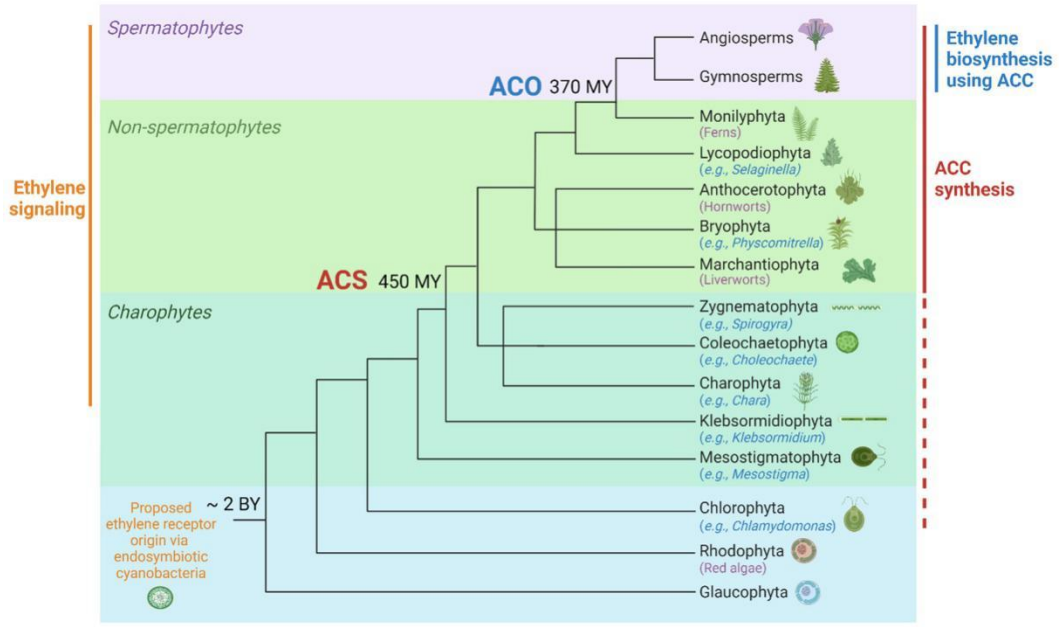
579



580
581

582 **Figure 2**

583



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585

Figure 3

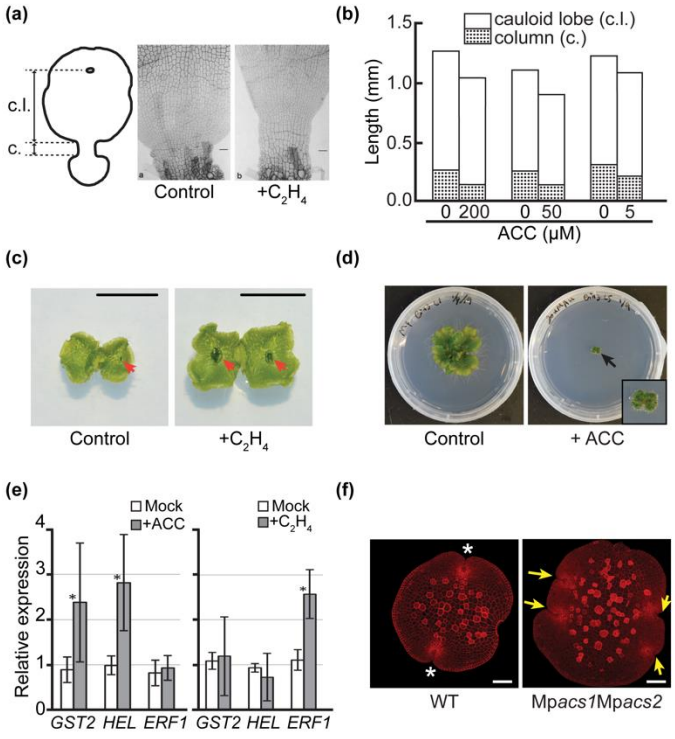
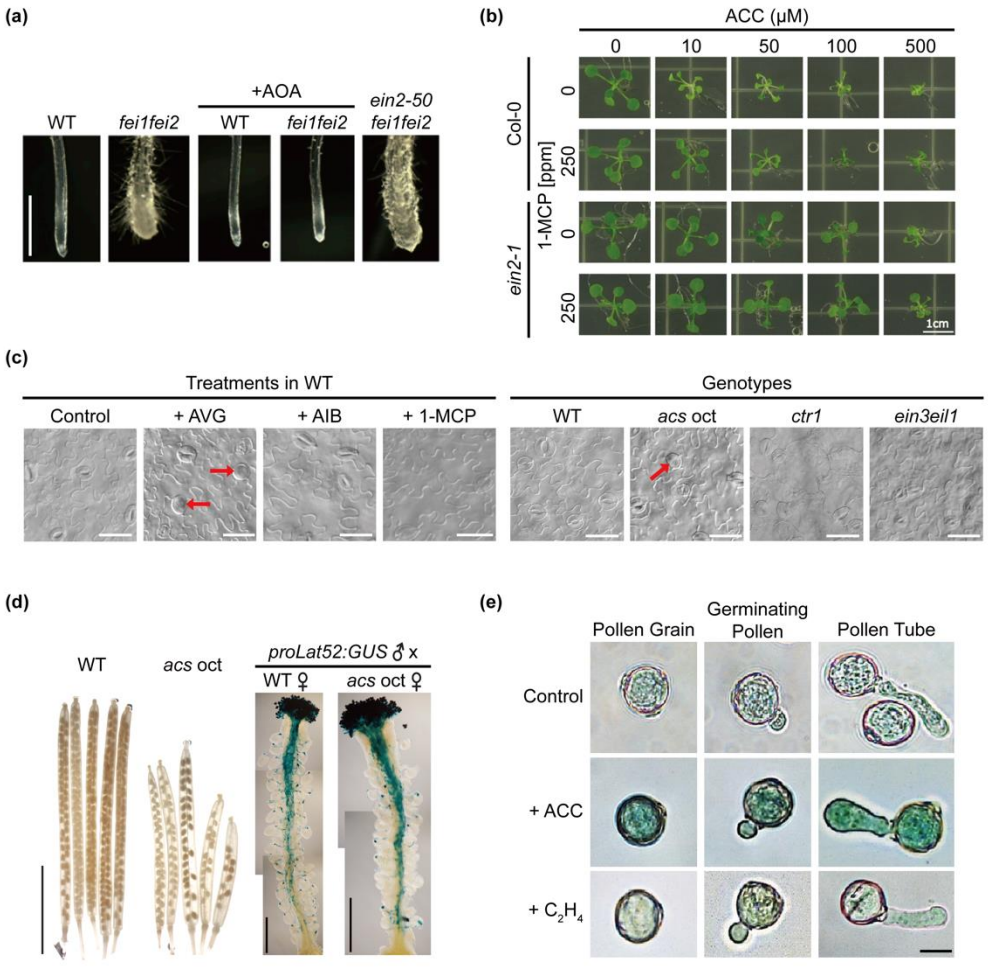

(a)
c.l.
c.
Control
+C2H4
(b)
cauloid lobe (c.l.)
column (c.)
Length (mm)
1.5
1.0
0.5
0.0
0 200
0 50
0 5
ACC (μM)
(c)
Control
+C2H4
(d)
Control
+ ACC
(e)
Mock
+ACC
Mock
+C2H4
Relative expression
4
3
2
1
0
GST2 HEL ERF1 GST2 HEL ERF1
(f)
WT
Mpacs1Mpacs2

Figure 4


(a)
WT
fei1fei2
+AOA
WT
fei1fei2
ein2-50 fei1fei2
(b)
ACC (μM)
0
10
50
100
500
Col-0
ein2-1
1-MCP [ppm]
0
250
0
250
1cm
(c)
Treatments in WT
Control
+ AVG
+ AIB
+ 1-MCP
Genotypes
WT
acs oct
ctr1
ein3eil1
(d)
WT
acs oct
proLat52:GUS ♂ x
WT ♀
acs oct ♀
(e)
Pollen Grain
Germinating Pollen
Pollen Tube
Control
+ ACC
+ C2H4

Highlights:

- 1-amino-cyclopropane-1-carboxylic acid (ACC) is known as the ethylene precursor
- Increasing evidence suggests ACC has signaling roles in plants
- Responses to ACC can be distinct from those induced by ethylene
- Evolutionarily, ACC may have been a signal before becoming an ethylene precursor
- Elucidation of the ACC signaling pathway is a topic for future research

Response to the Reviewers:

Reviewer 1: This review summarizes the current information available about ACC as a signaling molecule and its role independent of functioning as a precursor to the biosynthesis of ethylene. It does a good job of this and is well written. I suggest a few additional things to possibly be added and I have some minor comments for the authors to consider to improve clarity.

1. In the section "ACC is only weakly converted to ethylene in non-seed plants" I recommend adding a sentence or two with references about the alternative pathways/precursors for ethylene biosynthesis that are known to exist.
   Thank you for this helpful suggestion. On page 4, under the section "ACC is only weakly converted to ethylene in non-seed plants", we now include a separate short paragraph on the alternative pathways/precursors for ethylene biosynthesis in microbes as follows:

   "The alternative pathways of ethylene production in non-seed plants have yet to be identified. Certain bacteria and fungi produce ethylene from 2-oxoglutarate using the Ethylene Forming Enzyme (EFE) [19] or by a non-enzymatic conversion of α-keto ɣ-methylthiobutyric acid [20]. Still other bacteria convert carbon monoxide or sulfur-compounds to ethylene using vanadium nitrogenase or methylthio-alkane reductase enzymes, respectively [21-23]. However there is currently no evidence indicating that these particular mechanisms exist in plants." (lines 131-136)

2. Related to comment 1: There is good evidence that ethylene signaling evolved prior to plants. Is there anything to suggest this is true for the metabolism of ACC to ethylene? In other words, aquatic plants may lack this, but perhaps prokaryotes have this ability?
   This is an interesting idea, but we are unaware of good evidence for the metabolism of ACC to ethylene in prokaryotes. For example, the cyanobacteria *Hapalosiphon* can produce ethylene when treated with 1-10 mM ACC, but there is no evidence of an ACO homolog in this species, and these ACC concentrations are unlikely to be physiologically relevant. There is also an isolated case of a proposed ACC oxidase homolog in the fungus *Agaricus bisporus*, but it is very inefficient compared to ACO homologs of seed plants (with a Km for ACC in the mM range instead of the uM range). Given the murky evolutionary connections of such examples to plants, we prefer not to mention these examples.

3. Figure 3b- More description is needed here. There are two bars for each ACC concentration, but no description of what the right vs left bar represents. Is one a control?
   Thank you for catching this. For each dose, the left bar is the 0 uM ACC control while the right bar is the ACC treatment. We have added the missing labels to the figure.

4. "past and present evidence"- All the evidence is from the past, ie. published. I recommend removing "past and present". Perhaps "older and newer evidence" is what they mean.
   Thank you for catching this. We now simply say, "In this article, we review the evidence that ACC serves as a plant signaling molecule…." without specifying any time periods (line 84).

5 ."yeast overexpressing". Probably more accurate to say "heterologously expressing" since any expression in yeast would be more than they normally express since yeast does not

contain these genes.
Done. (line 101)

6. "polymporpha" spelling needs to be fixed.
Done. (line 102)

Reviewer 2: This is a well-written and logically structured review by the some of the leaders in the field of ACC signaling. This said, I have a few suggestions that I think could help to improve this review.

1. The fact that non-seed plants do not seem to have ACOs and that ACC treatment does not result in an increase in ethylene production suggests not only that ACC may have a signaling function but also indicates that an alternative ethylene biosynthesis pathway may exist. Although ethylene biosynthesis per se is not the topic of this review, it would be good to mention in a couple of sentences what is known about this possible alternative ethylene biosynthetic pathway(s).
Thank you for this helpful suggestion. On page 4, under the section "ACC is only weakly converted to ethylene in non-seed plants", we now include a short paragraph on the alternative pathways/precursors for ethylene biosynthesis in microbes as described in our response to Comment #1 by Reviewer 1.

"The alternative pathways of ethylene production in non-seed plants have yet to be identified. Certain bacteria and fungi produce ethylene from 2-oxoglutarate using the Ethylene Forming Enzyme (EFE) [19] or by a non-enzymatic conversion of α-keto ɣ-methylthiobutyric acid [20]. Still other bacteria convert carbon monoxide or sulfur-compounds to ethylene using vanadium nitrogenase or methylthio-alkane reductase enzymes, respectively [21-23]. However there is currently no evidence indicating that these particular mechanisms exist in plants." (lines 131-136)

2. I think it would be useful to indicate whenever appropriate whether the concentrations of ACC used in the different experiments are thought to be in the physiological range.
Thank you for this valuable suggestion. In response, we have made the following revisions:

In order to allow readers to quickly assess each experiment for themselves, we now provide the ACC doses used in every study (except for Tsang et al. 2011 where the dose for the particular cited experiment was not explicitly stated in the paper).

In the section titled "ACC is only weakly converted to ethylene in non-seed plants", we have added the following sentence after describing experiments that used 200 μM–10 mM: "Assuming there was uptake of the ACC in these experiments, such doses were likely above the physiological range." (lines 120-121)

In terms of ACC signaling in angiosperms, the doses of ACC that stimulated responses ranged between 0.1- 50 mM, which are likely to be in the physiological range. We have added the statement, "In this section, all of the studies in which exogenous ACC was applied used concentrations that were likely to be in the physiological range." (lines 181-183)

For the electrophysiological studies and imaging of a Ca^{2+} reporter, we have added the following sentence: "(The high concentrations of ACC used as a ligand in these studies are typical of electrophysiological studies and imaging in heterologous systems.)" (lines 257-258)

3. Also, somewhat related to this point, it would be useful to provide a list of the minimum requirements for a signaling molecule.
Thank you for this helpful suggestion. We have added the following sentence in the Introduction section, "By definition, a plant signal operates at low concentrations, evokes local or distant physiological responses, and can be transported." (lines 85-87).

We now also return to these points in the Conclusion section: "It is increasingly clear that ACC can function as a signaling molecule independent of its role as the ethylene precursor. ACC is active at low concentrations (e.g., 1 μM), can induce local or distant responses (e.g., Figure 4b), and can be transported via LHT1 and LHT2." (lines 297-299)

4. In general, when describing the experiments in support of, for example, the potential role of GLR as ACC receptors, the authors should mention any limitation of the studies (concentrations used, possible side effects of using inhibitors with potential off target effects, such as that of AVG, etc.)
Thank you for the valuable suggestion. We have added the following general and specific comments to the manuscript:

A general comment: "The untangling of responses that are due to ACC versus ethylene in *A. thaliana* has relied on an array of genetic tools (such as well-characterized ethylene signaling mutants) and pharmacological tools (including chemical inhibitors of ethylene biosynthesis and ethylene perception) [30], with the caveat that chemical inhibitors of ethylene biosynthesis or signaling can have unknown actions and side effects." (lines 178-181)

Specific comments:
"(Due to incomplete silencing of two of the *ACS* genes in this *acs* octuple mutant, a true knockout mutant of ACC synthesis has yet to be obtained.)" (lines 207-208).

"(The high concentrations of ACC used as a ligand in these studies are typical in electrophysiological studies and imaging in heterologous systems.)" (lines 257-258).

5. I find the hypothesis that ACC or ACC derivatives would work as an internal or external sensor very interesting, but at this point completely speculative. What are the actual data supporting this hypothesis? If there are none, the speculative nature of that proposition

needs to be clearly identified as such.
This is indeed speculation, as noted in the section title, “Speculation on ACC signaling mechanisms”. We have made modifications to the text (underlined portions) in order to make the speculative nature clearer: “We speculate that ACC, which is typically present at low concentrations, may conceivably reflect cellular metabolic homeostasis, similar to how the non-proteinogenic amino acid GABA is considered to be a proxy for the plant cell nitrogen or amino acid content [42], or how threhalose-6-phosphate, a low-abundant sugar-phosphate, mirrors sucrose levels in plants and controls the energy status [43]. Although this has not been investigated, ACC might have a nitrogen or metabolic sensing function, perhaps linked with changes in Ca^{2+}-fluxes caused by GLR channels.” (lines 267-272)

6. Does it make sense that a low-abundance ACC conjugate such as GACC could work as a significant source of nitrogen for plant-associated bacteria?
Thank you for this question. It probably does not make sense. Therefore in the sentence (line 278), “Secreted ACC (or GACC) could serve as a cellular messenger….” we have deleted the words “(or GACC)” so as not to imply that GACC is a significant source of nitrogen for plant-associated bacteria. Our thinking is that since it is known that ACC can be used as a nitrogen source by certain plant growth promoting bacteria, and since GACC is known to be synthesized in the apoplast, ACC might be secreted from the cell and used as a nitrogen source for microorganisms.

Minor comments

7. Please, rephrase this sentence, as it is unclear to me the message the authors are trying to convey. "It is unknown whether the evolution of ethylene signaling converged with the ability to synthesize ethylene".
We have deleted the sentence and replaced it with this clearer, more explicit statement: “Ethylene signaling may have evolved prior to the ability to synthesize ethylene, given that the ethylene receptor likely originated in cyanobacteria [3] and because environmental ethylene is produced by microorganisms as well as by chemical breakdown of organic matter.” (lines 64-67)

8. "Evolutionarily, ancestral aquatic species that later gave rise to land plants would not have benefitted from the biosynthesis of high ethylene levels, because ethylene cannot diffuse into water as readily as it diffuses in air." I do not follow this argument. Most signaling molecules do not freely diffuse in the environment. Why would this be a problem in the case of ethylene? Could not this fact help to trigger local responses wherever ethylene was produced?
Thank you for pointing this out. To clarify the rationale, we have revised the sentence as follows: “Evolutionarily, we believe aquatic species that subsequently gave rise to land plants would not have benefitted from the synthesis of high ethylene levels. This is because ethylene is removed only by diffusion and diffuses 1000 times slower in water than in air, thus precluding the rapid removal of cellular ethylene in aquatic organisms.” (lines 138-141)

9. "The concomitant presence of ACC and ethylene signaling suggests a possible interplay between the two signaling pathways, functioning perhaps as counteracting partners in some cases and as co-directional partners in others." I think this is an interesting idea that could

be reinforced by providing specific examples where ACC and ET counteract or synergize each other.
Instead of reinforcing the idea of an interplay between the two signaling pathways, we prefer to downplay this idea, because most effects of ACC and ethylene do not appear to be opposite or synergistic, making it unlikely that the central signaling pathways themselves intersect. We have replaced the sentence with the following: “There could also be feedback mechanisms based on the interplay of ACC and ethylene in terms of the downstream responses they each induce.” (lines 293-294).

Please also see our response to Comment #5 by Reviewer 3.

10. "Some other limitations of treating plants with ACC include not knowing the fate of the ACC once it is taken up by the plant, and distinguishing between ACC signaling versus a stress/toxicity response at particular doses of ACC." I think this is an important point not only when using ACC as an ethylene precursor, but also when investigating the signaling properties of ACC per se.
Yes, this was our intended meaning. To make the point clearer, we have added the underlined portion to the sentence: “Some other limitations of treating plants with ACC, particularly when investigating ACC signaling responses, include not knowing the fate of the ACC taken up by the plant and distinguishing between ACC signaling versus a stress/toxicity response at particular doses of ACC.” (lines 312-314)

Reviewer 3: This is a well-organized and comprehensive review of the recent advance in the role of ACC as a signaling molecule in plants. The paper presents the emerging role of ACC that acts as a signaling molecule independent of ethylene signaling in non-seed plants and in Arabidopsis. The paper also includes the speculation of potential ACC signaling mechanisms. The presented figures nicely inform the current status of the ACC research and the evolutionary aspects of ACS, ACO, and ethylene biosynthesis in the different plant lineage.

I have a few minor suggestions to help improve the article:

1. It would be helpful for the reader to understand ethylene biosynthesis in non-seed plants if the authors provided more information on whether non-seed plants produce ethylene or not. In the text, " ------many non-seed plants produce little or no additional ethylene when treated with ACC." This sentence is somewhat confusing as it is not clear if the non-seed plant can't produce ethylene naturally or it can produce it but it does not respond to ACC to increase ethylene biosynthesis.
Thank you for this comment. We have revised the sentence as follows: “….even though non-seed plants produce ethylene, non-seed plants that take up exogenous ACC produce little or no additional ethylene.” (lines 76-78)

2. On page 3, the authors mentioned that two ACS homologs in the physcomitrella exhibit Cβ-S lyase activity instead of ACS activity. One or two sentences regarding the general role of Cβ-S lyase activity in plants would be helpful.
Thank you for this suggestion. We have added the sentence, “C_{β}-S lyases catalyze the

cleavage of carbon–sulfur bonds of L-cystine and L-cysteine and are involved predominantly in amino acid metabolism." (lines 106-108)

3. On page 4, "Interestingly, angiosperms that have (re)adapted to a fully submerged lifestyle have lost ACO----------". Giving an example of the plant species with this characteristic would be more informative.
   We agree. We have expanded and revised this as follows: "Interestingly, *ACO* genes have been lost in *Potamogeton pectinatus* [24], and both the ethylene biosynthesis and signaling pathways have been lost in *Zostera muelleri* [25] and *Zostera marina* [26]. These are all angiosperms that have (re)adapted to a fully submerged lifestyle, in contrast to angiosperms that grow on the surface of water, such as *Spirodela polyrhiza*, which has retained the ethylene hormone pathways [27]." (lines 144-148)

4. On page 7, the authors describe the potential mechanism that underlies ACC signaling. Perhaps a paragraph can be added in relation to ACC transporters and potential sequestration mechanisms? In some plants, ACC has been shown to be transported from the roots to shoot, indicating the existence of ACC transporters. Similarly, ACC derivatives are shown to be stored in vacuoles, which may present a mechanism for controlling ACC availability in the cells.
   Thank you for this helpful suggestion. We have included these points in the sub-section titled "Dual ACC functions". The revised section states:

   "How might seed plants use ACC for both ethylene production and independent signaling? The function of ACC could be dependent on ACO expression/activity for ethylene production and the availability of ACC signaling components. A switch between ACC signaling and ethylene production could conceivably involve feedback mechanisms based on ACC and/or ethylene levels. ACC levels are controlled through ACC homeostasis, which is mainly regulated through synthesis (by ACS), conversion to ethylene, conjugation to malonyl, γ-glutamyl, or jasmonyl ACC [4], transport (e.g. via LYSINE-HISTIDINE TRANSPORTER1 (LHT1) and LHT2; [49, 50•]) and storage (e.g. in the vacuole [51])."

5. Fig. 1, the authors speculate a possible interplay between ACC and ethylene signaling. This interplay may be reflected in the figure.
   We considered this suggestion carefully and have concluded it would be better not to depict an interplay between ACC and ethylene signaling in the figure. First, an interplay between ACC and ethylene signaling is not a general observation. The evidence is limited to two downstream responses of ACC and ethylene – ACC inhibition vs. ethylene promotion of cell division in liverworts, and the promotion of pollen tube growth by both ACC and ethylene (*see new paper below) – whereas many other ACC and ethylene responses, including pollen tube attraction by Arabidopsis ovules and other ACC responses in *M. polymorpha* show no evidence of interplay. Secondly, the effect on liverwort cell division is not necessarily an interplay with the ethylene signaling pathway per se, but seems to be an effect on a downstream response to ethylene (cell division) that could be effected through other signaling pathways. Therefore we feel it would be too speculative, and would convey

a possibly inaccurate message, if we showed an interplay of ACC-ethylene signaling in the figure at this point in time.

Instead, in the concluding paragraph of the article we state that future directions "…will include how ACC signaling is regulated, particularly in seed plants that rely on ACC for ethylene production, and whether there is interplay with ethylene signaling and other plant hormones." (lines 318-320)

* We added a new reference to the manuscript: Althiab-Almasaud R, Sallanon H, Chang C, Chervin C: **1-aminocyclopropane-1-carboxylic acid (ACC) stimulates tomato pollen tube growth independently of ethylene receptors**. *Physiol Plant*, accepted. The description on lines 235-241 reads as follows:

"ACC might play a role in pollen tube growth as well. In *Solanum lycopersicum*, *in vitro* pollen tube growth was promoted by applying low concentrations of ACC (0.1-100 μM) when ethylene receptor signaling was inhibited [37•]. Unlike the ACC-specific responses discussed above, promotion of tomato pollen tube growth can be induced by both ethylene and ACC. Notably, treating pollen grains and pollen tubes with ACC stimulated the expression of an ethylene-responsive reporter fusion (*EIN3 Binding Site (EBS):GUS*) either in the ethylene-insensitive Never-ripe mutant (e.g., Figure 4e) or in presence of 1-MCP, suggesting that ACC might activate ethylene response signaling at a point downstream of the ethylene receptor [37•]."

;6. In Fig. 3b, it is not clear what the two columns represent. Additional information on the graph would be helpful and some differentiation between the column such as different color?? would work.
Thank you for pointing this out. For each dose in Fig. 3b, the left bar is the 0 uM ACC control while the right bar is the ACC treatment. We have added the missing labels to the figure in order to identify the left and right bars (without changing their colors).

In addition, we have added a diagram to Fig. 3a in order to show the "cauloid lobe" and the "column" regions of the Riella gemmaling, which are respectively represented by the open bar vs. dotted portions of the bars in the Fig. 3b graph.

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
